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Le

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**Titre :**

Identification et caractérisation d'un récepteur d'enveloppe au virus de leucémie T humaine (HTLV), le transporteur de glucose GLUT1

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*A mon frère Gildas.*

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# TABLE DES MATIERES

<b>REMERCIEMENTS .....</b>	<b>2</b>
<b>TABLE DES MATIERES.....</b>	<b>4</b>
<b>PREAMBULE.....</b>	<b>7</b>
<b>INTRODUCTION GENERALE .....</b>	<b>8</b>
<b>A. RETROVIRUS .....</b>	<b>8</b>
<i>A.1. Les rétrovirus, des rétroéléments.....</i>	<i>8</i>
<i>A.2. Biologie des rétrovirus .....</i>	<i>11</i>
A.2.a. Organisation physique et génétique des rétrovirus, le cas du HTLV.....	11
A.2.b. Cycle de réplication des rétrovirus.....	15
<i>A.3. Emergence des rétrovirus par acquisition du gène env.....</i>	<i>17</i>
A.3.a. La capture d'enveloppe.....	17
A.3.b. Une Env pour entrer ... et pour sortir ?.....	21
<b>B HTLV .....</b>	<b>22</b>
<i>B.2. HTLV est-il un virus pathogène ?.....</i>	<i>22</i>
<i>B.3. Une pandémie ou de nombreuses endémies ?.....</i>	<i>24</i>
<i>B.4. HTLV est un virus ancien .....</i>	<i>25</i>
<i>B.5. Tropisme.....</i>	<i>28</i>
<i>B.6. La réponse immunitaire au HTLV .....</i>	<i>29</i>
<b>C. ENV ET RECEPTEUR DU HTLV .....</b>	<b>30</b>
<i>C.1. Env.....</i>	<i>30</i>
C.1.a. Généralités.....	30
C.1.b. TM .....	31
C.1.c. SU .....	32
C.1.d. RBD .....	34
C.1.e. Dynamique d'entrée.....	34
<i>C.2. Attachement, liaison et récepteur cellulaire.....</i>	<i>35</i>
<i>C.3. Transmission cellule à cellule .....</i>	<i>36</i>
C.3.a. <i>In vitro</i> .....	36

C.3.b. <i>In vivo</i> .....	37
C.3.d. La synapse infectieuse.....	37
C.3.e. La transmission cellule à cellule : un paradigme.....	38
<b>RESULTATS .....</b>	<b>39</b>
A.    CARACTERISATION DE L'EXPRESSION DU RECEPTEUR HTLV A LA SURFACE DES LYMPHOCYTES HUMAINS .....	39
A.1. <i>Résumé article 1</i> .....	39
A.2. <i>Article original 1</i> .....	40
A.3. <i>Conclusion article 1</i> .....	41
B.    IDENTIFICATION DU TRANSPORTEUR DE GLUCOSE GLUT1 COMME RECEPTEUR HTLV.....	43
B.1. <i>Résumé article 2</i> .....	43
B.2. <i>Article original 2</i> .....	44
B.3. <i>Conclusion article 2</i> .....	45
C.    PROPRIETES DISTINCTES DE LIAISON ET D'INFECTION SUR GLUT1 .....	47
C.1. <i>Résumé article 3</i> .....	47
C.2. <i>Article original 3</i> .....	47
C.3. <i>Conclusion article 3</i> .....	48
<b>DISCUSSION.....</b>	<b>51</b>
A.    BIOLOGIE DE L'ENV HTLV.....	51
A.1. <i>Modulation de l'activité de l'Env HTLV</i> .....	51
A.2. <i>Env HTLV endogènes ?</i> .....	52
B.    GLUT1 COMME RECEPTEUR HLTV .....	53
B.1. <i>La famille des transporteurs de glucose</i> .....	53
B.2. <i>GLUT1 et tropisme HTLV</i> .....	54
B.3. <i>GLUT1 et la famille des récepteurs rétroviraux</i> .....	56
B.4. <i>Transporteurs de glucose, cycle cellulaire et trafic membranaire</i> .....	58
B.5. <i>L'Env HTLV, un marqueur de marqueur</i> .....	60
B.6. <i>Partenaires de GLUT1</i> .....	61
B.7. <i>Inhibition de l'activité de GLUT1 par l'Env HTLV</i> .....	63
C.    GLUT1 ET LES MODELES DE PATHOGENESE .....	64

<i>C.1. Résumé article 4</i> .....	64
<i>C.2. Article de revue 4</i> .....	64
<i>C.3. Discussion article 4</i> .....	65
C.1.a. La leucémie T de l'adulte.....	65
C.1.d. Considérations métaboliques.....	66
C.1.c. La paraparésie spastique tropicale.....	67
C.1.d. Effets directs et indirects de l'Env HTLV sur le transport du glucose.....	68
<b>BIBLIOGRAPHIE</b> .....	<b>70</b>
<b>ANNEXES</b> .....	<b>88</b>
ANNEXE 1 – AUTRES ARTICLES.....	88
<i>Article original 5</i> .....	88
<i>Article original 6</i> .....	88
<i>Article de revue 7</i> .....	88
<i>Article de revue 8</i> .....	88
<i>Article de revue 9</i> .....	88
<i>Article de revue 10</i> .....	89
ANNEXE 2 – BREVETS.....	90
<i>Brevet 1</i> .....	90
<i>Brevet 2</i> .....	90

# PREAMBULE

*- "L'objectivité scientifique n'est possible que si l'on a d'abord rompu avec l'objet immédiat, si l'on a refusé la séduction du premier choix, si l'on a arrêté et contredit les pensées qui naissent de la première observation. Toute objectivité dûment vérifiée dément le premier contact avec l'objet. Elle doit d'abord tout critiquer : la sensation, le sens commun, la pratique la plus constante, l'étymologie enfin, car le verbe qui est fait pour chanter et séduire rencontre rarement la pensée." Gaston BACHELARD, Psychanalyse du Feu.*

Comment le virus de la leucémie T humaine – HTLV – se propage-t-il ?

Quels composants, essentiellement liés au métabolisme cellulaire, sont impliqués dans cette propagation ?

Est-il possible de poser des questions de virologie fondamentale en utilisant HTLV comme modèle ?

Voici quelques questions auxquelles j'ai tenté d'apporter des éléments de réponse au cours de cette thèse.

*Convention pour le lecteur : Le sigle "HTLV" désigne à la fois HTLV-1 et HTLV-2. Lorsque des propriétés spécifiques l'exigent, la distinction entre HTLV-1 et HTLV-2 est explicitée.*

# INTRODUCTION GENERALE

## A. Rétrovirus

### A.1. Les rétrovirus, des rétroéléments

Les éléments transposables, un ensemble de composants génétiques hétérogènes, ont co-évolué de tout temps avec leurs hôtes et sont directement impliqués dans la plasticité des génomes. Ils sont de deux types principaux, transposons et rétroéléments.

Les transposons sont des éléments génétiques à intermédiaire uniquement ADN. Leur transposition peut être répllicative (l'élément génétique ADN est copié dans un site cible) ou conservative (l'élément génétique ADN est excisé et réintégré à un autre endroit du génome).

Les rétroéléments, dont font partie les rétrovirus, sont définis comme des fragments d'ADN dérivés par transposition répllicative d'un intermédiaire ARN. Une activité reverse-transcriptase est donc impliquée dans leur génération. Les rétroéléments constituent une famille large et hétérogène (Coffin, Hughes et al. 1997), au sein de laquelle la nomenclature des éléments varie selon les auteurs (Lower, Lower et al. 1996; Coffin, Hughes et al. 1997). Les types de rétroéléments les plus courants sont les SINE, les LINE, les rétrotransposons à LTR et les rétrovirus (Figure 1). Les SINE (*short interspersed nucleotide elements*) n'encodent pas d'activité rétrotranscriptase et dépendent donc d'un recrutement en *trans* d'une rétrotranscriptase. L'exemple type de SINE est la séquence Alu chez l'homme (environ 1 300 000 copies pour 1 800 000 éléments SINE), dont la longueur consensuelle est de 280 bp et qui ne possède pas de cadre de lecture ouvert (Strachan and Read 1999). Les SINE sont en général transcrits à partir d'un promoteur interne de type RNA pol II ou pol III, et dépendent d'une prise en charge pour leur transposition. Il a récemment été démontré que les LINE (*long interspersed nucleotide elements*), qui encodent une activité rétrotranscriptase, peuvent être mobilisés pour la transposition des SINE B1 et B2 murins (Dewannieux and Heidmann 2005)

## SINE



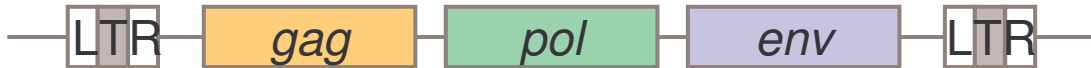
## LINE



## Rétrotransposon à LTR



## Rétrovirus



**Figure 1.** Organisation génétique des principaux rétroéléments.

P : promoteur. LTR : Long terminal repeat. *ORF2* possède l'activité de rétrotranscription.

et Alu humains (Dewannieux, Esnault et al. 2003). Notons également que, par un mécanisme similaire, environ 8000 éléments "pseudogènes traités" (*processed pseudogenes*) qui correspondent à des ARNm de gènes "cellulaires" rétrotranscrits puis intégrés dans le génome, ont été générés chez l'homme (Zhang, Harrison et al. 2003). Par contre, il n'est pas établi si la rétrotranscription des LINE s'exécute uniquement en *cis*, sur l'ARNm qui vient de servir à la traduction de cette rétrotranscriptase, ou si toute rétrotranscriptase de LINE peut prendre en charge tout ARNm LINE. Chez l'homme, le LINE prototype est L1 (environ 870 000 copies pour 1 400 000 éléments LINE), dont le consensus mesure 6,1 kb et encode deux polypeptides, ORF1 et ORF2, ce dernier possédant les activités endonucléase et rétrotranscriptase requises pour la rétrotransposition. L'extrémité 3' des SINE et LINE est en général caractérisée par une queue poly-A classique d'un ARNm cellulaire. Ceci suggère que ces éléments sont rétrotranscrits sous forme d'ARNm. Les rétrotransposons à LTR sont définis par la présence aux extrémités 5' et 3' de séquences terminales longues et répétées (LTR) et l'archétype de ces éléments est le Ty1 de la levure (Cameron, Loh et al. 1979). Les rétrotransposons à LTR encodent également, à la différence des SINE et LINE, un gène de structure *gag* (*group specific antigen*). Ce gène est responsable de l'assemblage dans la cellule de VLP (*virus like particles*) et est requis pour la rétrotransposition chez les rétrotransposons de type IAP chez la souris (Dewannieux, Dupressoir et al. 2004). Enfin, les rétrovirus possèdent une structure similaire aux rétrotransposons à LTR, avec l'adjonction d'un gène *env* codant pour une glycoprotéine d'enveloppe (Env). Il semble que les rétrovirus aient émergé des rétrotransposons à LTR par l'acquisition de ce gène (Temin 1980). Les rétrovirus se disséminent en trans, du génome d'une cellule à l'autre. Les infections rétrovirales ont généralement lieu dans la lignée somatique, ce qui rend les rétrovirus strictement dépendants du passage d'un hôte à un autre pour leur maintien. L'infection de la lignée germinale par les rétrovirus peut conduire à leur pérennisation chez leur hôte sous forme de rétrovirus dits endogènes (ERV, *endogenous retrovirus*), transmis verticalement (Stoye and Coffin 1987; Cohen and Larsson 1988; Martin, Herniou et al. 1997; Martin, Herniou et al. 1999). Les ERV



ont pour la plupart perdu leur capacité infectieuse et contiennent des mutations ou des délétions dans *env* qui les inactivent. Ces séquences *env* résiduelles distinguent donc les ERV des rétrotransposons à LTR. Lorsqu'ils sont sélectionnés, ou maintenus par absence d'effet délétère, les ERV transmis au cours des générations co-évoluent avec leur hôte initial en tant que partie intégrante du patrimoine génétique de l'espèce qui les porte (Qi, Bonhomme et al. 1998; Benit, Lallemand et al. 1999; Johnson and Coffin 1999). Ils vont transposer ou dériver au cours du temps, conduisant également à des polymorphismes entre les individus d'une même espèce (Mager and Goodchild 1989; Zhu, Hsieh et al. 1992; Boissinot, Chevret et al. 2000; Hughes and Coffin 2004; Jern, Sperber et al. 2004; Mamedov, Lebedev et al. 2004). Bien que ces polymorphismes peuvent servir de marqueurs de populations, ils restent rares au sein des populations humaines, et peu ont été associés à des phénotypes particuliers (Kambhu, Falldorf et al. 1990; Zhu, Hsieh et al. 1992).

Le génome humain contient un très grand nombre de rétrovirus endogènes répartis dans au moins 26 familles (Tristem 2000; Benit, Dessen et al. 2001). Les rétrovirus endogènes représentent environ 1% du génome humain (Lower, Lower et al. 1996), alors que les rétroéléments à LTR (rétrotransposons à LTR et rétrovirus endogènes) constituent quant à eux près de 8% du génome humain, soit environ 700 000 éléments (Li, Gu et al. 2001). De plus, il est important de noter qu'une majorité d'éléments transposables présentent des délétions, troncatures ou mutations et apparaissent donc comme "défectifs", *i.e.* ces éléments ne s'expriment plus ou ne possèdent plus d'activité de transposition.

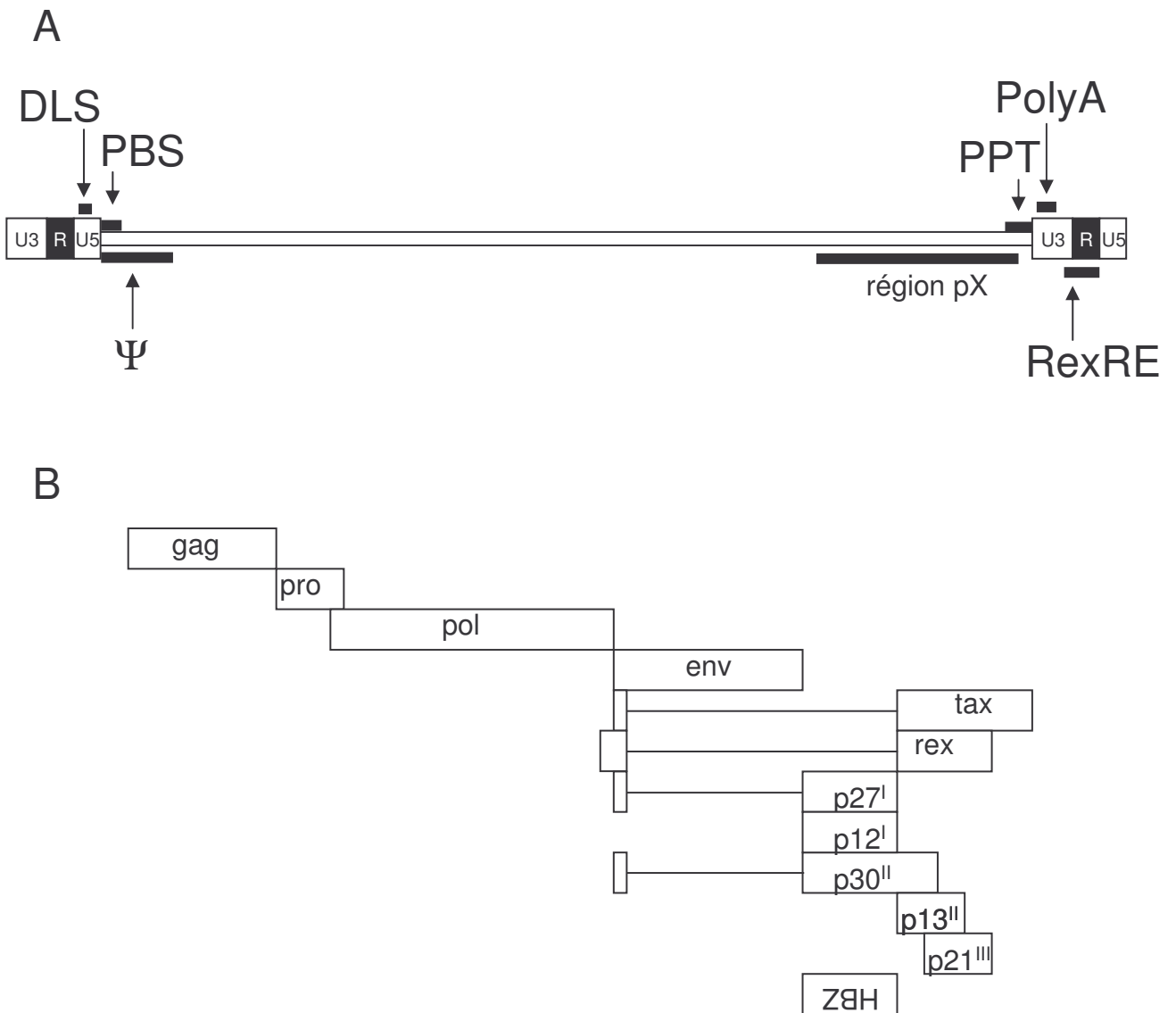
La plupart des rétrovirus endogènes semblent défectifs pour la réplication et la dissémination de cellule à cellule. Toutefois l'apport en trans de protéines Gag et Pol fonctionnelles restaure la capacité de transposition de ces éléments (Tchenio and Heidmann 1992).

## A.2. Biologie des rétrovirus

### A.2.a. Organisation physique et génétique des rétrovirus, le cas du HTLV

Les rétrovirus de vertébrés ont été récemment regroupés sur la base de leur gène *gag* en 7 genres, au sein de la famille des *Retroviridae* : *alpharetrovirus*, *betaretrovirus*, *deltaretrovirus*, *epsilonretrovirus*, *gammaretrovirus*, *lentivirus* et *spumavirus* (Van Regenmortel, Fauquet et al. 2000) et sont caractérisés par une grande variabilité génétique, cependant leur organisation est relativement homogène. Nous prendrons l'objet de ce travail, le HTLV-1 (*human T-cell leukemia/lymphoma virus type 1*), comme référence en soulignant ses particularités par rapport au consensus de la famille des *Retroviridae* auquel il appartient. Le génome du HTLV-1 sous forme proviral contient 8 685 nucléotides, alors que les autres rétrovirus compétents pour la réplication se situent entre environ 7 000 nucléotides (*e.g. avian leukosis virus*) et 13 000 nucléotides (*e.g. simian foamy virus*). Comme tous les rétrovirus, HTLV encode *gag*, *pol* et *env*, mais également une série de protéines accessoires (Figure 2).

*gag* encode un précurseur polypeptidique qui contient les protéines internes de structure : MA, CA, NC. La matrice MA est une protéine myristylée à son extrémité N-terminale, permettant son ancrage sous la membrane lipidique à l'interface avec la CA. La capsid CA se multimérise pour former une "coquille" protéique constituant, avec les composants qu'elle renferme, le "cœur" rétroviral. NC est une petite protéine basique à doigts de zinc impliquée dans l'interaction avec les deux ARN génomiques viraux (Corbin and Darlix 1996). MA, CA et NC sont issues de la maturation du précurseur Gag par clivage protéolytique effectué par la protéase (*pro*, PR). Chez HTLV, Pro est issu d'un saut de cadre lecture au niveau du codon stop de *gag*. Ce saut de -1 nucléotide s'effectue au niveau d'une séquence UUUAAC (Nam, Copeland et al. 1993) et requiert la présence d'une structure ARN en pseudonoeud en aval de cette séquence (Le, Shapiro et al. 1991).



**Figure 2.** Organisation du génome HTLV-1.

L'ADN proviral (A) ainsi que les différents cadres de lectures ouverts (B) sont indiqués. Les cadres de lectures répartis sur plusieurs exons sont séparés d'un trait horizontal. DLS : *dimer linkage signal*, impliqué dans l'interaction entre les deux ARN. PBS : *primer binding site*, site de fixation du tRNA<sup>Pro</sup> qui sert de première amorce à la rétrotranscription. Ψ : signal d'encapsidation des ARN viraux.. PPT : *poly purine tract*, séquence résistante à l'activité RNase H qui sert de seconde amorce à la rétrotranscription. PolyA : signal de polyadénylation. RexRE : *Rex responding element*, motif de liaison de Rex qui permet de maintenir un pool d'ARN pour l'encapsidation sous forme non épissée.

L'intégrase (IN) et la polymérase (RT) sont issues de la maturation, sous l'action de PR, d'un précurseur codé par *pol*. Alors que chez la majorité des rétrovirus *pol* est exprimé dans la continuité de *pro*, chez HTLV l'expression de *pol* nécessite un second saut de cadre de lecture. Un tel saut existe également chez HIV (*human immunodeficiency virus*), alors que chez les gammarétrovirus comme MLV (*murine leukemia virus*) *pol* est traduit par suppression d'un codon stop. *pol* est généralement schématisé comme codant pour les activités "enzymatiques" des rétrovirus. Ainsi, RT possède l'activité rétrotranscriptase associée à une activité RNase H qui permet la conversion des deux ARN génomiques en une molécule d'ADN complémentaire (on parle alors de provirus). IN est responsable du clivage de 2 nucléotides en 3' de chaque brin de la molécule d'ADN proviral (activité endonucléase) et de l'intégration de cet ADN proviral dans le génome cellulaire (activité de liaison d'ADN). Toutefois, ce schéma a été récemment réévalué dans les cas des spumavirus, et l'obtention d'un provirus compétent pour l'intégration semble nécessiter le clivage d'un intermédiaire circulaire à 2 LTR (Delelis, Petit et al. 2005). Les deux fragments simple-brins qui bordent le provirus sont supposés déclencher une réponse de type "dommages à l'ADN", cependant, au moins pour HIV, les senseurs de dommages à l'ADN ATM, ATR, DNA-PK et PARP-1 ne semblent pas impliqués dans ce processus (Ariumi, Turelli et al. 2005).

Au-delà de la structure même du virion et des activités enzymatiques qu'elles portent, il est clair que les protéines codées par *gag* et *pol* sont impliquées à plusieurs niveaux du cycle de répllication (bourgeonnement, décapsidation, trafic intracellulaire, interaction avec la chromatine, etc.) (Bieniasz 2003; Demirov and Freed 2004).

*env* encode l'Env (voir paragraphe Enveloppe), à partir d'un ARNm épissé qui élimine les cadres de lecture de *gag* et *pol*.

Le génome HTLV encode enfin toute une série de protéines dites "accessoires" : Rex, Tax, HBZ, p27<sup>I</sup> (Rof), p12<sup>I</sup>, p30<sup>II</sup> (Tof), p13<sup>II</sup>, p21<sup>II</sup>. Rex est responsable du trafic du noyau vers le cytoplasme d'ARN génomique non épissé. Le domaine RexRE (*Rex responsive element*) reconnu par Rex se situe dans les régions U3 et R (voir ci-dessous). Tax transactive

la transcription du génome HTLV à partir de la région U3. Tax reconnaît dans cette région un triple motif répété de 21 nucléotides. De plus HTLV encode une série de protéines accessoires dont la fonction est mal connue (Albrecht and Lairmore 2002). Parmi ces protéines, p13<sup>II</sup> semble être une protéine mitochondriale responsable d'un effet anti-prolifératif (Silic-Benussi, Cavallari et al. 2004). p30<sup>II</sup> est capable de lier les ARN épissés codant pour Rex et Tax induisant une baisse de la production virale (Nicot, Dunder et al. 2004). Le brin anti-sens du génome de HTLV encode la protéine HBZ (Gaudray, Gachon et al. 2002). HBZ interagit avec c-Jun et inhibe sa capacité de liaison à l'ADN, bloquant l'activité de transactivation de c-Jun. Il a donc été sélectionné au cours de l'évolution des protéines virales avec des effets opposés, positifs ou négatifs, sur la production rétrovirale. Ceci reflète au niveau du génome rétroviral la capacité adaptative des rétrovirus durant la co-évolution avec leur hôte, aboutissant à une réplication apparemment sub-optimale.

Le génome viral sous forme ARN est bordé à ses extrémités 5' et 3' (avant la queue polyA) de la même région R (*repeat*) de 228 nucléotides, et qui délimite donc l'initiation de la transcription et la polyadénylation (Figure 2). Deux copies ARN sont présentes dans le cœur viral. Après la région R en 5' se trouve la séquence U5 (*unique 5'*), impliquée notamment dans la rétrotranscription, suivie d'une séquence PBS (*primer binding site*). Le PBS, long de 18 nucléotides, permet la liaison au génome viral d'une molécule de tRNA cellulaire (tRNA<sup>Pro</sup> pour HTLV) qui est utilisée comme amorce pour la synthèse du brin d'ADN négatif par la rétrotranscriptase. Dans cette région va également se situer une séquence dite d'encapsidation,  $\Psi$ , qui permet l'empaquetage de l'ARN génomique viral dans la particule. La localisation de  $\Psi$  chez HTLV n'est pas connue, mais sur la base du travail effectué chez le BLV (*bovine leukemia virus*), le virus le plus apparenté,  $\Psi$  se situerait de manière discontinue en amont et dans gag, dans deux zones d'environ 150 nucléotides (Mansky, Krueger et al. 1995). Une séquence dite de dimérisation (DLS, *dimer linkage sequence*) située après  $\Psi$  permet aux deux molécules d'ARN génomique de s'associer. Chez HTLV, la DLS se situe au niveau d'un palindrome de 14 paires de base en amont du PBS

(Monie, Grotorex et al. 2001). Après toute la série de cadres de lectures, le dernier exon de *tax* se superpose à la séquence PPT (*polypurine tract*), une séquence courte (17 nucléotides pour HTLV) riche en purine, résistante à l'activité RNase H, qui est clivée puis utilisée par la rétrotranscriptase comme amorce pour la synthèse du brin d'ADN positif. Chez HIV, il existe également une séquence PPT centrale (cPPT), qui permet d'amorcer la synthèse de ce brin à plusieurs endroits. Le PPT est suivi de la région U3 (*unique 3'*) impliquée dans la rétrotranscription du génome viral et qui contient également les séquences promotrices de la transcription. Enfin, alors que pour la majorité des rétrovirus la séquence de polyadénylation AAUAAA se trouve dans R, chez HTLV elle est située dans U5, en amont de RexRE. En effet RexRE forme une structure secondaire de 255 nucléotides, qui juxtapose spatialement cette séquence avec une séquence riche en GU, permettant alors la polyadénylation (Ahmed, Gilmartin et al. 1991).

Les particules rétrovirales ne sont toutefois pas des structures homogènes. Tout d'abord, au sein d'une préparation rétrovirale, la constitution relative des particules va être extrêmement variable, notamment parce que ces particules sont relativement instables. De plus, une particule rétrovirale n'est pas constituée uniquement des composants viraux décrits ci-dessus. Ainsi, la double couche lipidique qui enveloppe la particule est arrachée à la membrane plasmique, et va pouvoir contenir une variété de protéines cellulaires. De plus, dans la particule, des molécules solubles (protéines, ARN cellulaires...) vont également être co-encapsidées avec le matériel viral. Parmi tous ces composants cellulaires, certains vont être incorporés de manière "obligatoire" en terme de réplication virale, l'exemple le plus évident étant le tRNA d'amorçage. D'autres éléments seront incorporés plus ou moins systématiquement (molécule du CMH, petits ARN nucléaires), l'influence respective de chacun de ces éléments sur le cycle de réplication viral et sur l'interaction entre virus/hôte n'étant pas établie de manière univoque.

### A.2.b. Cycle de réplication des rétrovirus

La première étape du cycle de réplication rétroviral consiste en l'expression de l'ARN génomique viral à partir du provirus ADN intégré dans le génome d'une cellule de l'hôte. Dans le contexte d'une émergence des rétrovirus à partir de rétroéléments, il est effectivement logique de considérer que la première phase de réplication s'effectue à partir du provirus intégré, plutôt qu'à partir de particules libres. Une partie des ARN transcrits va être épissée et permet ainsi la traduction de certains gènes, notamment *env*, tandis qu'une autre partie non épissée permettra à la fois l'expression de *gag* et *pol* et l'incorporation dans les particules virales. Les protéines Gag, Pol, Env, l'ARN viral, mais également d'autres composants viraux et cellulaires vont bourgeonner au niveau d'une bicouche lipidique, en général la membrane plasmique. Les particules virales ainsi constituées, d'environ 100 nm de diamètre, sont relarguées dans le milieu extracellulaire. Une étape extracellulaire de maturation va prendre place, incluant notamment le clivage du précurseur Gag et en conséquence le remaniement des éléments structuraux de la particule dont l'ARN (Muriaux, Mirro et al. 2001). De manière stochastique, ces particules vont s'attacher à la surface des cellules (la même cellule, une autre cellule, ou une cellule d'un nouvel organisme). Certaines interactions, entre l'Env et une molécule de la surface cellulaire (récepteur), conduisent à la fusion des membranes virales et cellulaires et donc au largage dans le cytoplasme du contenu de la particule. Le cœur viral va alors subir une série de remodelages, et la disponibilité de substrats cytoplasmiques, *e.g.* le pool de nucléotides cellulaires, va permettre le déroulement de la rétrotranscription. Après conversion du génome en ADN double brin, le cœur viral associé notamment à IN, correspond au complexe de préintégration (PIC, *preintegration complex*). Les molécules d'ADN synthétisées vont exister sous différentes formes (linéaire, circulaire à 1 ou 2 LTR) et la proportion, la localisation et le devenir de ces formes restent débattus. Une partie de ces molécules va s'associer à l'ADN chromatinien cellulaire généralement suite à la rupture de l'enveloppe nucléaire au cours de la mitose. Mais d'autres mécanismes existent, ainsi chez HIV l'ADN proviral peut passer au travers des pores nucléaires avant mitose (Lewis, Hensel

et al. 1992). L'intégration va alors se dérouler et conduire à la formation de l'ADN proviral dans le génome cellulaire.

Les activités cellulaires sont requises à toutes les étapes du cycle de réplication. Ainsi dans le cas de HTLV, l'activité acétyltransférase de CBP/p300 est recrutée par Tax permettant l'ouverture de la chromatine au niveau du promoteur et donc la transcription du virus (Georges, Giebler et al. 2003). Au cours du bourgeonnement de HTLV, Gag est ubiquitinylé par Nedd4.1, et la protéine Tsg101, impliquée dans le trafic des vésicules endosomiales, est recrutée. Ainsi, Gag est dirigé vers les corps multivésiculaires où le virus semble bourgeonner (Blot, Perugi et al. 2004).

Le cycle de réplication viral n'est cependant pas systématique et efficace. Ainsi, au cours du bourgeonnement viral, la voie cellulaire par défaut semble être la dégradation du cœur viral dans la voie lysosomiale (Basyuk, Galli et al. 2003). Seules les particules en formation qui auraient recruté l'Env conduiraient à un bourgeonnement productif. Après bourgeonnement, la demi-vie des particules extracellulaires dans un milieu liquide est extrêmement courte, de l'ordre de quelques heures à 37°C. De plus durant cette période, la disponibilité du récepteur n'est pas triviale (contraintes physiques et spatiales, expression et accessibilité du récepteur sur les cellules). En l'absence d'entrée, les virions attachés peuvent être dégradés et recyclés dans des structures de type phagosome (Marechal, Clavel et al. 1998; Marechal, Prevost et al. 2001). Ceci constitue donc un premier blocage, ou restriction, au niveau de l'entrée du virus. Une fois entré, d'autres restrictions vont également intervenir jusqu'à intégration. Ainsi, la restriction post-entrée exercée par APOBEC3G (Sheehy, Gaddis et al. 2002; Harris, Bishop et al. 2003; Mangeat, Turelli et al. 2003; Zhang, Yang et al. 2003) fait intervenir pour HIV une activité cytidine déaminase et conduit à la dégradation du génome viral. APOBEC3G semble également avoir un effet sur l'infection par HTLV, mais sans mettre en jeu l'activité de déamination (Sasada, Takaori-Kondo et al. 2005). Une autre restriction, médiée par TRIM5 $\alpha$  au niveau de la capsid virale, pourrait conduire à une dégradation dépendante de l'ubiquitine de la capsid (Stremlau, Owens et al. 2004). Décrite



pour HIV et MLV, l'activité de TRIM5 $\alpha$  sur HTLV n'est pas connue. L'ensemble de ces mécanismes permet de penser que la réplication des rétrovirus est en équilibre avec la dégradation et le recyclage des différents composants viraux. Les particules virales constitueraient donc aussi un substrat ribonucléoprotéique pour le métabolisme cellulaire. Ainsi, il pourrait être possible de proposer une vision alternative du cycle de réplication rétroviral, où la métabolisation du virus aux différentes étapes du cycle serait la voie "par défaut". D'un point de vue évolutif, les mécanismes de réplication virale productive pourraient avoir été sélectionnés sur la base d'un échappement à ces voies du catabolisme des complexes ribonucléoprotéiques.

### **A.3. Emergence des rétrovirus par acquisition du gène *env***

#### A.3.a. La capture d'enveloppe

- "*All DNA is recombinant DNA.*" Ioan NEGRUTIU

Howard Temin (Temin 1980) a originellement proposé que les séquences rétrovirales, notamment le gène codant pour l'Env, auraient été sélectionnées par mobilisation de séquences à partir de rétrotransposons. Le phénomène de "capture d'enveloppe" a été suggéré sur la base de cette hypothèse en référence à l'acquisition du gène codant pour une Env chez les rétrovirus (Kim, Battini et al. 2004; Kim, Manel et al. 2004).

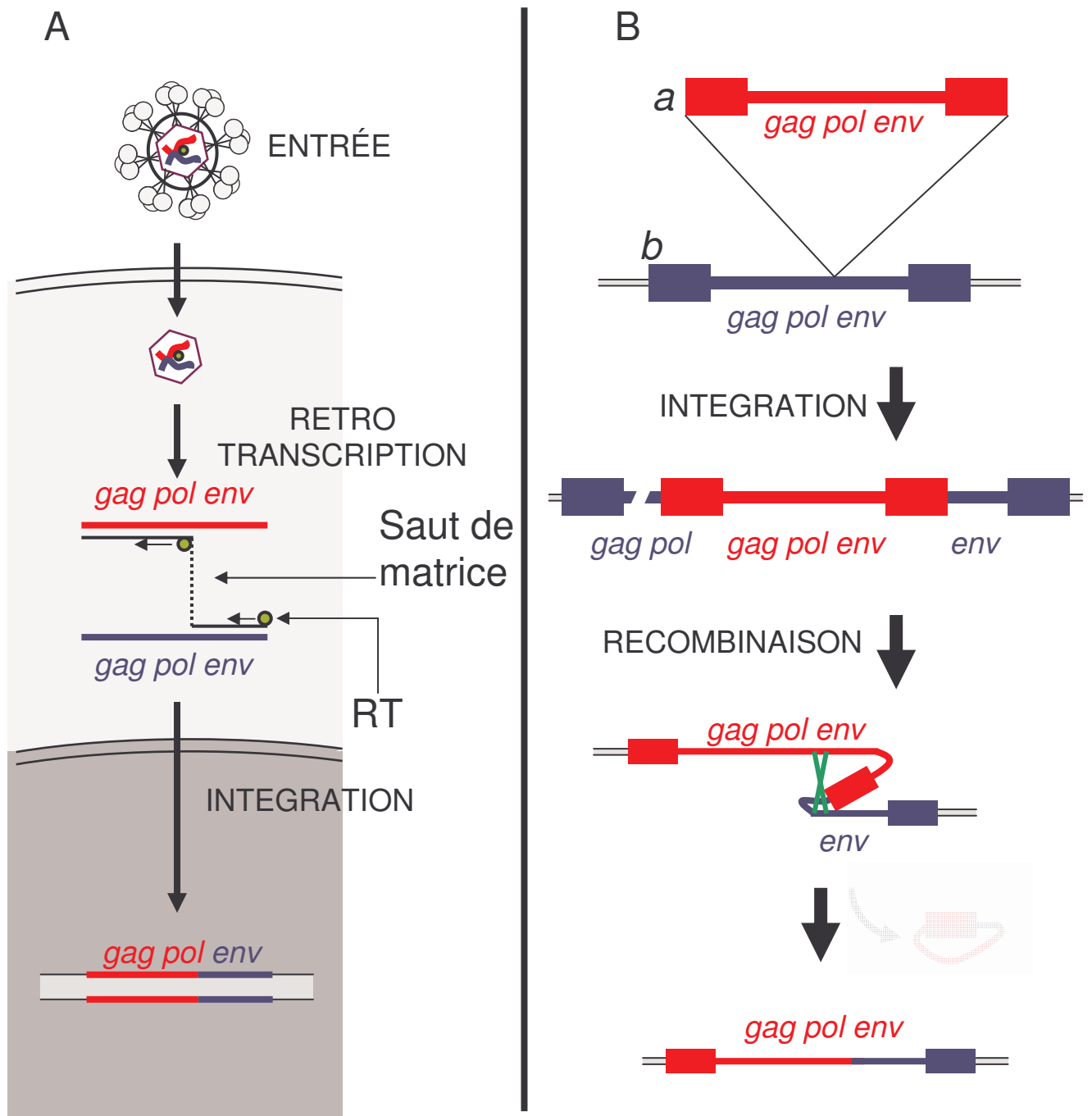
Plusieurs exemples d'émergence de rétrovirus infectieux d'invertébrés soutiennent l'hypothèse de la capture d'enveloppe. Gypsy, considéré comme un rétrotransposon de *Drosophila melanogaster*, possède un gène *env* et est un rétrovirus compétent pour la réplication (Song, Gerasimova et al. 1994; Chalvet, Teyssset et al. 1999). Des travaux fondateurs ont montré que le gène *env* de Gypsy est apparenté à un gène de baculovirus (famille *baculoviridae*), un virus d'insecte à ADN double-brin (Malik, Henikoff et al. 2000; Pearson and Rohrmann 2002). Le gène *env* du rétrovirus Cer de *Caenorhabditis elegans* quant à lui semble trouver son origine dans le gène codant pour la protéine de fusion d'un phlébovirus (famille *bunyaviridae*, virus à ARN simple brin négatif). Enfin, le gène *env* du

rétrovirus Tas de *Ascaris lumbricoides* semble avoir capturé le gène codant pour la glycoprotéine gB d'un virus herpétique ancestral (famille *herpesviridae*, virus à ADN double brin). Il est donc remarquable que des rétrovirus d'invertébrés phylogénétiquement très éloignés aient émergés par insertion d'un gène codant pour une Env héritée de virus de familles différentes infectant les mêmes hôtes.

Chez les rétrovirus mammifères, il n'a pas été identifié d'ancêtre non-rétroviral pour les gènes *env*. Cependant, des scénarios de capture de gène *env* entre rétrovirus ont bien été établis. Dans ce cas, ces événements sont généralement désignés comme recombinaisons. Ainsi le gène codant pour l'Env du rétrovirus endogène de souris RD114 suit le scénario de capture d'enveloppe. Dans un premier temps, il semble que le gène *env* du SERV (*simian endogenous retrovirus*) ait recombiné avec le PcEV (*papio cynocephalus endogenous virus*) pour conduire au BaEV (*baboon endogenous virus*). Dans un second temps, RD114 semble être issu d'une recombinaison entre le gène *env* du BaEV et le génome d'un FcEV (*felis catus endogenous virus*). L'émergence de RD114 suit une chaîne de prédation singe-singe, singe-chat, singe-souris. En plus des possibilités de maintien d'un virus transmis par zoonose chez une nouvelle espèce, cet exemple illustre bien les possibilités de recombinaison de séquences entre un rétrovirus introduit dans une nouvelle espèce. L'existence de séquences rétrovirales endogènes constitue en effet un substrat pouvant être recruté par des rétrovirus compétents pour la réplication conduisant à la génération de rétrovirus recombinants. Par exemple les rétrovirus de type MCF chez la souris ou FeLV-B (*feline leukemia virus*) chez le chat émergent par recombinaison de rétrovirus compétents, respectivement de type MLV écotropes ou FeLV-A avec des séquences endogènes de la souris (Fischinger, Nomura et al. 1975; Hartley, Wolford et al. 1977; Khan, Rowe et al. 1982) ou du chat (Elder and Mullins 1983; Overbaugh, Riedel et al. 1988). Les nouveaux rétrovirus recombinants émergents peuvent conduire à une pathologie distincte de la pathologie associée au virus initial (Hartley, Wolford et al. 1977). Des échanges sporadiques de matériel viral peuvent également être à la source de scénarios de capture d'enveloppe. Le JSRV (*Jaagsiekte retrovirus*), un rétrovirus

ovin et caprin responsable de l'adénocarcinome pulmonaire ovin, est un rétrovirus de type D dont le gène *env* est apparenté aux rétrovirus de type B suivant l'ancienne nomenclature (York, Vigne et al. 1992). Rétrovirus de type B et D sont désignés comme bêtarétrovirus suivant la nouvelle nomenclature. Ce genre comprend également les MMTV (*mouse mammary tumor virus*) dont l'Env et l'organisation génétique sont cependant très différentes des autres membres de cette famille. Cette apparente confusion illustre bien la dynamique évolutive de ces agents infectieux.

Le mécanisme conduisant à ces scénarios de capture d'enveloppe n'est pas connu. Comparé à une simple recombinaison, la capture d'enveloppe se distingue par la sélection systématique du gène *env* dans sa totalité. Cependant il n'est pas clair si cette observation correspond à une réalité mécanistique ou si elle n'est que le résultat de la sélection naturelle par élimination de recombinaisons non fonctionnelles. Le processus de capture d'enveloppe pourrait se dérouler dans plusieurs contextes. Tout d'abord, suite à une co-infection par deux rétrovirus, deux ARN viraux hétérologues peuvent se retrouver au sein d'une même particule virale. Bien que les modalités précises de co-encapsulation de deux ARN hétérologues, rétroviraux ou non, ne soit pas clairement établies, ce mécanisme est à l'origine des phénomènes de transduction d'oncogènes tels qu'ils ont été décrits pour plusieurs rétrovirus de vertébrés (Coffin, Hughes et al. 1997). Dans le cas d'HIV, il a été établi que la rétrotranscriptase effectue 2 à 3 "sauts" de matrice par cycle de réplication (Zhuang, Jetzt et al. 2002). L'émergence d'une copie cDNA recombinante au niveau de la séquence *env* est donc envisageable (Figure 3). De plus, il a été établi que certaines structures ARN sont des points privilégiés de recombinaison (Negroni and Buc 2001). Au contraire de *gag* et *pol*, les gènes *env* rétroviraux sont généralement bordés en 5' d'un site accepteur d'épissage, qui pourrait constituer un point privilégié ARN propice à la recombinaison. Il est également possible que la capture d'enveloppe intervienne après intégration dans le génome. Cette possibilité est soutenue par le cas du rétrovirus TED du lépidoptère *Trichoplusia ni*. Le gène *env* de TED est homologue au gène *F* de baculovirus, et de plus des formes intégrées de TED



**Figure 3.** Mécanismes hypothétiques de capture d'enveloppe.

(A) Après coencapsidation de deux ARN hétérologues, des sauts de matrice de la rétrotranscriptase permettent d'obtenir un rétrovirus recombinant, ayant "capturé" le gène *env* de l'autre rétrovirus. (B) Après intégration d'un rétrovirus "a" en amont du gène *env* d'un autre rétrovirus "b", un transcrit contenant à la fois le rétrovirus "a" suivi du gène *env* de "b" peut par recombinaison homologue donner un nouveau rétrovirus "a" ayant capturé l'*env* de "b". Dans les deux modèles, les séquences particulières en 5' du gène *env* (site accepteur d'épissage) contribueraient à leur sélection comme zone d'échange. La source de séquence d'enveloppe ("b") n'est pas nécessairement rétrovirale et peut mettre en jeu un pseudogène *env* encadré de LTR, ou un gène isolé apparenté à *env*. De plus le rétroélément "a" peut être dépourvu de gène *env*.

ont été trouvées dans le baculovirus ACNV. Dans ce contexte, il est possible d'envisager un mécanisme de capture d'enveloppe où un rétrovirus pourrait s'intégrer dans un autre rétrovirus, déjà intégré, et qui suite à excision par recombinaison homologue porterait un nouveau gène *env* (Figure 3). Encore une fois, les motifs accepteurs d'épissage pourraient constituer les substrats de la recombinaison. Ces deux mécanismes de capture d'enveloppe, l'un au cours de la rétrotranscription et l'autre après intégration, sont également envisageables à partir de différentes sources de séquences, par exemple le génome viral peut être issu d'un rétrovirus, d'un rétrotransposon à LTR, et le gène *env* peut être issu d'un autre rétrovirus, d'un pseudogène *env* encadrés de LTR ou d'un gène cellulaire isolé. Cependant, aucun gène *env* de rétrovirus de mammifère montrant une parenté avec un gène cellulaire, autre que les ERV humains, n'a été identifié.

Comme nous le verrons plus tard, certains arguments indiquent que le gène *env* du HTLV est dérivé par capture d'un gène *env* ancestral apparenté à celui des MLV (Tableau).

Les cas de mélange de gènes ont été décrits initialement dans le cadre de la génétique des immunoglobulines sous le terme de brassage d'exon (*exon shuffling*) (Maki, Traunecker et al. 1980). Par analogie, la capture d'enveloppe peut être considérée comme une forme de "brassage d'exon", bien qu'aucun argument ne permette à l'heure actuelle d'invoquer un mécanisme similaire au "brassage d'exons" des immunoglobulines.

**Tableau. Exemples de captures de gène *env* par des rétroéléments.**

Rétroélément	Espèce(s) hôte(s)	Origine du gène <i>env</i>	Origine du virus
<u>Invertébrés</u>			
Gypsy	<i>Drosophila melanogaster</i>	Baculovirus (dsDNA)	?
Cer	<i>Caenorhabditis elegans</i>	Phlebovirus (ssRNA-)	?
Ter	<i>Ascaris lumricoides</i>	gB d'un herpétovirus (dsDNA)	?
TED	<i>Trichoplusia ni</i>	F de baculovirus (dsDNA)	?
<u>Vertébrés</u>			
BaEV	Babouin	SERV	PcEV
RD114	Chat	BaEV	FcEV
MCF	Souris	<i>Env</i> polytrophe endogène murine	MLV écotrope
JSRV	Ovins, bovins, caprins	Rétrovirus de type B	Rétrovirus de type D
HTLV/STLV	Primates	type MLV ?	?

#### A.3.b. Une Env pour entrer ... et pour sortir ?

- "[...] la sélection naturelle ne planifie rien et n'invente rien. Elle ne peut qu'améliorer ce qui existe déjà. Les nouvelles fonctions dérivent de fonctions latentes et imprévisibles dont le potentiel sera révélé par les besoins de l'animal." Pierre SONIGO, *Ni Dieu Ni Gène*.

Dans le cadre de l'émergence des rétrovirus à partir de rétrotransposons, certains modèles proposent que c'est le gène *gag* qui acquiert les propriétés de migration vers la membrane cellulaire et de bourgeonnement de la particule (Lower, Lower et al. 1996). En effet, en conditions expérimentales de surexpression de Gag, des particules extracellulaires de type pseudo-virales sont relarguées dans le milieu extracellulaire. La fonction classiquement attribuée à l'Env est la liaison à un récepteur cellulaire à haute affinité qui permet l'entrée de la particule dans une cellule cible (Lower, Lower et al. 1996). Pour expliquer comment aurait été sélectionnée au niveau de l'Env la fonction d'entrée d'une particule virale, les modèles

proposent généralement un évènement rare de recrutement en *trans* d'une protéine transmembranaire possédant une activité de fusion, associé à la recombinaison d'une séquence codant pour une telle protéine dans le génome viral. Cependant, à ce modèle s'opposent des données récentes qui indiquent qu'en conditions de non-surexpression, situations naturelles des rétrovirus répliatifs, les protéines Gag virales en absence d'Env ne bourgeonnent pas à la surface cellulaire, mais sont dirigées vers des compartiments de dégradation (Basyuk, Galli et al. 2003). Par contre, l'addition de l'Env redirige le corps viral vers un compartiment sécrétoire (surface cellulaire ou corps multivésiculé) où le bourgeonnement est productif (Basyuk, Galli et al. 2003; Sandrin, Muriaux et al. 2004). Ainsi, la sélection des Env rétrovirales se serait produite sous l'effet combiné d'au moins deux propriétés principales : L'Env permettrait une sortie des particules virales et l'entrée de ces particules dans des cellules cibles.

## **B HTLV**

### **B.2. HTLV est-il un virus pathogène ?**

Deux pathologies sont clairement associées au HTLV de type 1. La première est une leucémie ou un lymphome rapide de l'adulte (*adult T-cell leukemia/lymphoma*, ATL). La seconde est une myélopathie (*tropical spastic paraparesis/HTLV-associated myelopathy*, TSP/HAM). Ces deux pathologies se déclarent généralement après une phase asymptomatique de plusieurs décennies. Toutefois, un développement extrêmement rapide de la TSP/HAM (de quelques semaines à quelques mois) a été rapporté suite à une transmission par transfusion sanguine, suggérant que le mode de transmission influence les conséquences pathogéniques du HTLV (Gout, Baulac et al. 1990; Kaplan, Litchfield et al. 1991; Gasmi, D'Incan et al. 1997). De plus, TSP/HAM et ATL ne concernent qu'un faible pourcentage de patients infectés, le risque cumulé de développer l'ATL ou la TSP/HAM étant de l'ordre de 5% (Edlich, Arnette et al. 2000). D'autres pathologies sont associées sporadiquement au

HTLV de type 1 : dermatite infectieuse (LaGrenade, Hanchard et al. 1990), uvéite (Mochizuki, Watanabe et al. 1992), myosite (Morgan, Rodgers-Johnson et al. 1989).

L'ATL est une leucémie agressive, qui lorsqu'elle se déclare est létale en moins d'une année (Siegel, Gartenhaus et al. 2001). L'ATL est également caractérisée par une hypercalcémie ainsi que des lésions de la peau associées à une infiltration lymphocytaire (Blayney, Jaffe et al. 1983). La réponse à la chimiothérapie n'est pas systématique et ne dure en général que quelques mois. De nouvelles stratégies de traitement anti-rétroviral de l'ATL montrent une certaine efficacité, en faisant notamment appel à une administration d'AZT ou de trioxide d'arsenic combinés à l'interféron  $\alpha$  ou à une immunothérapie à base d'anticorps monoclonal anti-récepteur à l'IL-2 (Bazarbachi and Hermine 2001).

La TSP/HAM est une paraparésie spastique, symétrique, avec ROT pyramidaux (réflexes ostéo tendineux) aux quatre membres et signe de Babinski uni ou bilatéral. Des troubles de la marche, une sensation de raideur, des douleurs des membres inférieurs, des troubles génito-sphinctériens (impériosité mictionnelle, pollakiurie) sont des signes pathologiques associés à la TSP/HAM. Les patients atteints de TSP/HAM présentent une démyélination sévère de la moëlle épinière associée à une réponse inflammatoire. Des anticorps anti-HTLV-I sont détectés dans le fluide cébrospinal des patients (Ceroni, Piccardo et al. 1988), et les cultures de cellules de ce fluide sont positives pour l'ADN HTLV (Bhagavati, Ehrlich et al. 1988; McKhann, Gibbs et al. 1989), ce qui indique que le virus est présent dans le système nerveux central.

A l'heure actuelle, aucune pathologie n'est associée à HTLV-2. Toutefois l'évolution rapide de ce virus dans certaines populations (voir Epidémiologie) appelle à la vigilance quant à l'émergence de pathologies associées.



### **B.3. Une pandémie ou de nombreuses endémies ?**

La prévalence mondiale du HTLV est estimée entre 15 à 20 millions d'individus infectés. Mais il est difficile d'obtenir une estimation précise de la distribution de HTLV dans le monde. Plusieurs raisons sont identifiables (i) les populations considérées varient d'une étude à l'autre (*e.g.* donneurs de sangs, population sélectionnée au hasard, utilisateurs de drogues, patients affectés de problème neurologiques) (Vrieling and Reesink 2004), (ii) les pays qui pratiquent la détection systématique de HTLV dans les produits sanguins sont minoritaires, (iii) les techniques de détection varient (ELISA, Immunoblots, PCR), (iv) les résultats des tests de détection ne sont pas toujours dichotomiques et une proportion substantielle de patients présente par exemple un profil sérologique indéterminé (Lal, Rudolph et al. 1992).

HTLV-1 est présent sous forme endémique dans la majorité des régions du globe : le Japon (notamment l'île de Kyushu), l'Afrique centrale, l'Océanie (Mélansiens de Papouasie Nouvelle Guinée (Imai, Terashi et al. 1990) et nord de l'Australie (Bastian, Gardner et al. 1993)), l'Amérique du Sud et les Caraïbes (Vrieling and Reesink 2004). HTLV-2 est présent sous forme endémique dans les populations natives d'Amérique du Nord et du Sud et d'Afrique tropicale (Vrieling and Reesink 2004), et sous forme épidémique soumise à évolution rapide (Salemi, Lewis et al. 1999; Vandamme, Bertazzoni et al. 2000) au sein des populations d'utilisateurs de drogues intraveineuses. Des foyers localisés d'infections HTLV-1 et HTLV-2 existent également de par le monde et sont liés aux migrations humaines (Slattery, Franchini et al. 1999). Récemment, HTLV-3 et HTLV-4 ont été rapportés chez des habitants d'Afrique centrale (Calattini, Chevalier et al. 2005; Wolfe, Heneine et al. 2005).

HTLV-2 a été introduit, il y a quelques décennies seulement, dans les populations d'utilisateurs de drogues intraveineuses à partir des populations amérindiennes infectées de façon endémique. Dans cette nouvelle population, HTLV-2 évolue 150 à 350 fois plus rapidement que dans les zones endémiques (Salemi, Lewis et al. 1999; Vandamme,

Bertazzoni et al. 2000). Cette accélération de l'évolution de HTLV-2 pourrait être associée au mode de transmission. En effet dans les zones endémiques pour HTLV-1 et HTLV-2, le virus se transmet principalement de la mère et l'enfant au cours de l'allaitement et par contact sexuel, alors que chez les utilisateurs de drogues intraveineuses la dissémination du virus est due à la transmission via des produits sanguins suite à échange de seringues, et ces modes de transmissions mettent en jeu des paramètres différents de multiplicité d'infection (*i.e.* nombre d'unités infectieuses par cellule), de cellules initialement infectées et de réponses immunes (mucosales *vs* réticulo-endothéliales).

#### **B.4. HTLV est un virus ancien**

Les mesures phylogénétiques indiquent que les premiers HTLV ont émergé il y a plus de 60 000 ans (Salemi, Desmyter et al. 2000). Des homologues simiens existent pour HTLV-1 et HTLV-2, respectivement STLV-1 et STLV-2, qu'on retrouve dans diverses espèces de singes africains et asiatiques. L'origine des STLV est encore plus ancienne, et semble remonter à près d'un million d'années (Salemi, Desmyter et al. 2000), indiquant que les STLV ont accompagné l'émergence d'*Homo Sapiens*. Ces datations doivent cependant être considérées avec précaution car bien que HTLV varie peu, son caractère viral rend difficile l'établissement d'une horloge moléculaire précise à cette échelle de temps. Il semble que l'origine de HTLV-1 soit associée à d'anciennes zoonoses entre singe et humain, chacune associée à l'émergence d'un des 4 sous-types de HTLV-1 connus (Vandamme, Salemi et al. 1998). HTLV-2 au contraire semble être issu d'un seul évènement de zoonose, les différents sous-types de HTLV-2 ayant émergé par séparation géographique de population infectées (Salemi, Vandamme et al. 1999). Il existe également un PTLV-L/STLV-3 chez certaines espèces de singe (Goubau, Van Brussel et al. 1994). L'identification d'un homologue humain, HTLV-3 (Calattini, Chevalier et al. 2005; Wolfe, Heneine et al. 2005), ainsi que d'un nouveau HTLV-4 vient d'être rapportée (Wolfe, Heneine et al. 2005).

La faible proportion de pathologies associées à HTLV, ainsi que leur occurrence tardive, associée à la faible évolution de HTLV, suggère que les pathologies dues au HTLV sont des "effets secondaires" apparus dans le contexte d'un allongement de l'espérance de vie humaine. Ainsi, la participation de ces pathologies à l'évolution contemporaine du HTLV est probablement marginale. Il est toutefois possible que les zoonoses initiales associées à l'émergence de HTLV aient été épidémiques à la manière du HIV et qu'elles aient été associées à des pathologies explosives et létales. Dans le contexte d'une coévolution hôte/virus, la sélection de HTLV marginalement pathogène a pu être associée à la co-sélection de certains caractères génétiques humains. Il est intrigant de constater que les premières migrations humaines hors du continent africain sont datées autour de 60 000 ans (Maca-Meyer, Gonzalez et al. 2001), correspondant à la datation actuelle du HTLV.

L'émergence du HTLV est-elle associée aux migrations humaines ? Un exemple intéressant est celui des différents HTLV présents dans les populations Amérindiennes. Par analyse phylogénétique, il a pu être montré que les souches de HTLV-2 qui y sont présentes sont issues d'au moins une migration via l'actuel détroit de Bering, il y a 15 000 à 30 000 ans, de populations asiatiques infectées (Vandamme, Bertazzoni et al. 2000), et ce en période interglaciaire lorsque ces régions bénéficiaient de climats moins rigoureux (Cavalli-Sforza, Menozzi et al. 1994). Par contre, les souches de HTLV-1 amérindiennes, apparentées à la souche 1a (ou transcontinentale) trouvée notamment en Afrique, suggèrent une introduction récente qui coïncide avec les migrations de populations africaines suite à la traite d'esclave (Van Dooren, Gotuzzo et al. 1998; Talarmin, Vion et al. 1999).

La dérivation de certaines sous-populations humaines pourrait-elle être décrite dans un cadre de co-évolution avec un HTLV ancestral particulièrement pathogène ? À ce titre, la population dite Caucasienne est la seule dans laquelle aucun HTLV n'a été identifié de manière endémique, à l'exception d'une population distincte de la région de Mashad, au nord de l'Iran (Meytes, Schochat et al. 1990).

Alors que HTLV est un virus ancien, HIV, "l'autre" rétrovirus humain pathogène, a été introduit beaucoup plus récemment dans les populations humaines à partir de souches simiennes SIV (Korber, Muldoon et al. 2000). En fait, les souches de HIV qui constituent l'épidémie mondiale sont issues de zoonoses récentes, mais il est parfaitement vraisemblable que des passages de SIV entre le singe et l'homme se déroulent depuis longtemps dans des zones endémiques d'Afrique. Le caractère fortement pathogène du HIV est généralement associé à la "jeunesse" de ce virus. Ainsi, il est possible qu'au cours du temps, l'apparition stochastique de mutants HIV et la sélection de mutants à pathogénicité diminuée, permettant une plus grande dissémination, pourrait conduire à un scénario similaire à celui que l'on observe actuellement pour HTLV. Le taux d'évolution des PTLV est estimé aux alentours de  $10^{-6}$  nucléotides par site par an, au niveau du troisième codon (Salemi, Desmyter et al. 2000), alors qu'il est estimé à environ  $10^{-3}$  nucléotides par site par an pour HIV-1 (Leitner and Albert 1999). Pour comparaison, le taux de mutation des génomes de mammifères est estimé à environ  $10^{-9}$  par nucléotide par an (Kumar and Subramanian 2002). Ainsi, la mutation individuelle de chaque nucléotide du génome des PTLV s'étend sur 100 ans, contre un peu plus d'un mois pour HIV. HTLV apparaît donc un virus relativement invariant, alors qu'HIV varie constamment à l'échelle des générations humaines. Au-delà d'une pression de sélection sur l'effet pathogène, les différences de taux d'évolution entre HIV et HTLV sont également associées à la pression de sélection par le système immunitaire, et au mode et à la fréquence de réplication au sein de l'hôte. Concernant ce dernier point, la réplication résultante de HTLV paraît se dérouler principalement sous forme provirale, par division cellulaire (Wattel, Vartanian et al. 1995). La rétrotranscription reste cependant importante pour la réplication, comme l'indique la baisse de la charge provirale suite à un traitement à l'AZT (Taylor, Hall et al. 1999). HIV, qui peut provoquer l'apoptose des cellules qu'il infecte avant leur division (Goh, Rogel et al. 1998), se réplique principalement sous forme virale via la rétrotranscriptase (Perelson, Neumann et al. 1996). La différence de fidélité entre la rétrotranscriptase et la

DNA polymérase cellulaire va donc contribuer aux différences de taux de mutation entre ces deux virus (Overbaugh and Bangham 2001).

## **B.5. Tropisme**

Les premières études décrivant la dissémination et l'infection HTLV *in vitro* ont été publiées peu de temps après l'identification du HTLV (Yamamoto, Okada et al. 1982; Clapham, Nagy et al. 1983; Hayami, Tsujimoto et al. 1984). Ainsi, il a été montré que des isolats primaires de HTLV-1 pouvaient se transmettre à des cellules gliales et monocytaires (Hoffman, Dhib-Jalbut et al. 1992), des cellules de l'épithélium basal mammaire (LeVasseur, Southern et al. 1998) ainsi que des cultures primaires de cellules endothéliales humaines (Ho, Rota et al. 1984; Hoxie, Matthews et al. 1984).

*In vivo*, HTLV-1 est aisément détecté dans les lymphocytes T CD4 à la fois chez les porteurs sains et malades (Grant, Barmak et al. 2002), indiquant clairement que le compartiment cellulaire CD4 constitue un composant privilégié du tropisme *in vivo* de HTLV-1 (Richardson, Edwards et al. 1990). Cette observation, associée à la prolifération cellulaire induite par HTLV-1 dans le compartiment cellulaire T CD4 au cours de la leucémie, a conduit à diminuer, sinon évincer, le tropisme HTLV-1 dans de nombreux autres types cellulaires *in vitro*. Ainsi, plusieurs cas d'infection de lymphocytes B par HTLV-1 *in vivo* ont été rapportés (Yamamoto, Matsumoto et al. 1982; Longo, Gelmann et al. 1984; Franchini, Mann et al. 1985). Toutefois, ces résultats obtenus par caractérisation de cellules transformées en culture, ne permettent pas d'exclure une contamination par des lymphocytes T. Plus récemment, des expériences de détection de séquences HTLV par PCR et RT-PCR après fractionnement cellulaire et sans culture cellulaire ont été réalisés (Koyanagi, Itoyama et al. 1993). Chez des porteurs de HTLV-1 asymptomatiques, ou atteints de leucémie ou de myélopathie, l'ADN proviral HTLV-1 et sa transcription active ont pu être fortement détectés dans les lymphocytes T CD4, mais également dans les lymphocytes T CD8, les lymphocytes

B et les monocytes (Koyanagi, Itoyama et al. 1993). L'infection des cellules T CD8 a été par la suite largement confirmée (Eiraku, Hingorani et al. 1998; Hanon, Stinchcombe et al. 2000; Nagai, Brennan et al. 2001; Grant, Barmak et al. 2002). La recherche de séquences HTLV par hybridation *in situ* dans des cerveaux post-mortem de patients atteints de myélopathie a démontré que les astrocytes peuvent aussi être infectés (Lehky, Fox et al. 1995). L'infection *in vivo* de cellules adhérentes sans mise en culture a été ponctuellement décrite, notamment dans l'épithélium de la glande salivaire (Setoyama, Kerdel et al. 1998; Setoyama, Mizoguchi et al. 1999; Tangy, Ossondo et al. 1999), les cellules endothéliales vasculaires (Setoyama, Kerdel et al. 1998) et les glandes mammaires (Loureiro, Southern et al. 2000).

En conclusion, bien que le compartiment CD4 semble être le réservoir le plus important, d'autres cellules hématopoïétiques, gliales et autres contribuent également à la définition du tropisme de HTLV (voir "*HTLV-1 tropism and envelope receptor*" en annexe). Ces observations permettent de relativiser le paradoxe apparent et classiquement mis en avant entre un tropisme large *ex vivo* et restreint *in vivo*.

## **B.6. La réponse immunitaire au HTLV**

La réponse des lymphocytes T CD4 est difficile à mettre en évidence. En effet après mise en culture *in vitro*, la présence de HTLV dans les lymphocytes T CD4, qui sont la cible principale de l'infection, induit la prolifération et l'expression de nombreux gènes cibles dont l'IFN- $\gamma$ . Toutefois, une approche récente basée sur une mesure effectuée quelques heures après la mise en culture suggère que la réponse helper de type Th1 (réponse cellulaire, activation des macrophages), dirigée surtout contre Tax et Env, semble prédominer sur la réponse Th2 (réponse humorale, production d'anticorps neutralisants) (Goon, Hanon et al. 2002).

La réponse cytotoxique des lymphocytes CD8 est principalement dirigée contre Tax (Jacobson, Shida et al. 1990; Kannagi, Harada et al. 1991), mais il existe également chez les

patients des lymphocytes CD8 spécifiques pour des épitopes de Gag, Pol, Env et certaines protéines accessoires (Jacobson, Shida et al. 1990; Parker, Daenke et al. 1992; Pique, Ureta-Vidal et al. 2000). Cette réponse CD8 est abondante puisque la proportion de lymphocytes CD8 qui reconnaissent un épitope de HTLV excède 1% (Bangham 2000). Notamment, l'étude de la variation du gène  $V_{\beta}$  du TCR chez les patients infectés a démontré une expansion clonale étendue des lymphocytes T CD8 (Furukawa, Mori et al. 1994; Hoger, Jacobson et al. 1997).

Les anticorps détectés dans les premiers mois après une infection par HTLV sont dirigés contre Gag, puis les anticorps anti-Env et anti-Tax constituent la majorité (Manns, Murphy et al. 1991).

L'ensemble de ces paramètres ont amené les auteurs à décrire la réponse immunitaire anti-HTLV comme forte et chronique (Bangham 2003). Comment l'infection HTLV se maintient-elle ? Bien qu'une expression continue de HTLV, notamment de Tax, semble nécessaire pour maintenir cette forte réponse, l'absence d'expression de HTLV dans la majorité des cellules infectées semble permettre d'éviter leur éradication (Bangham 2003).

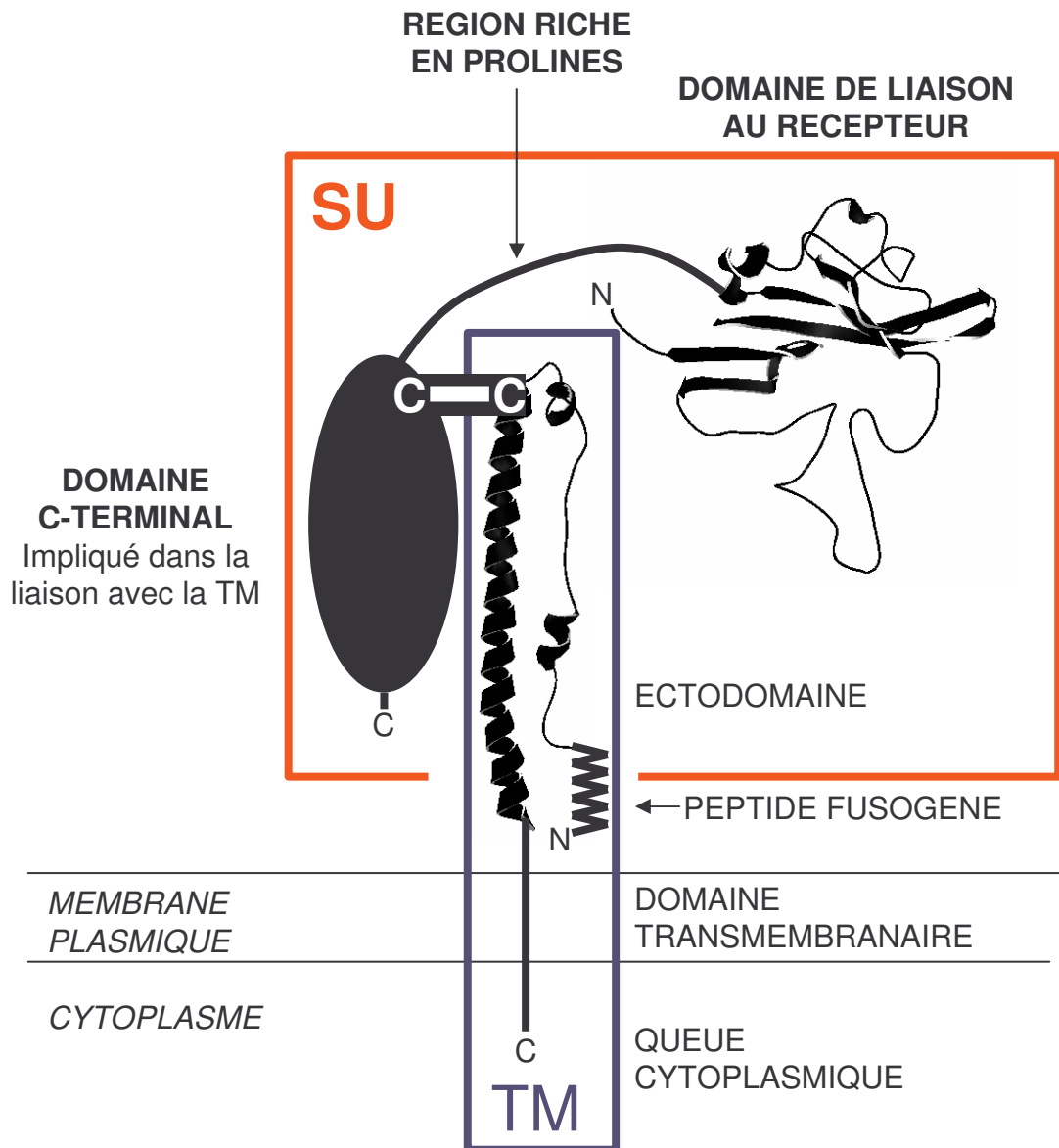
## **C. Env et Récepteur du HTLV**

### **C.1. Env**

#### C.1.a. Généralités

Comme toutes les Env rétrovirales, L'Env du HTLV est synthétisée sous forme d'un précurseur transmembranaire de type I. Le peptide signal de l'Env HTLV-1 contient 20 acides aminés (Gray, White et al. 1990). Le précurseur est clivé dans l'appareil de Golgi sous l'action d'une furine en une sous-unité de surface gp46 (SU) et une sous-unité transmembrane gp21 (TM) (Figure 4).

Le gène *env* qui encode l'Env se superpose à d'autres gènes viraux. Les deux derniers codons de *pol* chevauchent le début d'*env*. De plus, alors que le premier exon de *rex* débute 56 nucléotides avant le début d'*env*, le premier exon de *tax* coïncide avec *env*. Ces deux exons



**Figure 4.** Topologie de la glycoprotéine d'enveloppe du HTLV.

La sous-unité trans-membranaire gp21 (TM) permet l'ancrage de la glycoprotéine dans la membrane plasmique, et possède à son extrémité N-terminale un peptide fusogène qui pénètre dans la membrane de la cellule cible et induit la formation de pores de fusion des membranes virales et cellulaires. La sous-unité de surface gp46 (SU) est composée de 3 modules (i) un domaine N-terminal de liaison au récepteur cellulaire, (ii) une région centrale riche en prolines, probablement impliquée dans les changements de conformation de l'Env et (iii) un domaine C-terminal qui interagit avec l'ectodomaine de la TM par un pont disulfure (C-C) conservé parmi les Env rétrovirales. La représentation en ruban du domaine de liaison au récepteur a été obtenue par modélisation moléculaire (voir paragraphe Résultats) et celle de l'ectodomaine de la TM a été dérivée de la structure 1MG1 de la PDB (qui représente cependant à un état post-fusion) (Kobe et al., 1999)

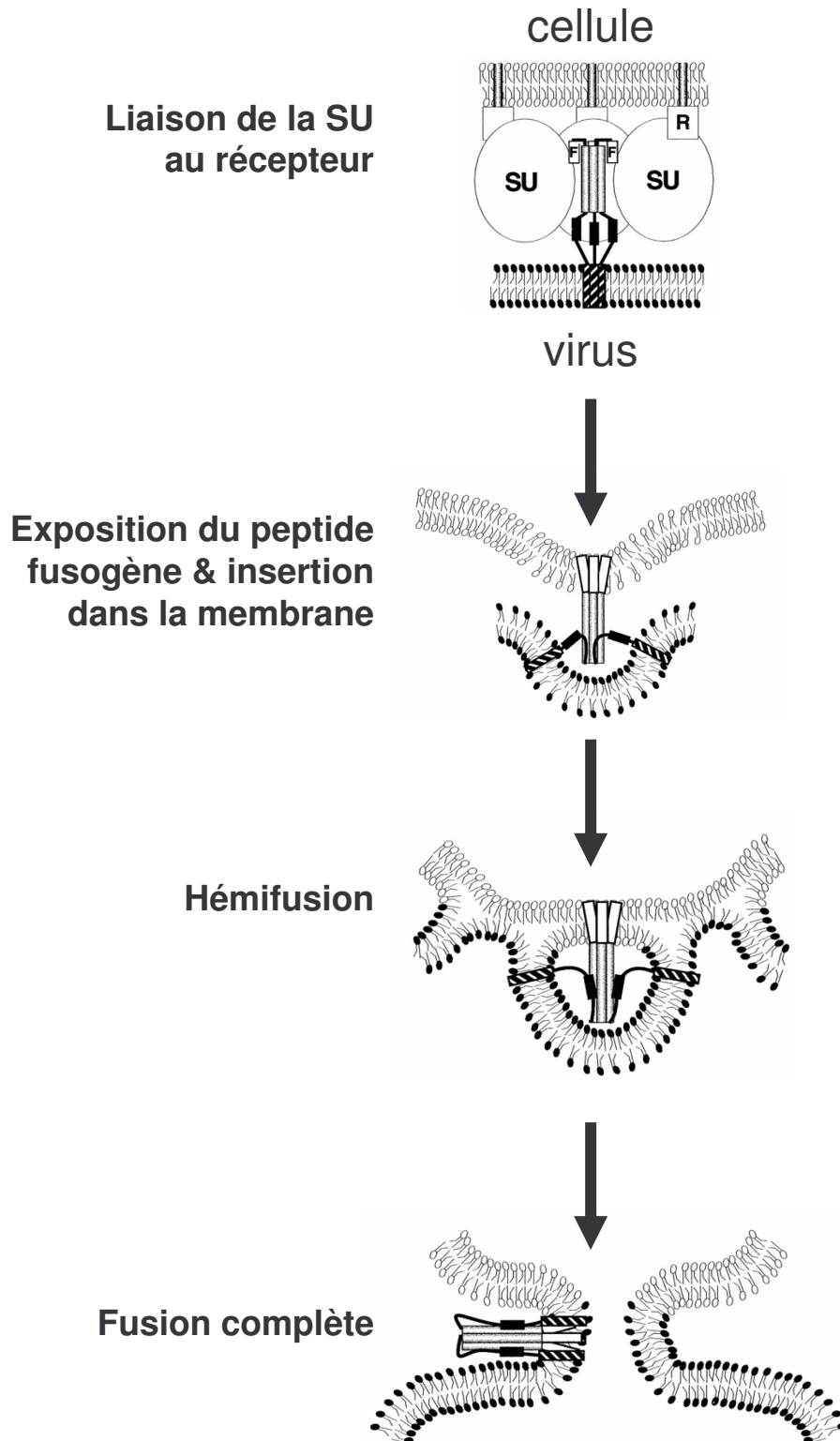


sont épissés, après le 4<sup>ème</sup> nucléotide du cadre de lecture d'*env* et *tax*, avec un exon situé dans la région pX, après *env*, qui encode le reste des cadres de lecture de *tax* et *rex*. Pour des raisons encore obscures, nous avons observé que l'expression hétérologue de l'Env HTLV (1 ou 2), ou de sa seule SU, dans un contexte classique (*e.g.* plasmide à promoteur CMV) n'est pas possible à partir du seul cadre de lecture. La région correspondant au premier exon de *rex* et la région pX sont ainsi nécessaires. Il serait intéressant d'évaluer si d'éventuels sites cryptiques d'épissage dans *env*, démasqués en l'absence du premier exon de *rex* et de la région pX, pourraient expliquer cette observation.

### C.1.b. TM

La TM est responsable de la fusion des membranes virales et cellulaires. Un peptide fusogène, situé à l'extrémité N-terminale de la TM est responsable de cette activité. Il est suivi d'un ectodomaine, d'un domaine transmembranaire et d'une queue cytoplasmique, qui semble jouer un rôle dans la capacité fusogène de l'Env (voir "*Human T-cell leukemia virus type 1 envelope-mediated syncytium formation can be activated in resistant Mammalian cell lines by a carboxy-terminal truncation of the envelope cytoplasmic domain*" en annexe).

Dans l'Env HTLV mature, la TM semble se trouver dans une conformation linéaire qui masque le peptide fusogène (Figure 5). Après reconnaissance du récepteur par la SU, le peptide fusogène, exposé, pénètre dans la membrane plasmique de la cellule cible. Le peptide fusogène déstabilise la bicouche lipidique, et cette instabilité est tout d'abord résolue par le mélange de la première couche de lipide de la membrane de la cellule avec celle de la membrane virale, constituant l'intermédiaire d'hémifusion. Enfin, les deuxièmes couches de lipides se mélangent également, complétant le processus de fusion en formant un pore permettant de libérer le contenu du virus. Cette activité de fusion est également observable lorsque l'Env HTLV est exprimée à la surface cellulaire. La fusion cellule-cellule qui a alors lieu conduit à la formation d'une cellule multinucléée (syncytium) (Nagy, Clapham et al. 1983).



**Figure 5.** Modèle de fusion membranaires induite par la TM.

L'Env est représentée sous forme trimérique. F : peptide fusogène. R : récepteur cellulaire. Adapté de Kobe et al., 1999.

L'ectodomaine de la TM de HTLV a pu être cristallisé et se présente sous la forme d'un trimère d'hélices enroulées (*coiled coil*) en N-terminal, conservé chez les rétrovirus, suivi d'un domaine C-terminal replié de manière anti-parallèle sur le domaine *coiled coil* (Kobe, Center et al. 1999). Cette conformation semble être celle de la TM après fusion des membranes.

La TM de HTLV, comme la majorité des TM rétrovirales (à l'exception de HIV, MMTV et HERV-K), possède également un motif homologue au domaine immunosuppresseur CKS17 de la TM de Moloney-MLV (Cianciolo, Copeland et al. 1985). Un peptide de l'Env HTLV correspondant au motif CKS17 présente une activité immunosuppressive *in vitro* (Ruegg, Monell et al. 1989). Il serait intéressant d'évaluer si dans le contexte de l'Env entière ce motif présente également une activité immunosuppressive *in vivo*, comme c'est le cas pour l'Env de MPMV (Blaise, Mangeney et al. 2001), toutefois ce type d'étude est limitée dans le cas de HTLV par l'impossibilité, liée à l'ubiquité de l'effet syncytial et au blocage du transport de glucose par cette Env (voir paragraphe Résultats), d'établir de lignées de mammifère exprimant stablement l'Env HTLV.

### C.1.c. SU

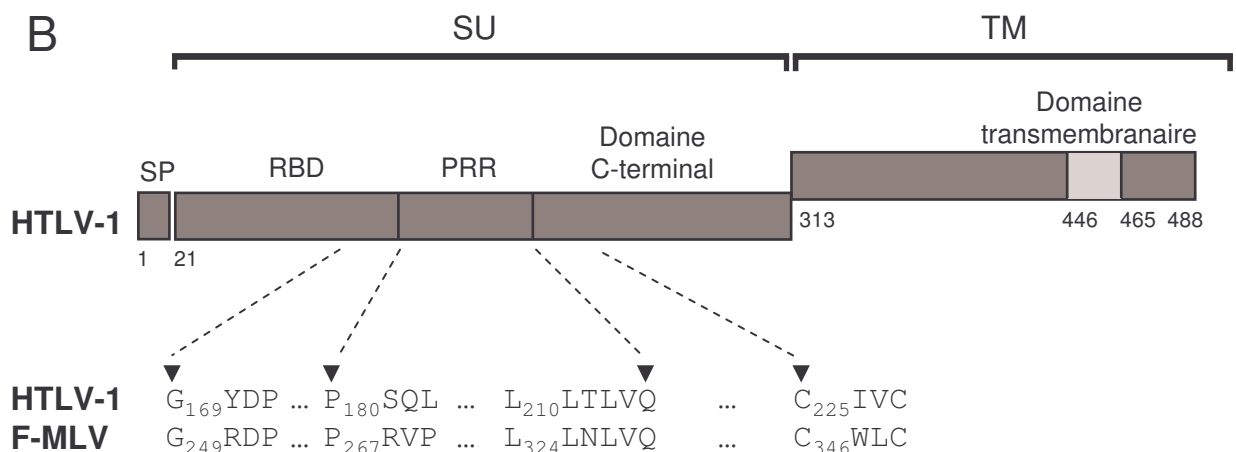
La SU est responsable de la reconnaissance du récepteur à la surface des cellules. La SU de HTLV possède plusieurs motifs peptidiques qui la rapprochent de celle des MLV (Figure 6). Le motif G<sub>169</sub>YDP de HTLV-I est homologue au motif G<sub>249</sub>RDP de F-MLV. Chez MLV, ce motif délimite la fin du domaine de liaison au récepteur (RBD, *receptor binding domain*) (Heard and Danos 1991; Battini, Danos et al. 1995) et le début d'une région riche en prolines, charnière entre le RBD et le reste de l'Env (P<sub>180</sub> chez HTLV, P<sub>267</sub> chez MLV) (Koch, Hunsmann et al. 1983). Cette région se termine par le motif L<sub>209</sub>LTLVQ chez HTLV-I et L<sub>323</sub>LNLVQ chez MLV, délimitant le début de la région C-terminale, qui est impliquée dans la liaison entre la SU et le TM (Pinter and Honnen 1983). A ce titre, le motif C<sub>225</sub>IVC de HTLV-1 est homologue au motif C<sub>346</sub>WLC, conservé chez les oncorétrovirus (Sitbon,

A

	peptide signal				
Fr-MLV	<u>MACSTLPKSP</u>	<u>KDKIDPRDLL</u>	<u>IPLILFSLK</u>	<u>GARSAAPGSS</u>	PHQVYNIWE 50
HTLV-1	MG-----K--	-----FL	ATLILFFQF-	--CPLILGDY	SPSCCTLTVG 32
Consensus	M.....K..	.....L	..LILF....	.....G..	.....T..
Fr-MLV	VTNGDRETVW	AISGNHPLWT	WWPVLTPDLC	<u>MLALSGPPHW</u>	GLEYQAPYSS 100
HTLV-1	VSSYHSK---	PCNPAQPVCS	W--TL--DL-	- <u>LALS</u> -----	ADQALQ-- 64
Consensus	V.....	.....P...	W...L..DL.	. <u>LALS</u> .....	....QA....
Fr-MLV	PPGPPCCSGS	SGSSAGCSR	CDEPLTSLTP	RCNTAUNRLK	LDQVTHKSSE 150
HTLV-1	---PPCPNLV	SYSSYHATY-	-----SLYL	FPH--WIK-K	----PNRNGG 97
Consensus	...PPC....	S.SS.....	.....SL..	.....W...K	.....
Fr-MLV	GFYVCPGSHR	PREAKSCGGP	DSFYCASWGC	ETTGRV---Y	WKPSSSWDYI 197
HTLV-1	GYI-SASYS	PCSLK-C--P	-YLGCSWTC	PYTGAUVSSPY	<u>WK</u> ----- 134
Consensus	G.Y.....	P...K.C..P	....C.SW.C	..TG.V...Y	<u>WK</u> .....
Fr-MLV	TVDNNLTTSQ	AVQVCKDNKW	CNPLAIQFTN	AGKQVTSWTT	GHYWGLR--L 245
HTLV-1	-----	-----	-FQODVNF	TQ EVSHLNINLH	FSKCGFSFSL 163
Consensus	.....	.....	.....FT.	.....	....G....L
Fr-MLV	YVS--GRDPG	LTFGIRLRYQ	NLGPRVPIGP	NPVLADOLSL	PRPNLPKPA 293
HTLV-1	LVDAPGYDP-	IWF---L---	NTEP-SQLPP	--TAPLLSH	----- 193
Consensus	.V...G.DP.	..F...L...	N..P.....P	.....LS.	..... 300
Fr-MLV	KSPPASNSTP	TLISPSPTPT	OPPPAGTGDR	<u>LLNLVQGAYQ</u>	<u>ALNLTMPDKT</u> 343
HTLV-1	-----SNLDH	-ILEPSI-PW	KS-----K	<u>LLTLVQLTLQ</u>	<u>STNYT</u> ----- 224
Consensus	.....SN...	....PS..P.	.....	.. <u>LL.LVQ</u> ..	.Q... <u>N.T</u> ....
Fr-MLV	<u>QECWLC</u> LVSG	PPYYEGVAVL	GTYSNHTSAP	ANCSVASQHK	LTLSEVTGRG 393
HTLV-1	-- <u>CIVCIDRA</u>	S-LSTWH-VL	--YSPNVSV	SPSSTP---L	LYPSLA---- 261
Consensus	.. <u>C..C</u> ....	.....VL	..YS...S.P	...S... ..L.....	.....
Fr-MLV	LCIGTVPKTH	QALCNTTLKI	DKGSYYLVAP	TGTTWACNTG	LTPCLSATVL 443
HTLV-1	-----LPAPH	LTL-----	-----P	F--NW-----	-THCFDPQIQ 282
Consensus	.....P..H	..L.....	.....	...W.. ..T.C.....	.....
Fr-MLV	NRTTDYCVLV	ELWPRVITYHP	PSYVYSQFEK	<u>SYRHKR</u>	480
HTLV-1	AIVSSPCHNS	LILPPFSLSP	VPTL-----	<u>GSRSR</u>	312
Consensus	.....C...	...P.....P	.....	.. <u>R..R</u>	

clivage SU/TM

B



**Figure 6.** (A) Alignement entre les SU de Friend MLV et HTLV-1. Le peptide signal et le site de clivage SU/TM délimitent la SU. Les motifs conservés sont soulignés. (B) Organisation de l'Env HTLV-1, et motifs conservés entre Friend-MLV et HTLV-1. SP : Peptide signal. RBD : Domaine de liaison au récepteur. PRR : Région riche en prolines.

d'Auriol et al. 1991) et impliqué dans la liaison SU-TM via un pont disulfure (Pinter, Kopelman et al. 1997). L'identification de ces motifs a permis de mettre en évidence l'existence d'une organisation modulaire de l'Env HTLV au même titre que celle des MLV. En effet, l'échange du RBD de l'Env MLV par celui de HTLV confère à cette nouvelle Env chimérique une activité de fusion étendue aux cellules humaines, caractéristique de l'Env HTLV (Kim, Seiliez et al. 2000). Exprimée à la surface de virions MLV, elle ne conduit pas à un titre infectieux significatif lors d'infection avec des surnageants viraux acellulaires, mais elle est parfaitement fonctionnelle par transmission de cellule à cellule (Felix Kim et Marc Sitbon, observations non publiées). Par ailleurs, l'expression du seul RBD de l'Env HTLV sous forme tronquée et soluble, et fusionnée au fragment constant des immunoglobulines de lapin, inhibe aussi bien la fusion induite par la coculture avec des cellules exprimant l'Env HTLV que l'infection par des virions MLV pseudotypés par l'Env HTLV (Kim, Manel et al. 2004). Toutefois, le motif P<sub>41</sub>HQ retrouvé chez MLV, mais également chez tous les gammarétrovirus, est absent de l'Env HTLV. Ce motif est impliqué dans une interaction avec la partie C-terminale de la SU qui peut intervenir à deux niveaux : en *cis*, au cours du processus classique d'entrée au sein d'une même molécule d'Env, mais également en *trans* entre un RBD soluble et l'Env rétrovirale correspondante mutée dans le motif PHQ (Lavillette, Boson et al. 2001). Alors qu'une mutation dans le motif PHQ rend l'Env défective, l'ajout de RBD restaure l'infection. La stringence de cette reconnaissance est relativement souple, ainsi divers domaines de liaisons au récepteur de gammarétrovirus sont capable d'interagir avec des Env mutées (Lavillette, Ruggieri et al. 2000). La capacité des domaines de liaison au récepteur HTLV solubles à restaurer des Env MLV mutées dans le motif PHQ reste à déterminer. Toutefois l'absence du motif PHQ dans l'Env HTLV permet de soulever l'hypothèse de l'existence d'un autre facteur, cellulaire ou viral, capable de fournir un motif PHQ. Alternativement, les cystéines C<sub>26</sub> et C<sub>27</sub> de la SU HTLV pourraient interagir avec le domaine C-terminal via un pont disulfure, à la place de l'interaction non covalence d'un motif PHQ.

### C.1.d. RBD

Le RBD du HTLV est contenu dans les 180 premiers acides aminés de l'Env : il a été montré qu'un domaine tronqué au résidu E<sub>179</sub> de l'Env HTLV-1 et au résidu Q<sub>178</sub> de l'Env HTLV-2 possède les propriétés de liaison au récepteur HTLV (Kim, Seiliez et al. 2000). Dans l'Env des MLV, ce domaine est divisé en régions variables VRA, VRB et VRC. VRA et VRB ont été initialement identifiées comme deux segments de séquences variables entre les différents types d'Env MLV (Battini, Heard et al. 1992). Les déterminants stricts de liaison au récepteur sont localisés dans VRA. VRB est responsable de la stabilisation des déterminants de VRA. VRC est un sous-domaine en C-terminal de VRA situé à l'opposé de VRA et VRB dans la structure tridimensionnelle du RBD de MLV (Fass, Davey et al. 1997). Nous verrons dans la partie Résultats les structures retrouvées, ou non, dans un modèle de structure du RBD de HTLV.

### C.1.e. Dynamique d'entrée

La fusion des membranes virales et cellulaires, suite à la pénétration du peptide fusogène, conduit à la formation de pores de fusions. Mais le nombre et la distribution des pores qui se sont formés ne sont pas connus, deux mécanismes sont donc possibles. Soit les complexes Env/récepteurs s'organisent avant fusion en anneau autour du futur pore fusion, soit plusieurs petits pores sont initialement formés, nécessitant par la suite une étape de fusion des pores pour aboutir à un pore suffisamment large pour permettre la libération du corps viral. Dans les deux cas une mobilité des complexes Env/récepteur ou SU/récepteur apparaît nécessaire pour libérer l'espace permettant la formation d'un pore large, mais aucun mécanisme de ce type n'a pour l'instant été démontré. Une coopérativité positive a été mise en évidence entre les molécules de récepteur pour la liaison des RBD du MLV amphotrope (Battini, Rodrigues et al. 1996), mais une éventuelle association entre cette coopérativité de liaison et la mobilité des récepteurs n'est pas connue.

Après entrée du virus, le devenir des TM et des complexes SU/récepteur n'est pas connu, et une éventuelle colocalisation de ces complexes avec la capsid entrante au niveau de vésicules d'endocytose n'est pas établie.

## **C.2. Attachement, liaison et récepteur cellulaire**

L'identité du récepteur HTLV n'ayant pas été découverte pendant plus de vingt ans, l'existence même d'un récepteur était mise en doute. En revanche, l'influence des microdomaines lipidiques et des protéines impliquées dans l'adhésion cellulaire sur la capacité fusogène de l'Env HTLV a été largement rapportée. Les intégrines LFA-1 et VLA-4, CD44, la E-sélectine et la P-sélectine jouent un rôle dans l'adhésion entre des cellules infectées par HTLV (cellules d'ATL ou lignées établies) et des cellules endothéliales de cordon ombilical (HUVEC) (Uchiyama, Ishikawa et al. 1996). Par ailleurs, il a été montré que les molécules d'adhésion ICAM-1, ICAM-3 et VCAM-1 modulent la formation de syncytia alors que des anticorps dirigés contre les intégrines  $\beta 2$  et  $\beta 7$  inhibent la formation de syncytia (Hildreth, Subramaniam et al. 1997; Daenke, McCracken et al. 1999). La participation de molécules non-protéiques au récepteur HTLV a également été évaluée.

Le palmitoyl(16:0)-oleoyl(18:1)-phosphatidylglycerol et les radeaux lipidiques participent ainsi à la formation de syncytia (Niyogi and Hildreth 2001; Sagara, Inoue et al. 2001). Il a enfin été récemment démontré que les protéoglycans d'héparan sulfate (HSPG) sont impliqués dans la fusion et l'infection relayées par l'Env HTLV (Pinon, Klasse et al. 2003). Notamment, le traitement de cellules exprimant les HSPG par l'heparitinase I (également appelée héparinase III ou HS lyase) conduit à une baisse significative de la liaison de la SU de l'Env HTLV à la surface des cellules. Cependant, cette baisse n'est pas totale, et l'effet de ce traitement sur l'infection assurée par l'Env HTLV n'a pas été évalué directement – seul un effet indirect de polyanions a été évalué. Les HSPG sont constitués d'un cœur protéique (*e.g.* syndecan, glypican, agrine, etc.) ancré dans la membrane plasmique sur lequel sont branchés des polymères linéaires de disaccharides (GlcNAc $\alpha$ 1-4GlcA $\beta$ 1-4) au niveau des

résidus sérine (Varki, Cummings et al. 1999; Varki, Cummings et al. 1999). Les polysaccharides subissent au cours de leur polymérisation des modifications conformationnelles (épimérisation) et de sulfatation. La grande variabilité des arrangements de chaînes de polysaccharides et de leurs modifications permettent aux HSPG d'interagir avec un large spectre de ligands (Varki, Cummings et al. 1999). Ainsi les HSPG sont impliqués dans l'attachement et l'entrée d'un grand nombre de virus enveloppés ou non, humains et animaux, *e.g.* les virus herpes simplex, les flavivirus, les picornavirus (Spillmann 2001) ainsi que HIV (Bobardt, Saphire et al. 2003). Il est donc vraisemblable qu'à l'instar des autres enveloppes virales, les HSPG soient impliqués dans un attachement non spécifique de l'Env HTLV, et probablement également des virions mêmes. De tels facteurs d'attachement doivent être clairement distingués des récepteurs d'entrée. En amont de l'entrée virale propre, l'attachement via les HSPG ou d'autres composantes de la matrice extracellulaire module nécessairement la liaison distincte aux récepteurs d'entrée. Ce sont ces récepteurs d'entrée qui permettent (i) de lier de manière spécifique le virus et (ii) d'induire dans le confinement de la surface cellulaire les changements de conformations de l'Env responsable de la fusion des membranes virales et cellulaires.

### **C.3. Transmission cellule à cellule**

HTLV se dissémine particulièrement mal sous forme de particule libre, et l'on considère en général que HTLV infecte préférentiellement par transmission de cellule-à-cellule, avec contacts directs entre cellules. Cependant, ce concept général fait la confusion entre deux types d'infection : *in vivo* et *in vitro*, et deux modèles : le virus HTLV entier et l'Env HTLV.

#### C.3.a. *In vitro*

*In vitro*, aucune infection HTLV n'a pu être établie et entretenue à partir d'un inoculum acellulaire, ou d'un surnageant de cellules infectées mises en culture. Mais le maintien de



l'infection par transmission cellule à cellule, par exemple par coculture de cellules cibles et de cellules infectées, n'a pas non plus été établi. Seule la culture de cellules chroniquement infectées par HTLV a été établie (*e.g.* les lignées MT-2, C91/PL). Une seule étude a démontré une préférence formelle pour la transmission cellule à cellule pour HTLV (Landau, Page et al. 1991). Dans cette étude, des virions HIV pseudotypés par l'Env HTLV, mais également dans une moindre mesure d'autres Env rétrovirales, sont plus infectieux par coculture de cellules cibles avec des cellules productrices que par inoculation de cellules cibles par un surnageant viral.

### C.3.b. *In vivo*

*In vivo*, une préférence pour la transmission cellule à cellule est soutenue par l'observation que des infections HTLV sont transmissibles par des produits sanguins cellulaires seulement, alors qu'HIV est transmissible par des produits sanguins cellulaires comme acellulaires (Donegan, Lee et al. 1994). Cependant, cette observation peut être simplement liée à l'absence de virémie périphérique dans le cas d'infection HTLV, au contraire d'HIV, et non à une "préférence" intrinsèque à la transmission de cellule à cellule. Mais, par ailleurs, les conditions naturelles d'infection par HTLV tout comme HIV font intervenir des produits cellulaires : rapports sexuels, accouchement, allaitement.

### C.3.d. La synapse infectieuse

La synapse immunologique est une structure qui s'établit entre une cellule présentatrice d'antigène et une cellule T. Cette structure transitoire se caractérise par le recrutement des médiateurs de l'activation (TCR sur la cellule T et MHC classe II sur la cellule présentatrice) au cœur d'un anneau de molécules d'adhésion (LFA-1 sur la cellule T et ICAM-1 sur la cellule présentatrice) (Monks, Freiberg et al. 1998; Grakoui, Bromley et al. 1999). Une structure similaire a été observée suite à la coculture de cellules T CD4 et CD8 de patients infectés par HTLV avec des cellules T de patients sains (Igakura, Stinchcombe et al. 2003). Dans cette structure dite "synapse virologique", le centre organisateur des microtubules

ainsi que le cœur HTLV (Gag et ARN viral) se trouvent polarisés à la zone de contact avec la cellule cible. Cette structure pourrait donc constituer un lieu privilégié de la transmission cellule à cellule. Cependant, la transmission cellule à cellule du HTLV est plus efficace que la transmission par surnageant acellulaire dans des lignées cellulaires qui ne sont pas de type lymphoïde (Landau, Page et al. 1991). Il est donc important d'évaluer si les structures de contact impliquées dans ces lignées sont similaires à la synapse virologique telle que décrite (Igakura, Stinchcombe et al. 2003).

#### C.3.e. La transmission cellule à cellule : un paradigme

En conclusion, l'Env HTLV dans un système hétérologue *in vitro* semble être plus infectieuse par contact cellule-cellule que via un surnageant acellulaire, mais les causes biochimiques de cet avantage ne sont pas connues. La préférence *in vivo* quant à elle semble être plus liée aux contraintes d'expression du virus et à la disponibilité de particules virales extracellulaires libres (*e.g.* aucune virémie HTLV n'est détectable) qu'à une propriété de l'Env. Ceci illustre la limitation des infections acellulaires qui, bien que fournissant d'excellents modèles expérimentaux, ne reflètent pas le système écologique dans lequel les rétrovirus évoluent, *i.e.* se répliquent et se disséminent. Par extension, la transmission cellule-à-cellule des rétrovirus doit être considérée comme le paradigme des infections rétrovirales naturelles.

# RESULTATS

## **A. CARACTERISATION DE L'EXPRESSION DU RECEPTEUR HTLV A LA SURFACE DES LYMPHOCYTES HUMAINS**

### **A.1. Résumé article 1**

Le RBD de l'Env HTLV peut être produit de manière ectopique, fusionné à une étiquette rapporteuse (étiquette GFP, HA, Fc de lapin). Ce RBD, produit de manière soluble dans le surnageant de culture de cellules 293T transfectées, peut alors être utilisé comme ligand de surface du récepteur HTLV, alors inconnu. Les lymphocytes T CD4 sont les principales cibles de l'infection HTLV. Nous avons donc dans un premier temps cherché à caractériser l'expression du récepteur HTLV sur ces cellules, afin de définir les modalités de leur infection. De manière inattendue, l'expression du récepteur est indétectable sur les lymphocytes T CD4 au repos isolés du sang périphérique de l'adulte. Cependant, après activation par des anticorps anti-CD3, un complexe de 5 chaînes associées au TCR, et anti-CD28, le ligand des molécules co-stimulatrices CD80 et CD86, l'expression du récepteur est rapidement induite (détectable à partir de 4h), et suit celle de CD25, qui est un marqueur classique d'activation correspondant à la chaîne  $\alpha$  du récepteur à l'IL-2. L'induction de CD25 et du récepteur HTLV, et pas celle de CD69, un autre marqueur d'activation qui apparaît plus précocement que CD25, requièrent une synthèse protéique car elle est inhibée par la cycloheximide. L'expression du récepteur HTLV culmine après 48h puis décroît progressivement jusqu'à ne plus être détectable, coïncidant avec le retour au repos des lymphocytes. L'expression du récepteur HTLV accompagnant l'activation T, nous avons cherché à déterminer si le récepteur HTLV localise avec les structures effectrices de

l'activation T (CD3 du TCR). Cependant, un capping du TCR induit par des anticorps anti-CD3 ne modifie pas la localisation du récepteur HTLV, celui-ci étant réparti sur toute la surface cellulaire. L'activation des cellules T CD4 conduit également à leur prolifération. Nous avons souhaité déterminer si l'expression du récepteur HTLV était plutôt associée à l'état activé ou à l'état prolifératif. L'IL-7 induit la prolifération partielle des cellules T CD4 de sang de cordon sans expression de tous les marqueurs d'activation par le TCR. Dans ces cellules, nous avons pu démontrer que la prolifération est concomitante de l'expression du récepteur HTLV. Ainsi l'apparition du récepteur HTLV à la surface des lymphocytes accompagne la prolifération, qu'elle soit obtenue par le TCR ou pas.

## **A.2. Article original 1**

# The HTLV receptor is an early T-cell activation marker whose expression requires de novo protein synthesis

Nicolas Manel, Sandrina Kinet, Jean-Luc Battini, Felix J. Kim, Naomi Taylor, and Marc Sitbon

**The human T-cell leukemia virus type 1 (HTLV) is the first isolated human retrovirus, but its receptor has yet to be identified, in part due to its ubiquitous expression. Here we report that quiescent CD4 and CD8 T lymphocytes do not express this receptor, as monitored with a soluble receptor-binding domain derived from the HTLV envelope. However, HTLV receptor is an early activation marker in neonatal and adult T lymphocytes, detected as early as 4 hours following T-cell-receptor (TCR) stimulation. This induced surface expression of the HTLV**

**receptor requires de novo protein synthesis and results in a wide distribution on the surface of activated lymphocytes. Moreover, the distribution of the HTLV receptor is independent of TCR/CD3-capped membrane structures, as observed by confocal immunofluorescence microscopy. To determine whether HTLV receptor up-regulation specifically requires TCR-mediated signals or, alternatively, is dependent on more generalized cell cycle entry/proliferation signals, its expression was monitored in interleukin 7 (IL-7)-stimulated neonatal and adult T cells.**

**Neonatal, but not adult, lymphocytes proliferate in response to IL-7 and HTLV receptor expression is restricted to the former population. Thus, HTLV receptor expression appears to be an early marker of cell cycle entry. Up-regulation of the HTLV receptor, via signals transmitted through the IL-7 cytokine receptor as well as the TCR, is likely to contribute to the mother-to-infant transmission and spreading of HTLV-1. (Blood. 2003; 101:1913-1918)**

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## Introduction

The human T-cell leukemia virus type 1 (HTLV-1), the first characterized human retrovirus,<sup>1</sup> is present in all areas of the world as either an endemic or a sporadic infectious agent.<sup>2</sup> In endemic areas, HTLV-1 transmission seems to occur mostly from mother to infant through breast-feeding.<sup>3</sup> The exceptionally broad tropism of HTLV-1 *in vitro*<sup>4,5</sup> contrasts with the finding that *in vivo*, HTLV-1 is found primarily in CD4<sup>+</sup> lymphocytes and less frequently in other mononuclear blood cells.<sup>6,7</sup> Studies of this apparent discrepancy have been hindered by the high cytotoxicity of HTLV envelopes and their dependence on cell-to-cell contact for infection and spreading.<sup>4,8,9</sup> Moreover, investigations have been limited because the HTLV envelope receptor remains unidentified, even though multiple cell surface components including adhesion molecules,<sup>10,11</sup> matrix-associated proteins,<sup>12</sup> lipids,<sup>13</sup> and lipid rafts,<sup>14</sup> have been implicated in HTLV envelope (Env)-mediated membrane fusion and virus transmission.

The HTLV-1 Env receptor-binding determinants are entirely contained within the extracellular surface component (SU).<sup>4,15</sup> Recently, we demonstrated that the amino-terminal 215 amino acids of the SU harbors the receptor-binding domain (RBD) of HTLV Env.<sup>16</sup> The binding specificity of a soluble tagged construct encompassing this region (H<sub>RBD</sub>) has been demonstrated by its efficient competition with HTLV Env-mediated cell fusion and infection (F.J.K., E. N. Garrido, N.M., M.S., J.-L.B., manuscript in preparation).

Using the RBD of HTLV Env, we have now tracked HTLV receptor expression on T lymphocytes, which have been reported to be a major HTLV-1 reservoir *in vivo*. Circulating T lymphocytes are almost entirely in the G<sub>0</sub> phase of the cell cycle. Activation of these cells via their cognate antigen receptor is the predominant feature of an efficient immune response. On activation, expression of numerous surface markers is modulated,<sup>17,18</sup> cytokines are secreted, and cells can undergo as many as 6 to 8 divisions. Here, we have assessed whether expression of the HTLV receptor on T lymphocytes is modulated by their activation state. Although it has previously not been possible to identify mammalian cell types that do not express the HTLV receptor,<sup>4,15</sup> we now report that quiescent T lymphocytes do not express the HTLV receptor. Rather, receptor expression on T lymphocytes is induced by T-cell-receptor (TCR) engagement and requires de novo protein synthesis. Furthermore, interleukin 7 (IL-7)-stimulated neonatal and adult T lymphocytes demonstrate distinct cell surface HTLV receptor levels, with significantly higher expression in the immature neonatal T-cell population.

## Materials and methods

### Generation of HTLV and amphotropic MLV Env fusion proteins

The construction of a pCSI expression vector<sup>19</sup> encoding the amino terminal 215 amino acids of the SU region of HTLV Env comprising the

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signal peptide and the SU RBD fused to a carboxy-terminal rabbit immunoglobulin Fc (rFc) tag, herein referred to as  $H_{RBD}$ , is detailed elsewhere (F.J.K., E. N. Garrido, N.M., M.S., J.-L.B., manuscript in preparation). A similar construct, comprising the N-terminal 379 amino acids of the amphotropic murine leukemia virus (MLV) Env SU and fused to the carboxy-terminal rFc tag, was also generated and is herein referred to as  $A_{RBD}$ . The RBD of HTLV-2 comprising the N-terminal 178 amino acids was fused to the enhanced green fluorescence protein (EGFP)-coding sequence (from pEGFP-N3; Clontech, Palo Alto, CA) lacking the ATG initiation codon by polymerase chain reaction (PCR) amplification in the pCSI expression vector and is herein referred to as  $H_{RBD}$ -EGFP. Cloning details are available on request.

$H_{RBD}$ ,  $A_{RBD}$ , and  $H_{RBD}$ -EGFP proteins were produced by transfecting 293 T cells with the appropriate constructs or with the empty control vector using the calcium phosphate method. After transfection, cells were washed twice with phosphate-buffered saline (PBS) and fresh medium was added. Media containing the various soluble RBDs were harvested 1 day later and clarified by a 5-minute centrifugation at 13 000 rpm at 4°C.

### Cell isolations and culture conditions

Adult peripheral blood (APB), obtained from healthy adult donors, as well as umbilical cord (UC) blood, obtained immediately after delivery of full-term infants, was collected in heparinized tubes.  $CD4^+$  T cells were purified by negative selection using tetrameric complexes in which one antibody recognizes a surface antigen on B cells, monocytes, natural killer (NK) cells or  $CD8^+$  cells and the other recognizes glycophorin A on the surface of red blood cells (RosetteSep, Stemsep Technologies, Vancouver, BC, Canada). Non- $CD4^+$  T cells were then pelleted on Ficoll-Hypaque separation. The purity of each cell isolation was monitored on a FACScalibur (Becton Dickinson, San Jose, CA) following staining with fluorescein isothiocyanate (FITC)-conjugated  $\alpha CD3$  and phycoerythrin (PE)-conjugated  $\alpha CD4$  monoclonal antibodies (mAbs; Immunotech, Marseille, France). Lymphocytes were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin. Cells were stimulated with immobilized  $\alpha CD3$  (UCHT1; a generous gift from D. Cantrell, Imperial Cancer Research Fund, London, United Kingdom) and  $\alpha CD28$  (9.3; kindly provided by C. June, University of Pennsylvania, Philadelphia) mAbs (1  $\mu g/mL$ ) and after 2 days, cultured in the presence of recombinant IL-2 (100 U/mL; Chiron, Emeryville, CA). Alternatively, cells were cultured in the presence of recombinant IL-7 (10 ng/mL; Peprotech, London, United Kingdom). When indicated, cycloheximide was added to cell cultures at a concentration of 5  $\mu g/mL$  (Sigma, St Louis, MO).

The Jurkat T-cell clone 77-6.8, generously provided by Dr K. A. Smith (New York, NY), was maintained in RPMI 1640 medium supplemented with 10% FCS.

### Flow cytometry for Env binding, surface markers, and cell cycle analysis

To evaluate binding of  $H_{RBD}$  and  $A_{RBD}$  experiments,  $5 \times 10^5$   $CD4^+$  T cells were washed with PBA (PBS containing 1% bovine serum albumin [BSA] and 0.1% sodium azide), incubated with 0.3 mL control,  $H_{RBD}$ , or  $A_{RBD}$  supernatants for 30 minutes at room temperature, washed, and labeled for 20 minutes on ice with an FITC-conjugated sheep anti-rFc antibody (1:500 dilution; Sigma). To detect expression of CD4, CD8, CD25, CD69, CD45RA, and CD45RO, cells were incubated for 20 minutes on ice with the appropriate PE-conjugated mAbs or PE-conjugated isotype control mAbs (Immunotech). In all cases, cells were immediately analyzed on a FACScalibur (Becton Dickinson) and data analysis was performed using CellQuest software (Becton Dickinson).

The percentage of cells in the S-G<sub>2</sub>/M phases of the cell cycle was determined by propidium iodide (PI) staining. At the indicated time point, cells were resuspended in PI (50  $\mu g/mL$ ) diluted in PBS with 5% glycerol and 0.1% Triton X-100 and incubated for 15 minutes prior to analysis. Cell cycle was analyzed on the FL2-A wavelength after gating out signals due to cell debris.

### Confocal immunofluorescence microscopy

$CD4^+$  T cells ( $5 \times 10^5$ ) were washed with PBS and incubated with  $H_{RBD}$ -EGFP supernatants as well as either  $\alpha CD4$  or  $\alpha CD3$  mAbs (0.5  $\mu g$ ) for 30 minutes at room temperature. Cells were then fixed with 3.7% paraformaldehyde in PBS, washed with ice-cold PBS containing 0.1% BSA, and labeled with Cy3-conjugated sheep  $\alpha$ mouse-IgG (1:500; Sigma) for 20 minutes on ice. Following PBS washes, cells were seeded on slides (Superfrost; Menzel-Glaser, Braunschweig, Germany) that were coated with poly-L-lysine (0.01%; Sigma) and mounted in Mowiol (Calbiochem, La Jolla, CA). Slides were analyzed on a Leica confocal microscope and acquisitions were performed using an Agfa CoolSpan camera and the MetaMorph software. For comparative analyses, all photographs were taken using the same exposure conditions.

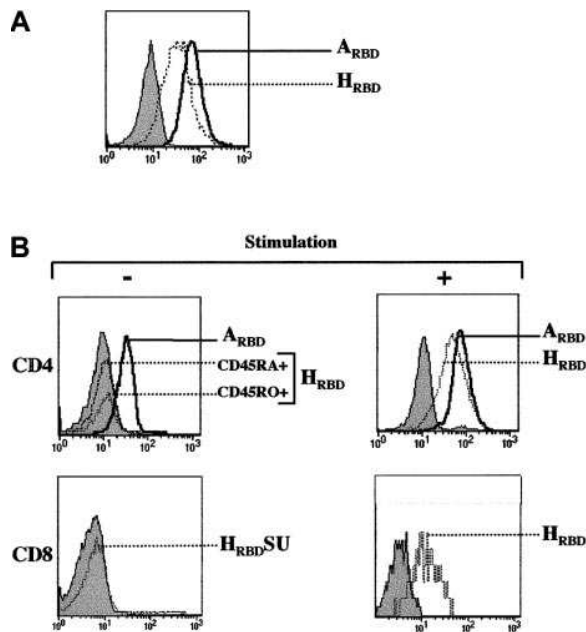
## Results

### Expression of the HTLV receptor on human T lymphocytes is induced by TCR engagement

A 215-amino acid truncation of the SU of HTLV Env, herein referred to as  $H_{RBD}$ , retained the capacity to bind the HTLV Env receptor as monitored by its ability to specifically interfere with HTLV Env-mediated cell binding, cell fusion, and infection (F.J.K., E. N. Garrido, N.M., M.S., J.-L.B., manuscript in preparation).<sup>16</sup> This truncated fragment fused at its C-terminus to a rabbit Fc-tag was used to study HTLV receptor expression on human T-cell subsets. Binding experiments were first validated in the Jurkat T-cell leukemia cell line. As previously demonstrated using the entire HTLV SU,<sup>15</sup>  $H_{RBD}$  bound efficiently to these cells (Figure 1A). pIT-2, the receptor for amphotropic MLV Env,<sup>20</sup> is expressed on all T-cell subsets,<sup>21</sup> and as such was used as a control throughout this study. As expected, binding of an Fc-tagged amphotropic MLV Env SU fragment ( $A_{RBD}$ ) was readily detectable in Jurkat cells (Figure 1A). Significant binding of the  $H_{RBD}$  to Jurkat T cells was observed as early as 30 minutes after incubation at either 21°C or 37°C, but not at 4°C. In contrast, binding of the amphotropic RBD ( $A_{RBD}$ ) was observed at all 3 temperatures (data not shown). Whether these differences involve conformational changes of the receptors, the envelopes, or other membrane components remains to be determined.

The *in vivo* profile of the HTLV receptor is not known and, specifically, its expression on T lymphocyte populations has not yet been elucidated. Using the  $H_{RBD}$  construct, we were unable to detect binding to freshly isolated naive (CD45RA) or memory (CD45RO)  $CD4^+$  or  $CD8^+$  lymphocyte subsets, although in some donors low-level expression could be detected on a maximum of 5% of lymphocytes (Figure 1B and data not shown). The lack of binding was specific to  $H_{RBD}$  because  $A_{RBD}$  binding was readily detectable on all lymphocyte subsets (Figure 1B and data not shown). Significant differences between Jurkat cells and primary T lymphocytes include cell cycle progression and their general "activation" state. Thus, unlike Jurkat cells, primary T cells are almost entirely in the G<sub>0</sub> phase of the cell cycle and do not express activation markers. To induce cell cycle progression and an "activated" profile, T lymphocytes were activated through their cognate antigen receptor (TCR) using  $\alpha CD3$  and  $\alpha CD28$  mAbs. In marked contrast with quiescent T lymphocytes, there was a significant level of  $H_{RBD}$  binding to TCR-stimulated  $CD4^+$  as well as  $CD8^+$  lymphocytes.

To directly visualize the distribution of the HTLV receptor on  $CD4^+$  T lymphocytes, we used an  $H_{RBD}$  fused directly to EGFP, referred to herein as  $H_{RBD}$ -EGFP. In agreement with the FACS data,



**Figure 1. HTLV receptor expression on CD4<sup>+</sup> and CD8<sup>+</sup> lymphocyte subsets requires T-cell-receptor stimulation.** (A) Expression of the HTLV Env receptor on Jurkat T cells was assessed using a truncated receptor-binding domain (RBD) of HTLV Env tagged with the Fc domain of rabbit IgG ( $H_{RBD}$ ). Cells were then incubated with an FITC-conjugated sheep  $\alpha$ rabbit IgG antibody and binding was detected by fluorescence-activated cell sorting (FACS) analysis. Similarly, binding of the amphotropic MLV envelope to its receptor was monitored with an amphotropic SU fragment fused to the Fc domain of rabbit IgG ( $A_{RBD}$ ). Filled histograms depict binding in the presence of the secondary FITC-conjugated sheep antirabbit antibody alone. (B) CD4<sup>+</sup> lymphocytes were purified by negative selection and expression of the HTLV Env and amphotropic MLV Env receptors was immediately assessed on the naive (CD45RA<sup>+</sup>) and memory (CD45RO<sup>+</sup>) populations as indicated. Expression of the HTLV Env and amphotropic MLV Env receptors was then monitored on CD4<sup>+</sup> lymphocytes that had been prestimulated with immobilized  $\alpha$ CD3 and  $\alpha$ CD28 mAbs (+) for 4 days (upper panels). HTLV Env receptor expression on freshly isolated CD8<sup>+</sup> lymphocytes and  $\alpha$ CD3/ $\alpha$ CD28-stimulated CD8<sup>+</sup> lymphocytes (+) is shown (lower panels). Control binding with the secondary FITC-conjugated antibody is shown in all histograms (filled). Data are representative of results obtained in 5 independent experiments.

$H_{RBD}$ -EGFP binding was not detected on unstimulated lymphocytes but was widely distributed on the surface of activated lymphocytes (Figure 2A). The lack of  $H_{RBD}$ -EGFP binding was not due to an intrinsic difficulty in detecting staining in small quiescent T cells because equivalent binding of an  $\alpha$ CD4 mAb was observed on unstimulated and TCR-stimulated CD4<sup>+</sup> lymphocytes (Figure 2A). Using an  $\alpha$ CD3 mAb, a redistribution of TCR/CD3 complexes to capped membrane structures was induced.<sup>22</sup> In contrast, this process did not result in a redistribution of the HTLV receptor (Figure 2B). These data were observed whether or not cells were fixed immediately following addition of the primary  $\alpha$ CD3 mAb. It should nevertheless be noted that some variation of  $H_{RBD}$ -EGFP staining on the surface of cells was observed both in the absence and presence of the  $\alpha$ CD3 mAb, most likely due to the convoluted state of the image. Altogether, these data demonstrate a high expression of the HTLV receptor in stimulated T lymphocytes and strongly suggest that the receptor does not partition with CD3/TCR clusters.

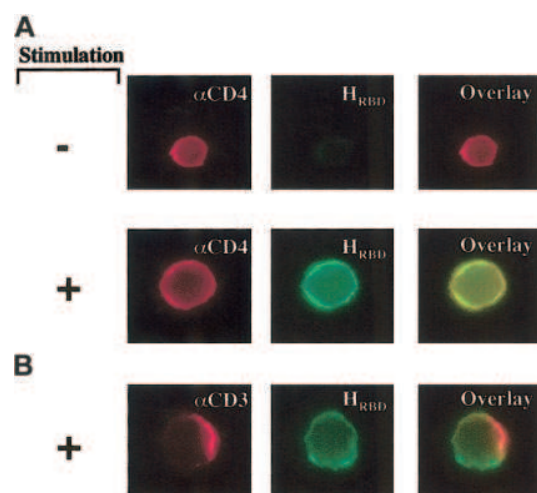
#### TCR-induced HTLV receptor expression on CD4<sup>+</sup> T lymphocytes precedes proliferation and requires de novo protein synthesis

To determine the kinetics of HTLV receptor expression at the surface of activated CD4<sup>+</sup> T lymphocytes, cells were stimulated

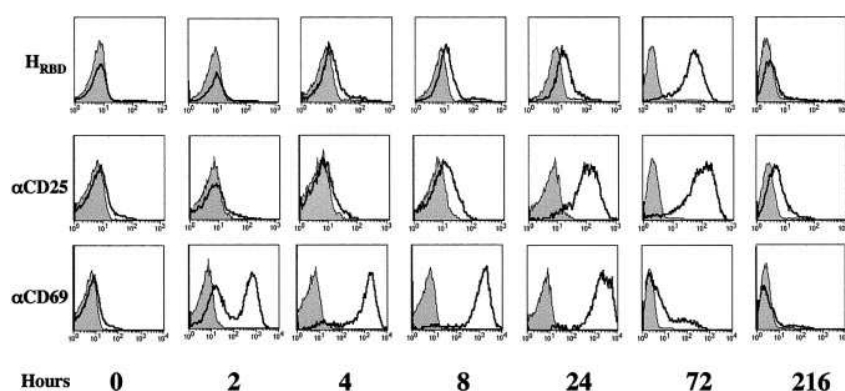
with immobilized  $\alpha$ CD3/ $\alpha$ CD28 mAbs and binding of  $H_{RBD}$  was assessed at 2, 4, 8, 24, 72, and 216 hours following stimulation (Figure 3). Cell surface expression of the HTLV receptor was compared with expression of 2 early activation markers, CD25 (IL-2R $\alpha$  chain) and CD69 (Figure 3). Cell surface CD69 and CD25 are detected at approximately 4 and 16 hours after stimulation,<sup>17</sup> respectively, whereas T lymphocytes enter S phase at approximately 36 hours following TCR engagement<sup>23</sup> (J. Garcia-Sanz, personal oral communication, April 2002). As expected, CD69 expression was detected on the vast majority of cells within 8 hours after activation and reached maximal levels at approximately 24 hours. The profile of CD25 induction was slower; levels increased gradually between 4 and 24 hours and plateau levels were maintained for 72 hours. The kinetics of HTLV receptor expression was fairly comparable with that observed for CD25, with a steady increase in receptor levels occurring between 4 and 72 hours after stimulation: the HTLV receptor was expressed on 30% to 40% of cells at 24 hours, whereas high receptor levels were detected on the vast majority of cells at 72 hours (Figure 3).

HTLV receptor expression did not remain stable following TCR activation but diminished significantly as T lymphocytes returned to their resting state. In experiments performed with CD4<sup>+</sup> T lymphocytes isolated from 4 individual donors, HTLV receptor expression decreased to basal levels between 8 and 14 days after stimulation. Expression of the CD69 and CD25 activation markers also decreased, albeit with different kinetics; CD69 levels were largely decreased by 72 hours, whereas CD25 levels returned to baseline with a longer lag time than the HTLV receptor (12-24 days).

The TCR-induced proliferation of T lymphocytes is preceded by a 7- to 10-fold increase in protein synthesis.<sup>24</sup> Indeed, translation is crucial for the propagation of TCR-induced signals because in its absence, activation is inhibited.<sup>25</sup> To assess whether surface expression of the HTLV receptor required de novo protein synthesis, freshly isolated quiescent CD4<sup>+</sup> lymphocytes were stimulated with  $\alpha$ CD3/ $\alpha$ CD28 mAbs in the presence of the protein synthesis inhibitor, cycloheximide (CHX). Although a 24-hour culture in the presence of 5  $\mu$ g/mL CHX did not reduce cell viability (not



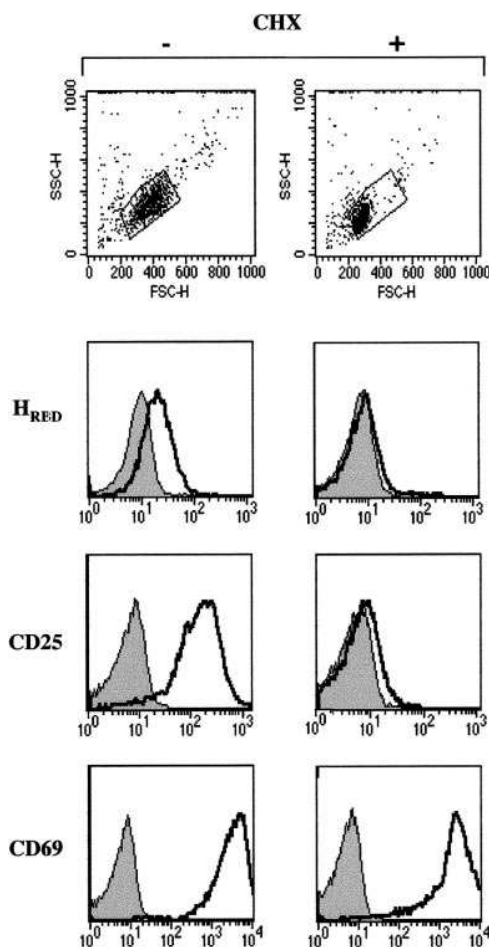
**Figure 2. Distinct localizations of the TCR and HTLV Env receptor in activated CD4<sup>+</sup> lymphocytes.** (A) Unstimulated or  $\alpha$ CD3/ $\alpha$ CD28-stimulated CD4<sup>+</sup> lymphocytes were incubated with an  $\alpha$ CD4 mAb, detected with a goat antimouse IgG-Cy3, and the HTLV Env SU RBD fused to EGFP ( $H_{RBD}$ -EGFP). Cells were examined by confocal immunofluorescence microscopy. (B) Capping was induced in prestimulated CD4<sup>+</sup> lymphocytes using an  $\alpha$ CD3 mAb. After a 30-minute induction at room temperature, cells were fixed and incubated with a goat  $\alpha$ mouse IgG-Cy3 and  $H_{RBD}$ -EGFP to visualize the localization of CD3 and the HTLV receptor, respectively. Original magnification,  $\times 100$ .



**Figure 3. Kinetics of HTLV Env receptor expression following TCR engagement.** Freshly isolated CD4<sup>+</sup> lymphocytes were activated with immobilized αCD3 and αCD28 mAbs and expression of the HTLV Env receptor was followed after 2, 4, 8, 24, 72, and 216 hours of culture using H<sub>RBD</sub>. Cells were simultaneously evaluated for expression of the CD25 (IL-2Rα) and CD69 activation markers using PE-conjugated mAbs. Staining profiles are shown and filled histograms depict staining with an FITC-conjugated secondary mAb alone or with isotype-matched PE-conjugated control mAbs. Results are representative of data obtained in 4 independent experiments.

shown), it inhibited TCR-activated blast formation as demonstrated by significantly lower forward and side angle light scatters (Figure 4). Up-regulation of the HTLV receptor as well as CD25 expression were completely abrogated by CHX treatment (Figure 4). In contrast, CD69 was induced to levels observed in control T cells,

suggesting that the rapid kinetics of CD69 up-regulation is due mainly to translocation of intracellular stores of CD69 to the cell membrane. Expression of the amphotropic envelope receptor PiT-2 does not require TCR activation and in agreement with this observation, PiT-2 was present on CHX-treated lymphocytes (not shown). Altogether, these data demonstrate that distinct metabolic pathways regulate cell surface expression of the HTLV receptor and CD25, on the one hand, and CD69 on the other hand.



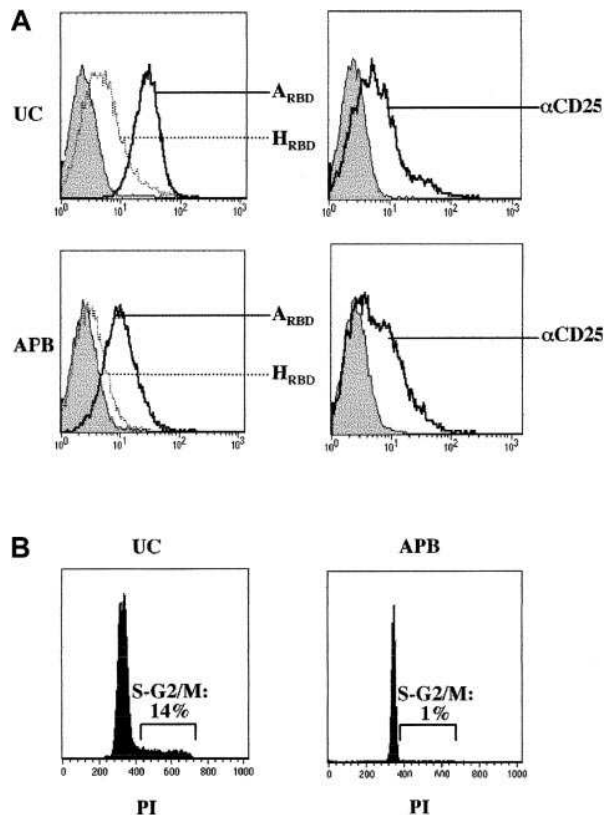
**Figure 4. Expression of the HTLV receptor on TCR-stimulated lymphocytes requires de novo protein synthesis.** Freshly isolated CD4<sup>+</sup> lymphocytes were activated with immobilized αCD3 and αCD28 mAbs in the absence or presence of the protein synthesis inhibitor cycloheximide (CHX; 5 μg/mL) for 24 hours. The size and density of viable cells were monitored by forward angle light scatter (FSC) and side angle scatter (SSC) on a flow cytometer. HTLV receptor expression as well as CD25 and CD69 levels were assessed using H<sub>RBD</sub> and the appropriate conjugated mAbs, in the absence and presence of CHX. Filled histograms depict control binding. Data are representative of results obtained in 2 independent experiments.

#### Induction of HTLV receptor expression in IL-7-stimulated neonatal T cells

TCR engagement in primary T lymphocytes leads to the induction of an “activated” profile with high expression of CD25 and CD69 at the cell surface as well as an acquisition of a memory phenotype (Figure 3 and data not shown). Because the IL-7 cytokine serves as a T-cell survival factor without inducing an extensive activation profile,<sup>26-28</sup> we assessed its effects on HTLV receptor expression. In T cells from APB, relatively low levels of CD25 were induced by IL-7 and cell surface expression of the HTLV receptor was marginal as monitored by binding of H<sub>RBD</sub> (Figure 5A). In contrast, surface expression of the HTLV receptor was detected in IL-7-treated neonatal CD4<sup>+</sup> lymphocytes isolated from UC blood, albeit at significantly lower levels than those observed in TCR-activated lymphocytes (Figure 5A). UC-derived lymphocytes are particular in that they are naive T cells that have recently emigrated from the thymus and intriguingly IL-7 induces their proliferation<sup>29,30</sup> but not the proliferation of APB CD4<sup>+</sup> T cells (Figure 5B).

Expression of the HTLV receptor in IL-7-stimulated UC lymphocytes was not directly associated with expression of the CD25 activation marker as demonstrated by the findings that CD25 was induced to similar levels in IL-7-treated neonatal and adult CD4<sup>+</sup> lymphocytes, whereas only the former bound H<sub>RBD</sub> at significant levels, and HTLV receptor expression in UC lymphocytes was not restricted to the CD25-expressing population (not shown). Expression of the HTLV receptor was not a constitutive characteristic of UC lymphocytes but was directly dependent on IL-7 treatment; H<sub>RBD</sub> binding was not observed in freshly isolated quiescent UC T lymphocytes. Moreover, on TCR engagement, H<sub>RBD</sub> binding to UC lymphocytes was induced to equivalent levels and with comparable kinetics as that observed in adult CD4<sup>+</sup> lymphocytes (not shown). Thus, the HTLV receptor, which demonstrates a differential expression profile in IL-7-stimulated lymphocyte populations, represents a distinct activation marker in neonatal and adult CD4<sup>+</sup> lymphocytes.





**Figure 5. Induction of HTLV receptor expression on IL-7-stimulated neonatal CD4<sup>+</sup> lymphocytes.** (A) Adult and neonatal CD4<sup>+</sup> T cells were isolated from adult peripheral blood (APB) and umbilical cord blood (UC), respectively, and cultured for 7 days in the presence of recombinant IL-7 (10 ng/mL). Expression of the HTLV and amphotropic MLV receptors as well as CD25 levels were monitored by FACS using H<sub>RBD</sub>, A<sub>RBD</sub>, and a PE-conjugated αCD25 mAb. Filled histograms depict control binding. (B) Cell cycle entry of IL-7-stimulated APB and UC CD4<sup>+</sup> lymphocytes was monitored at day 7 by assessing the DNA content of PI-stained cells on FACS. Results are representative of data obtained in 3 independent experiments.

## Discussion

Previous to this study, vertebrate cell lines that do not express the HTLV receptor had not been identified. Paradoxically, although T cells appear to constitute a major HTLV reservoir *in vivo*, we report here that quiescent T lymphocytes lack cell surface expression of the HTLV receptor. However, we demonstrate that HTLV receptor expression is induced under conditions where lymphocytes are stimulated to enter into cell cycle, either via TCR engagement or in the case of neonatal T cells, following IL-7 stimulation. Notably though, expression of the HTLV receptor demonstrates a profile that is distinct from that of other activation markers. The kinetics of HTLV receptor expression was less rapid than that of the CD69 activation marker in TCR-stimulated cells. Moreover, the induction and down-regulation of the IL-2Rα subunit (CD25) did not correlate with HTLV receptor expression in either IL-7-stimulated neonatal or adult T-cell subsets. Whereas low levels of CD25 induction were observed in both IL-7-induced T-cell populations, HTLV receptor expression was detected only in the proliferating neonatal T cells. Thus, HTLV receptor expression appears to be a novel cell cycle entry/proliferation marker on human T cells.

In the context of TCR engagement, expression of the HTLV receptor was not limited to any particular T-cell subset; rather, it was detected on naive and memory lymphocytes as well as on both

CD4<sup>+</sup> and CD8<sup>+</sup> populations. Additionally, it should be noted that induction of HTLV receptor expression on naive T lymphocytes largely preceded the acquisition of a memory phenotype as acquisition of the memory CD45RO marker<sup>31</sup> generally requires between 4 and 6 days under the conditions of TCR stimulation used here.<sup>32</sup> The timing of HTLV receptor expression also preceded DNA synthesis, because the former was detected at 4 to 8 hours after TCR stimulation, whereas the latter generally begins at approximately 36 hours.<sup>23</sup> Indeed, we found that HTLV receptor expression was independent of DNA synthesis. Treatment of TCR-stimulated T cells with an agent that blocks cell cycle progression at the end of the G<sub>1</sub> phase (aphidicolin) did not abolish up-regulation of the HTLV receptor (S.K. and N.M., unpublished observations, September 2002).

Expression of the HTLV receptor was strictly dependent on *de novo* protein synthesis. It has been shown that prior to T-lymphocyte proliferation, there is a 7- to 10-fold increase in protein synthesis and a 30- to 40-fold augmentation in mRNA synthesis.<sup>33</sup> This "pre-proliferation" characteristic appears to be a particularity of T lymphocytes and is not observed in other cell types. The required pre-proliferation burst in mRNA/protein synthesis likely results from the extremely low metabolic rate of resting G<sub>0</sub> T lymphocytes, which make up the vast majority of the circulating T-lymphocyte pool. Thus, it is intriguing to speculate that it is this low metabolic rate that accounts for the fact that T lymphocytes are the first and only cell type, identified to date, which do not constitutively express the HTLV receptor. Notably, Wodarz and Bangham have recently used a mathematical model to suggest that the rate of evolution of HTLV-1 is limited by the restricted availability of activated uninfected T cells, irrespective of the viral load.<sup>34</sup> Distinctions between CD4<sup>+</sup> and CD8<sup>+</sup> T cells in serving as a reservoir for HTLV *in vivo* are probably not due to expression of the receptor itself, because we found that binding of H<sub>RBD</sub> depended on similar activation requirements and kinetics in these 2 cell types. Therefore, the distinct *in vivo* characteristics of these 2 cell types with regard to HTLV might be due to differences in the postentry steps of the infection. The precise associations between HTLV receptor expression, T-cell cycle progression, and infection remain to be elucidated, but our data suggest that quiescent and activated CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes will provide important models in which to evaluate these questions.

Because of the previous lack of adequate tools and the cytotoxicity of the HTLV envelope, few of the molecular mechanisms regulating HTLV binding at the cell surface have been elucidated. On the other hand, a great deal of work has been performed with the HIV retroviral envelope and it is generally accepted that binding of the SUgp120 HIV-1 envelope glycoprotein to its receptors results in receptor cocapping.<sup>35</sup> Notably, HTLV Env was widely distributed on target cells, as assessed by confocal microscopy (Figure 2), and no capped structures were observed. Moreover, under conditions where capping was induced with an antibody directed against the CD3ε epitope of the TCR, neither capping nor a coaggregation of the HTLV receptor was detected. Because TCR engagement results in the clustering of rafts,<sup>36</sup> domains of lateral lipid clusters that are enriched in sphingolipids, cholesterol, and glycosylphosphatidylinositol (GPI)-anchored proteins as well as signal transduction assemblies,<sup>37</sup> this suggests that HTLV envelope-receptor complexes do not partition in CD3-associated raft structures. The finding that HTLV-1-mediated syncytium formation is dependent on raft integrity,<sup>14</sup> is likely due to the fact that the mechanisms regulating HTLV Env binding and syncytia formation are not identical.<sup>8,38</sup>

In conclusion, the absence of a functional HTLV receptor on quiescent CD4<sup>+</sup> lymphocytes and its expression for a short period of time following TCR stimulation are likely to be crucial for the constitution of these cells as HTLV-1 reservoirs in vivo. In the case of neonatal T cells, our results suggest that a narrow window of susceptibility to HTLV-1 infection would result from either TCR stimulation or on contact with the IL-7 cytokine. Neonatal lymphocytes differ from their adult counterparts in that the former represent, almost solely, recent thymic emigrants. Thus, our finding that IL-7-stimulated neonatal T cells express the HTLV receptor suggests a possible mechanism via which HTLV can be transmitted from an infected mother to "nonactivated" recent thymic emigrants in the infant. The data presented here lay the groundwork for

further in vitro and in vivo studies directed toward elucidating the pathophysiology of HTLV-1 infection and the role of the HTLV receptor in the T-cell activation process.

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### A.3. Conclusion article 1

Alors que le HTLV est détecté majoritairement dans les lymphocytes T circulants au repos, nos résultats indiquent que le récepteur HTLV n'est pas exprimé à la surface de ces cellules, ou tout du moins n'y est pas détectable. Ceci suggère que les cellules circulantes, en l'état, ne peuvent pas être la cible directe de l'infection par HTLV. Cette observation corrèle avec la faible capacité des cellules T circulantes à être transduites par des vecteurs rétroviraux, qui nécessitent une certaine activité métabolique induite par divers traitements à base d'interleukines (Dardalhon, Jaleco et al. 2000). Dans ce contexte, il paraît vraisemblable que l'infection HTLV a lieu dans des cellules "activées" en prolifération, *i.e.* exprimant le récepteur HTLV, que ces cellules subissent une expansion proliférative, et que les produits de cette expansion donnent naissance au pool de cellules T au repos infectés. Ainsi, la cible cellulaire de l'infection par HTLV pourrait être de deux types, soit des lymphocytes T périphériques ayant été précédemment activés et donc exprimant le récepteur HTLV et ayant une activité métabolique suffisante, soit des précurseurs de différenciation des lymphocytes T circulants.

Dans le cas du SIV il a récemment été montré que des lymphocytes T CD4 à phénotype mémoire au repos sont infectés dans la lamina propria de l'intestin (Li, Duan et al. 2005; Mattapallil, Douek et al. 2005). Il semble que ces cellules viennent d'entrer en phase de repos, et possèdent une activité métabolique résiduelle suffisante pour supporter l'infection. Le phénotype naïf *vs* mémoire des cellules infectées par HTLV n'est pas clairement décrit. Cependant, il a été observé que chez les patients infectés par HTLV la proportion de cellules T à phénotype mémoire augmente, et donc que la proportion des cellules T à phénotype naïf diminue (Yasunaga, Sakai et al. 2001). Ceci pourrait indiquer que les cellules infectées sont de phénotype mémoire, et donc qu'elles ont déjà subi un état d'activation au cours duquel a pu se dérouler l'infection HTLV. Alternativement, il est possible que la diminution de la

proportion des cellules T naïves soit due à leur élimination sélective suite à l'infection par HTLV.

L'expression du récepteur HTLV à la surface des divers types cellulaires qui composent la différenciation des lymphocytes T n'a pas été rapportée à l'heure actuelle. Cependant des résultats récents de nos collaborateurs (Louise Swainson et Naomi Taylor, résultats non publiés) indiquent que le récepteur HTLV est exprimé dans le thymus, mais seulement dans certaines sous-populations thymocytaires. Notamment, une sous-population particulièrement susceptible à la transduction par des vecteurs rétroviraux (et donc probablement à l'infection par des rétrovirus) présente la plus forte expression de GLUT1 du thymus. Il pourrait donc exister des niches cellulaires dans le thymus propices à l'infection par HTLV. Les cellules souches CD34+, qui se situent à un niveau plus précoce de la différenciation, sont infectables *ex vivo* par HTLV (toutefois cette observation n'a pas pu être confirmée chez les patients (Nagafuji, Harada et al. 1993)), et leur inoculation dans des souris SCID humanisées conduit à l'apparition de thymocytes infectés (Feuer, Fraser et al. 1996). Ces cellules CD34+ pourraient donc également constituer une cible de l'infection.

Des deux scénarios (infection de lymphocytes périphériques ou infection de précurseurs), l'infection de précurseurs du lignage T semble la plus probable. En effet, il a été établi que la population de cellules infectées par HTLV dans le sang périphérique est issu d'un nombre restreint, en général de l'ordre de la dizaine, d'évènements d'infections (Wattel, Vartanian et al. 1995; Cavrois, Gessain et al. 1996), qui aboutissent par expansion oligoclonale à une charge provirale entre 1 et 10 copies pour 100 PBMC (Wattel, Vartanian et al. 1995; Cavrois, Gessain et al. 1996). Dès lors, l'infection de quelques cellules précurseurs, qui subissent une expansion au cours de leur différenciation, est compatible avec un profil oligoclonal dans le sang périphérique, alors que l'expansion dans de telles proportions de cellules T mémoires infectées, à partir du sang périphérique, est très improbable. Bien que l'élimination de lymphocytes infectés suite à une infection peut rendre compte de l'oligoclonalité, il est difficile d'expliquer l'expansion périphérique qui lui serait associée.

L'identité du récepteur HTLV n'était toutefois pas élucidée. La caractérisation de ce récepteur comme un marqueur d'activité métabolique des lymphocytes, associée à un faisceau d'autres expériences nous a permis comme nous allons le voir d'identifier la nature de ce récepteur.

## **B. IDENTIFICATION DU TRANSPORTEUR DE GLUCOSE GLUT1 COMME RECEPTEUR HTLV**

- "*Trouver d'abord, chercher ensuite.*" Jean COCTEAU.

### **B.1. Résumé article 2**

L'expression de l'Env HTLV ou de son seul RBD bloque l'acidification du milieu de culture des cellules. Ce blocage est accompagné d'un blocage de production de lactate, catabolite de la glycolyse anaérobie qui est la principale source d'ATP des cellules en culture *in vitro*. Nous avons démontré que ce blocage est en fait causé en amont par le blocage du transport de glucose, substrat de la glycolyse anaérobie.

Par ailleurs, nous avons démontré que le récepteur HTLV est associé à la prolifération des lymphocytes T. De plus, l'organisation commune entre l'Env HTLV et celle des MLV suggère que le récepteur HTLV est de la même famille que celui des MLV, *i.e.* un transporteur de nutriments à multiples domaines transmembranaires. Nous avons donc émis l'hypothèse qu'un transporteur de glucose est le récepteur du HTLV, et que son activité est bloquée par l'expression de l'Env HTLV. Parmi les transporteurs de glucose, seul GLUT1 répond à l'ensemble de ces critères. Nous avons donc cloné l'ADN complémentaire de GLUT1, ainsi que celui de son plus proche homologue GLUT3. La surexpression de GLUT1, mais pas GLUT3, augmente la capacité de liaison des cellules au RBD de HTLV et GLUT1 co-immunoprécipite avec le RBD de HTLV. La cytochalasine B, un inhibiteur de GLUT1, inhibe la liaison du RBD de HTLV ainsi que l'infection par des virions pseudotypés par l'Env HTLV. La surexpression du RBD de HTLV dans des cellules inhibe l'infection de ces cellules

par ces mêmes virions ainsi que la liaison du RBD à l'extérieur de ces cellules (mécanisme d'interférence d'Env à la surinfection). GLUT1, mais pas GLUT3, lève ces inhibitions. Cependant, ces données sont compatibles avec la possibilité que GLUT1 ne soit qu'une molécule de liaison à haute affinité, titrant le RBD au profit du récepteur canonique. Cette expérience ne démontre donc pas que GLUT1 exprimé de manière ectopique est le médiateur de l'infection. Afin d'éclaircir ce point, nous avons mis au point une expérience d'interférence à l'ARN dirigée contre GLUT1. Le traitement de cellules par un mélange d'ARN interférents induit une baisse de l'expression de GLUT1 de l'ordre de 50%, accompagnée par une baisse du transport de glucose du même ordre. De plus, la liaison du RBD ainsi que l'infection par des virions pseudotypés par l'Env HTLV est inhibée par ce traitement. La coexpression du cDNA codant pour GLUT1 et GLUT3 restaure le transport de glucose. Cependant, seul GLUT1 permet de rétablir la liaison au RBD ainsi que l'infection par des virions pseudotypés par l'Env HTLV. L'ensemble de ces expériences démontre donc que le transporteur de glucose GLUT1 est un récepteur pour HTLV.

## **B.2. Article original 2**

# The Ubiquitous Glucose Transporter GLUT-1 Is a Receptor for HTLV

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## Summary

The human T cell leukemia virus (HTLV) is associated with leukemia and neurological syndromes. The physiopathological effects of HTLV envelopes are unclear and the identity of the receptor, present on all vertebrate cell lines, has been elusive. We show that the receptor binding domains of both HTLV-1 and -2 envelope glycoproteins inhibit glucose transport by interacting with GLUT-1, the ubiquitous vertebrate glucose transporter. Receptor binding and HTLV envelope-driven infection are selectively inhibited when glucose transport or GLUT-1 expression are blocked by cytochalasin B or siRNAs, respectively. Furthermore, ectopic expression of GLUT-1, but not the related transporter GLUT-3, restores HTLV infection abrogated by either GLUT-1 siRNAs or interfering HTLV envelope glycoproteins. Therefore, GLUT-1 is a receptor for HTLV. Perturbations in glucose metabolism resulting from interactions of HTLV envelope glycoproteins with GLUT-1 are likely to contribute to HTLV-associated disorders.

## Introduction

The human T cell leukemia virus (HTLV) type 1 and 2 are present in all areas of the world as endemic or sporadic infectious agents (Slattery et al., 1999). The etiological role of HTLV-1 in adult T cell leukemia (ATL) and tropical spastic paraparesis/HTLV-associated myelopathy (TSP/HAM) has been well established (Gessain et al., 1985; Osame et al., 1986; Poiesz et al., 1980; Yoshida et al., 1982). The apparently restricted tropism of HTLV to T lymphocytes in infected patients (Cavrois et al., 1996; Hanon et al., 2000) contrasts with the ability of the viral-encoded envelope glycoprotein (Env) to bind to and direct entry into all vertebrate cell types tested in vitro (Kim et al., 2003b; Sutton and Littman, 1996; Trejo and Ratner, 2000). Retroviral infections depend on early interactions between Env and cellular receptors. Identification of cellular receptors and coreceptors for other retroviral envelopes have helped to elucidate certain aspects of retrovirus physiopathology as well as their transmission and spreading within organisms and populations (Overbaugh et al., 2001). However, no clear association between HTLV Env and HTLV-associated

diseases has been established and the identity of the receptor(s) for HTLV-1 and HTLV-2 Env has remained elusive.

Numerous cell surface components have been shown to play a role in HTLV Env-mediated syncytia formation (Daenke et al., 1999; Hildreth et al., 1997) and in enhancing virus adsorption and infection (Pinon et al., 2003). Nevertheless, HTLV Env-dependent cell membrane fusion and syncytia formation appear to be distinct from receptor binding per se (Daenke and Booth, 2000; Denesvre et al., 1996; Kim et al., 2000, 2003b). The search for the HTLV Env receptor has been hindered in part by its ubiquitous presence (Jassal et al., 2001; Kim et al., 2003b; Sutton and Littman, 1996). Additionally, the induction of rampant syncytium formation in cell culture upon expression of HTLV Env has prevented its efficient and persistent expression. Based on our observation that the HTLV Env amino-terminal domain shares striking structural and functional homology with that of murine leukemia viruses (MLV) (Battini et al., 1995; Kim et al., 2000, 2003a), we defined the HTLV Env receptor binding domain (RBD) and derived HTLV Env-based tools that overcome the problem of syncytia formation (Kim et al., 2000, 2003b). We were thus able to follow specific interactions between the Env RBD and the HTLV receptor. We found that the HTLV receptor is not expressed on the surface of quiescent T lymphocytes, the major HTLV reservoir in vivo, but is induced following T cell activation (Manel et al., 2003).

Here, we describe striking metabolic alterations in cells expressing HTLV envelopes as well as truncated HTLV receptor binding domains. These alterations are independent of syncytia formation and are characterized by a defect in the acidification of the cell culture medium associated with decreased extracellular lactate production and diminished glucose uptake and consumption. These observations, as well as the knowledge that Env receptors for the related MLV and most gammaretroviruses belong to the family of multimembrane-spanning transporters (Overbaugh et al., 2001), prompted us to test ubiquitous lactate and glucose transport-associated molecules that are upregulated on activated T cells, as receptors for HTLV Env. We show that the ubiquitous glucose transporter GLUT-1 is a specific component of the HTLV receptor and plays an essential role in HTLV-envelope mediated infection. Our observation that interaction of GLUT-1 with the entire HTLV-1 and HTLV-2 envelopes as well as truncated HTLV-1 and HTLV-2 RBD alters glucose metabolism provides new leads regarding the mechanisms implicated in HTLV-associated physiopathological disorders.

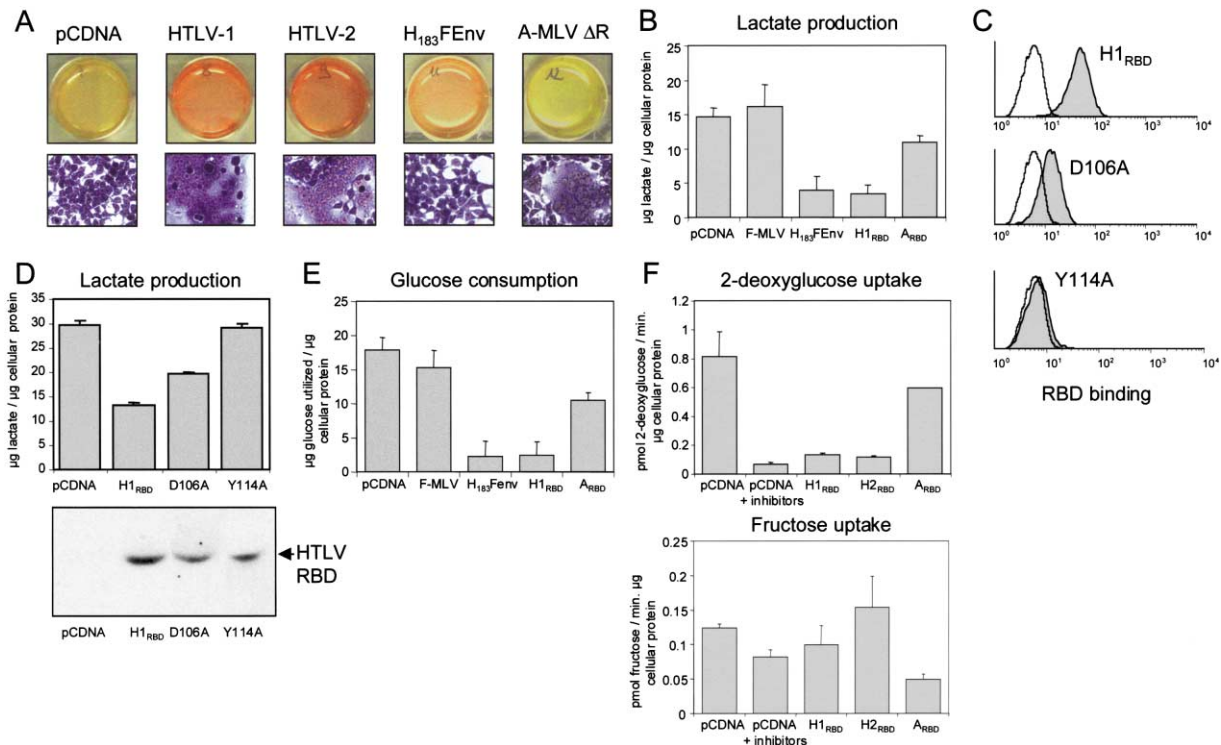
## Results

### HTLV Envelopes and Lactate Metabolism

Cell proliferation in standard culture media is accompanied by acidification of the milieu that translates into a color change from red to yellow tones in the presence of the phenol-red pH indicator. Upon transfection of

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**Figure 1. HTLV Envelope-Receptor Binding Alters Extracellular Lactate Production and Glucose Transport**

(A) Medium acidification and syncytia formation (Giemsa staining) in 293T cells one day post-transfection with control DNA (pCDNA) or Env expression vectors, including syncytial wild-type HTLV-1 Env and HTLV-2 Env, a non-syncytial chimeric HTLV/MLV Env harboring the HTLV receptor binding domain (H<sub>183</sub>FEnv), and a syncytial amphotropic-MLV Env (A-MLV ΔR). Yellow medium indicates normal acidification during cell culture.

(B) Extracellular lactate production in the culture medium of 293T cells was measured two days following transfection with control DNA (pCDNA), with expression vectors containing Friend-MLV (F-MLV) Env, chimeric H<sub>183</sub>FEnv, HTLV-1 (H<sub>1</sub>RBD), or amphotropic MLV RBD (A<sub>RBD</sub>). Mean extracellular lactate production normalized to cellular protein content of triplicate samples is shown.

(C) Binding of rFc-tagged H<sub>1</sub>RBD, D106A, and Y114A H<sub>1</sub>RBD mutants on 293T cells was monitored by FACS analysis following incubation with a FITC-conjugated anti-rabbit IgG antibody.

(D) Extracellular lactate production in the medium of 293T cells one day post-transfection with an irrelevant DNA (pCDNA), H<sub>1</sub>RBD, or the D106A and Y114A H<sub>1</sub>RBD mutants. Data are representative of three independent experiments. The secretion or presence of HTLV Env mutants in the 293T-transfected culture medium was monitored by immunoblotting with a peroxidase-conjugated anti-rabbit IgG antibody.

(E) Extracellular glucose in the culture medium of 293T cells was measured two days following transfection with pCDNA, F-MLV Env, H<sub>183</sub>FEnv, H<sub>1</sub>RBD, or A<sub>RBD</sub>. This measure was used to calculate mean glucose consumption after normalization to cellular protein content.

(F) 2-deoxy[1-<sup>3</sup>H]glucose and [U-<sup>14</sup>C]fructose uptake following either transfection of 293T cells with control DNA (pCDNA), H<sub>1</sub>RBD, H<sub>2</sub>RBD, or A<sub>RBD</sub> expression vectors, or treatment with the cytochalasin B and phloretin glucose transporter inhibitors (pCDNA+inhibitors). Data are the means of triplicate measures and are representative of two to three independent experiments.

293T cells with either highly syncytial HTLV-1 and HTLV-2 envelopes, or a non-syncytial chimeric envelope that harbors the HTLV-1 RBD in an MLV Env backbone (H<sub>183</sub>FEnv), culture medium did not readily become acidic, and harbored red tones for several days post-transfection (Figure 1A). Moreover, expression of truncated soluble HTLV RBD proteins fused with either EGFP, an influenza hemagglutinin peptide tag (HA), or a rabbit immunoglobulin fragment (rFc) also inhibited medium acidification (not shown). In contrast, envelope constructs that lacked HTLV RBD did not have this effect, including different MLV group envelopes, feline, porcine, lentiviral, and Jaagsiekte retroviral Env, as well as VSVG and Ebola glycoproteins (not shown). Syncytium formation was not in itself responsible for this effect; medium acidification was observed in cells expressing a syncytial amphotropic-MLV (A-MLV ΔR) Env (Figure 1A), and was blocked when HTLV Env was ex-

pressed in cells that are resistant to HTLV-Env mediated syncytia formation (NIH3T3 TK<sup>-</sup> cells) (Kim et al., 2003b) (not shown).

The acidification of cell culture media is associated with the extracellular accumulation of lactate (Warburg, 1956). Lactate is the major byproduct of anaerobic glycolysis *in vitro* and its excretion is mediated by an H<sup>+</sup>/lactate symporter (Halestrap and Price, 1999). We monitored lactate content in culture supernatants following transfection of various retroviral envelopes and RBD. Extracellular lactate production was consistently 3-fold lower in H<sub>183</sub>FEnv- and HTLV RBD-transfected cells than in control- or MLV Env-transfected cells (Figure 1B). This difference was detected as early as 4 hr after the addition of fresh media (not shown). Moreover, this decrease in extracellular lactate production after HTLV RBD transfection was DNA dose dependent (not shown).

To examine whether there is a direct relationship be-



tween binding of the HTLV envelope receptor and the subsequent diminution in extracellular lactate production and acidification, we used HTLV RBD mutants with impaired receptor binding capacities. For this purpose, mutations resulting in single alanine substitutions were introduced in H1<sub>RBD</sub>, at either the D106 or Y114 positions, both of which are highly conserved among primate T-lymphotropic viruses (F.J.K., unpublished observations). D106A and Y114A RBD mutants exhibited significantly reduced (D106A) or non-detectable (Y114A) binding to the HTLV receptor as monitored by FACS analysis (Figure 1C) and did not lead to detectable infection (not shown). Moreover, extracellular lactate accumulation was not reduced in cells expressing the non binding Y114A RBD mutant and was only partially reduced in cells harboring the D106A RBD, although both D106A and Y114A RBD mutants were expressed and secreted as efficiently as the wild-type H1<sub>RBD</sub> (Figure 1D). Similar results were obtained with H2<sub>RBD</sub> harboring the same allelic mutations (not shown), following transfection of these mutants in HeLa cells. Therefore, perturbations in lactate metabolism directly correlated with binding of HTLV RBD to the HTLV receptor.

Extracellular lactate accumulates in cell cultures following its transport across cellular membranes by the multi-membrane-spanning MCT1 monocarboxylate transporter (Garcia et al., 1994). Because HTLV and MLV envelopes share a common modular organization (Kim et al., 2000, 2003a) and the receptors for MLV Env are multi-membrane-spanning metabolite transporters (Overbaugh et al., 2001), we assessed whether the HTLV RBD bound to MCT1. Moreover, expression of the MCT1 chaperone CD147 (Kirk et al., 2000) increases during T cell activation (Kasinrerk et al., 1992), a characteristic of the HTLV receptor (Manel et al., 2003; Nath et al., 2003). Nevertheless, separate and combined overexpression of MCT1 and CD147 did not result in increased H1<sub>RBD</sub> binding (not shown), arguing against a role for these molecules as receptors for HTLV Env.

#### HTLV Envelopes Alter Glucose Metabolism

In addition to a decrease in extracellular lactate accumulation, expression of the HTLV RBD also led to decreased intracellular lactate content (not shown), indicative of metabolic alterations upstream of lactate transport. In cell cultures, lactate production results from the degradation of glucose during anaerobic glycolysis. Therefore, we assessed whether the decreased accumulation of lactate observed upon expression of HTLV RBD was linked to glucose metabolism. We measured glucose consumption as an indicator of the rate of glycolysis. Glucose consumption of cells expressing an HTLV RBD within the context of the H<sub>183</sub>FEnv entire envelope or the tagged H1<sub>RBD</sub> was significantly decreased as compared to that of control cells (Figure 1E) and this defect was detected as early as 8 hr post-transfection (not shown). Notably, the transfection efficiencies in the experiments ranged from 80%–95% (not shown).

We found that this decrease in glucose consumption correlated with a decrease in glucose transport across the cellular membrane. Transfection of either HTLV-1 or HTLV-2 RBD resulted in an approximately 4-fold decrease in 2-deoxyglucose uptake (Figure 1F), compara-

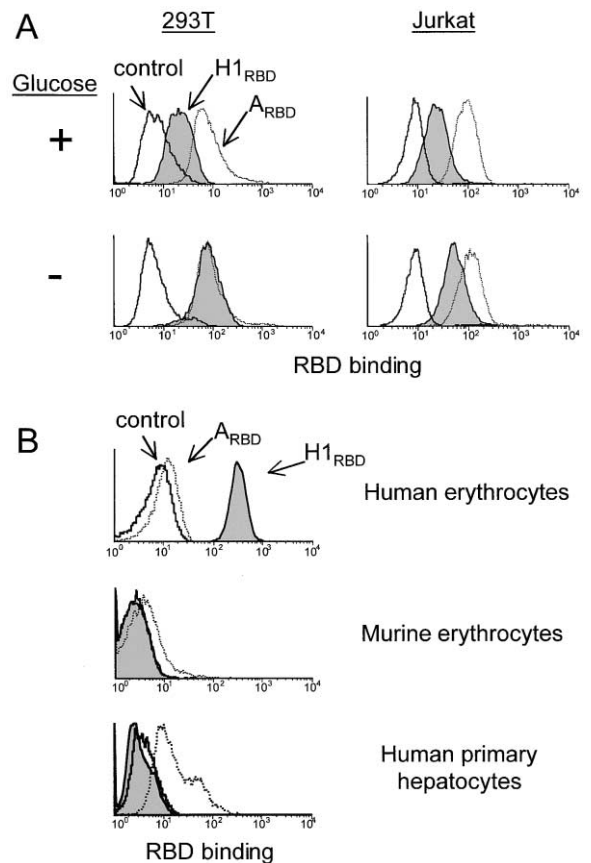


Figure 2. Expression of the HTLV Receptor on Glucose-Starved Cell Lines and Primary Cells

(A) Expression of the HTLV and amphotropic-MLV receptors on 293T and Jurkat T cells cultured overnight in the presence (1 g/l) or absence of glucose was monitored by binding of H1<sub>RBD</sub> and A<sub>RBD</sub>, respectively.

(B) Expression of the HTLV and amphotropic-MLV receptors on the surface of freshly isolated human and murine erythrocytes, and primary human hepatocytes was monitored by binding of H1<sub>RBD</sub> and A<sub>RBD</sub>, respectively. Binding of the HTLV envelope RBD is shown in filled histograms, A<sub>RBD</sub> binding is shown in dotted histograms, and control immunofluorescence is shown in solid line histograms.

ble to the decrease observed when glucose uptake was inhibited by cytochalasin B and phloretin. The envelope-mediated inhibition was specific to HTLV, since the A-MLV RBD (A<sub>RBD</sub>) control envelope had only a minor effect on glucose uptake. Moreover, the inhibitory effect of HTLV envelopes was specific to glucose uptake, since 3-O-methylglucose transport was similarly inhibited (not shown) whereas fructose uptake was not altered by the presence of either HTLV-1 or HTLV-2 RBD (Figure 1F). The decrease in glucose transport was likely due to the steady state expression of the HTLV RBD following transfection, as the level of the secreted RBD was insufficient to modulate glucose metabolism.

We next evaluated the effect of glucose deprivation on the availability of the HTLV receptor in both adherent 293T cells and suspension Jurkat T cells. After overnight culture of cells in the absence of glucose, binding of H1<sub>RBD</sub> was surprisingly increased by 2-fold in both cell types (Figure 2A). This effect of glucose deprivation was

specific to HTLV as A<sub>RBD</sub> binding was only marginally altered under the same conditions (Figure 2A). This phenomenon is reminiscent of a general metabolite transport feedback loop, whereby transporter availability at the cell surface increases upon substrate starvation.

#### GLUT-1 Expression Parallels HTLV RBD Binding

Inhibition of glucose consumption via binding of the HTLV RBD to a glucose transporter is compatible with the metabolic effects described above. Upon evaluation of the different glucose transporter candidates, GLUT-1 was the only one to present all the known properties of the HTLV receptor. Indeed, GLUT-1 expression is increased upon glucose deprivation and is involved in glucose transport in all vertebrates (Mueckler et al., 1985). Furthermore, GLUT-1 is not expressed on quiescent primary T cells and its expression is induced upon T cell activation (Chakrabarti et al., 1994; Rathmell et al., 2000) with kinetics that are strikingly similar to what we and others have reported for the HTLV receptor (Manel et al., 2003; Nath et al., 2003). Since human, but not murine, erythrocytes have been described to exhibit the highest concentration of GLUT-1 (Mantych et al., 1991; Mueckler, 1994), we compared HTLV receptor availability on freshly isolated human and murine red blood cells. Binding of H1<sub>RBD</sub> to human erythrocytes was remarkably efficient, reaching levels higher than those observed on any other tested cell type (Figure 2B and not shown). In marked contrast, no significant H1<sub>RBD</sub> binding was detected on murine erythrocytes although comparably low levels of A<sub>RBD</sub> binding were observed on murine and human erythrocytes. Similarly, primary human hepatocytes which do not express GLUT-1 (Mueckler, 1994) did not bind H1<sub>RBD</sub>, while A<sub>RBD</sub> binding on these cells was readily detected (Figure 2B). Thus, the expression of GLUT-1 parallels that of the HTLV receptor.

#### HTLV Envelopes Bind GLUT-1

In order to directly test the ability of HTLV envelopes to bind GLUT-1, we derived a tagged GLUT-1 expression vector and overexpressed this protein in 293T cells. Binding of both H1<sub>RBD</sub> and H2<sub>RBD</sub> were dramatically increased upon GLUT-1 overexpression (Figure 3A). This interaction required a functional HTLV RBD as binding of the D102A HTLV-2 mutant did not increase following overexpression of GLUT-1 (Figure 3A). Moreover, this interaction was specific to GLUT-1, since H1<sub>RBD</sub> and H2<sub>RBD</sub> binding remained at background levels upon overexpression of the A-MLV envelope receptor, the inorganic phosphate transporter PIT-2 (Miller et al., 1994; van Zeijl et al., 1994). However, as expected, binding of A<sub>RBD</sub> was specifically increased upon transfection of PIT-2, but remained unchanged after GLUT-1 overexpression (Figure 3A).

The specificity of the interaction between GLUT-1 and HTLV RBD was further confirmed by experiments using GLUT-3, the closest isoform to GLUT-1 whose glucose transport kinetics are similar to that of GLUT-1 (Asano et al., 1992; Joost and Thorens, 2001). GLUT-3 is expressed at similar levels as GLUT-1 (See Supplemental Data available at <http://www.cell.com/cgi/content/full/115/4/449/DC1>), and like GLUT-1, alleviates the block in glucose metabolism induced by the HTLV RBD (see

below). However, unlike GLUT-1, overexpression of GLUT-3 did not increase H1<sub>RBD</sub> and H2<sub>RBD</sub> binding (Figure 3A). These data demonstrate that increased HTLV RBD binding is specific to GLUT-1 expression and not an indirect consequence of increased glucose uptake. To confirm that increased H1<sub>RBD</sub> binding was directly due to those cells that overexpressed GLUT-1, we derived DsRed2 fluorochrome-tagged GLUT-1 and GLUT-3 expression vectors to unequivocally identify GLUT-overexpressing cells. In this context, we found that those cells overexpressing DsRed2-tagged GLUT-1 displayed significantly increased H1<sub>RBD</sub> binding, as demonstrated by FACS analysis (Figure 3B). H1<sub>RBD</sub> binding was not altered in cells overexpressing the tagged-GLUT-3.

A direct interaction between GLUT-1 and HTLV envelopes was demonstrated by coimmunoprecipitation of H1<sub>RBD</sub> and GLUT-1. As shown in Figure 4, GLUT-1 was detected upon immunoprecipitation of H1<sub>RBD</sub>-rFc with anti-rabbit-IgG-coated beads. The coimmunoprecipitation of GLUT-1 with the H1<sub>RBD</sub> Y114A receptor binding defective mutant was dramatically diminished. No coimmunoprecipitation was observed in the absence of HTLV RBD.

#### GLUT-1 Mediates HTLV Infection

As demonstrated above, HTLV envelopes physically associate with GLUT-1 and this interaction modulates glucose metabolism. To determine whether this interaction is essential for infection, it would have been opportune to use GLUT-1-deficient cell lines. However, since there are no GLUT-1 null cell lines and since quiescent primary cells lacking GLUT-1 are not amenable to HTLV infection assays, we developed three different models of HTLV envelope-driven infection in which either receptor availability or GLUT-1 expression were limiting.

In the first model, we monitored HTLV envelope-driven infection in the presence of cytochalasin B which inhibits glucose transport (Kasahara and Hinkle, 1977) and which we found to inhibit HTLV RBD binding. Indeed, treatment of Jurkat T cells with cytochalasin B dramatically inhibited binding of H1<sub>RBD</sub>, whereas binding of A<sub>RBD</sub> remained unchanged (Figure 5A). To determine whether this effect was due to the role of cytochalasin B as a ligand for GLUT-1 or, alternatively, was indirectly due to its indirect effects on microfilaments, cells were treated with the related molecule cytochalasin D. This latter molecule, which is not a ligand of GLUT-1, led to a low-level, nonspecific decrease in both HTLV and A<sub>RBD</sub> binding (see Supplemental Data at above URL). Thus, these data suggest that the cytochalasin B-mediated inhibition of HTLV RBD binding is largely due to its interaction with GLUT-1 and not to an impact on actin microfilaments.

To determine whether the cytochalasin B-mediated decrease in receptor availability would translate into an effect on HTLV envelope-driven infection, control and cytochalasin B-treated cells were infected with virions harboring either the HTLV or A-MLV envelopes (Figure 5B). A 75% inhibition of HTLV envelope-driven infection was observed after cytochalasin B treatment. In contrast, A-MLV envelope-driven infection was not reduced, but was in fact increased.

In the second model, HTLV receptor availability was

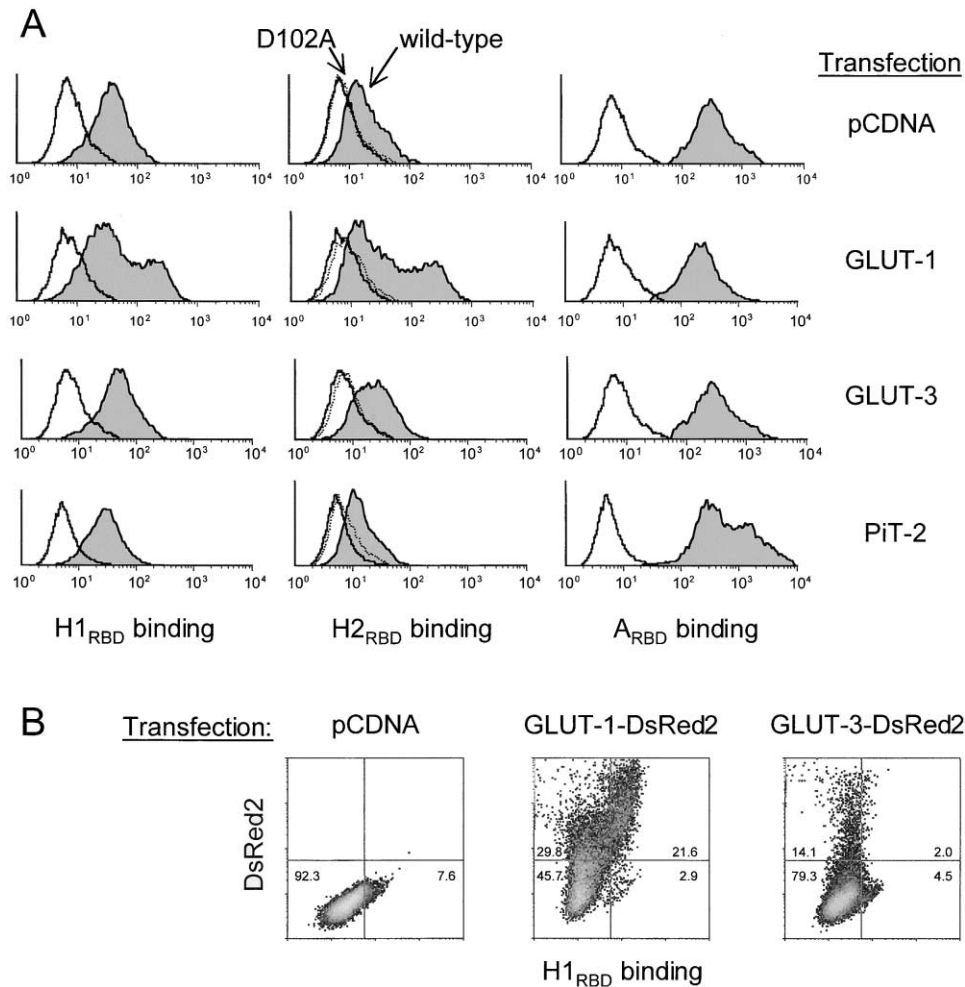


Figure 3. HTLV Envelope-Receptor Binding Increases in GLUT-1 Overexpressing Cells

(A) Binding of H1<sub>RBD</sub>, H2<sub>RBD</sub>, H2<sub>RBD</sub> D102A mutant, and A<sub>RBD</sub> to control 293T cells or 293T cells overexpressing either GLUT-1, GLUT-3, or PiT-2. Intrinsic fluorescence (solid line histograms), wild-type RBD binding (filled histograms), and mutant D102A binding (dotted lines histograms) are shown.

(B) Correlation between GLUT-1 expression and HTLV RBD binding in individual cells. FACS analyses of H2<sub>RBD</sub>-EGFP binding to 293T cells transfected with control DNA (pCDNA), GLUT-1-DsRed2, or GLUT-3-DsRed2 expression vectors are shown. Quadrants were drawn to separate cells with basal HTLV RBD binding from cells with high binding. The percentage of cells in each quadrant is indicated.

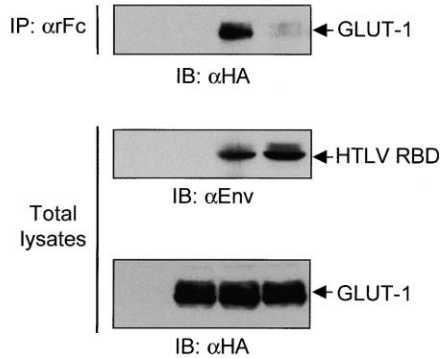
specifically limited by the intracellular expression of an interfering HTLV envelope RBD (H2<sub>RBD</sub>). In these conditions, both extracellular binding of HTLV envelope (Figure 6A) and infection by HTLV envelope-harboring virions (Figure 6B) were dramatically reduced. Overexpression of GLUT-1 in these cells rescued HTLV envelope-mediated binding and infection. Although overexpression of GLUT-3 in these cells reversed defects in lactate and glucose metabolism (Figure 6C and not shown), HTLV envelope binding and viral titers remained significantly reduced. Expression of the interfering H2<sub>RBD</sub> was equivalent in all conditions of glucose transporter overexpression (Figure 6D). These results confirmed the specific relationship between HTLV receptor availability and GLUT-1 expression.

In the third model, we used siRNAs directed against the GLUT-1 3'UTR to specifically down-modulate endogenous GLUT-1 expression. Transfection of those GLUT-1 siRNAs led to a concomitant decrease in HTLV

RBD binding (Figure 7A) and a 75% inhibition of HTLV envelope-mediated infection (Figure 7B). As observed in the receptor interference experiments, overexpression of GLUT-1, but not GLUT-3, restored HTLV RBD binding and HTLV envelope-mediated infection (Figures 7A and 7B). The effect of the GLUT-1 siRNAs on HTLV envelope-driven infection was not due to metabolic perturbations per se as both GLUT-1 and GLUT-3 overexpression reestablished glucose transport and lactate production (Figure 7C and data not shown). Furthermore, GLUT-1 siRNAs did not inhibit A-MLV envelope-mediated binding and infection. Down-modulation of GLUT-1 by siRNAs, and overexpression of ectopic GLUT-1 following transfection were directly verified in these different cell culture conditions by immunoblotting with an anti-GLUT-1 polyclonal antibody (Figure 7D). Altogether, these data demonstrate that HTLV envelope-mediated viral entry occurs through a direct interaction with GLUT-1.

**Transfection**

GLUT-1-HA	-	+	+	+
H1 <sub>RBD</sub> -rFc	-	-	+	-
H1 <sub>Y114A</sub> -rFc	-	-	-	+



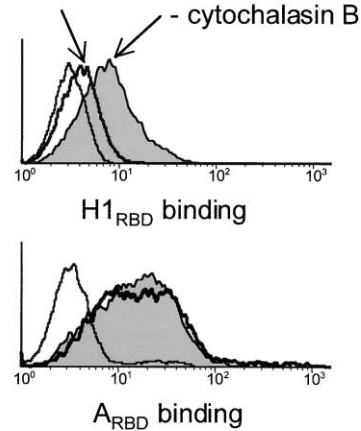
**Figure 4. GLUT-1 Physically Associates with the HTLV Envelope**  
Immunoprecipitation of HA-tagged GLUT-1 from 293T cells transfected with expression vectors carrying control DNA, GLUT-1 alone, or a combination of GLUT-1 with either wild-type H1<sub>RBD</sub> or the Y114A H1<sub>RBD</sub> mutant. Immunoprecipitations were performed using anti-rabbit-IgG beads and blots were probed with an anti-HA antibody. Total cell extracts were blotted using a monoclonal anti-HTLV envelope antibody (1C11) and an anti-HA antibody to monitor HTLV RBD and GLUT-1 levels, respectively.

**Discussion**

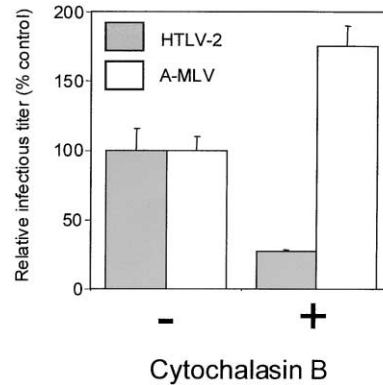
Here we show that the HTLV-1 and -2 receptor binding domains physically associate with the glucose transporter GLUT-1. This interaction strongly inhibited glucose uptake and consumption, leading to decreased lactate production and a drop in the acidification of the extracellular milieu. This effect was specific as HTLV-1 and 2 envelopes with mutations that interfered with receptor binding did not block glucose consumption (not shown). There was a rapid increase in HTLV envelope binding upon glucose starvation, highlighting a nutrient-sensing negative feedback loop between glucose availability and cell surface HTLV receptor expression. In this context, we explored whether GLUT-1, the ubiquitous glucose transporter, was a receptor for HTLV Env-mediated viral entry. Because antibodies that reliably interact with all the glycosylated forms of GLUT-1 have been raised against internal determinants, they could not be used to inhibit HTLV Env-mediated viral entry (M. Mueckler, personal communication). Nevertheless, extensive evidence converged to identify GLUT-1 as the receptor for HTLV envelope-mediated binding and infection: HTLV envelopes bind all vertebrate cell lines but not insect cells and GLUT-1 orthologs are conserved among vertebrates but are highly divergent between vertebrates and insects (Escher and Rasmuson-Lestander, 1999); highest binding of HTLV RBD is observed on human erythrocytes, where GLUT-1 is the major glucose transporter isoform (Concha et al., 1997); HTLV RBD binding is not detected on human primary hepatocytes and murine erythrocytes where GLUT-1 is known to be only minimally expressed (Mantych et al., 1991; Mueckler, 1994);

**A**

+ cytochalasin B



**B**



**Figure 5. Cytochalasin B Inhibits HTLV Envelope Binding and Infection**

(A) H1<sub>RBD</sub> and A<sub>RBD</sub> binding were monitored on Jurkat cells pretreated with or without 20 μM of the GLUT-1 inhibitor cytochalasin B. (B) Relative infectious titers of virions pseudotyped with either HTLV-2 (shaded) or A-MLV (open) envelopes were assessed in control and cytochalasin B-treated HeLa cells. Relative infectious titers were calculated from input virions ranging from 10 to 80 PFU per well for both HTLV-2 and A-MLV.

HTLV RBD binding is increased upon overexpression of GLUT-1 but not GLUT-3; and GLUT-1 coimmunoprecipitates with H1<sub>RBD</sub>. Moreover, as we previously demonstrated for the HTLV receptor (Manel et al., 2003), GLUT-1 is not expressed on resting T lymphocytes whereas the GLUT 2-4 isoforms are present (Chakrabarti et al., 1994; Korgun et al., 2002), and GLUT-1 is induced upon immunological (Frauwirth et al., 2002; Yu et al., 2003) or pharmacological (Chakrabarti et al., 1994) activation. Finally, GLUT-1 overexpression specifically restores HTLV envelope-mediated infection after receptor blocking with either an interfering HTLV envelope or GLUT-1 siRNAs. GLUT-1 is thus a new member of the multimembrane spanning metabolite transporter family known to serve as receptors for retroviral envelopes. Interestingly, until now, all envelopes that recognize such receptors have been shown to be encoded by retroviruses that have a “simple” genetic organization,

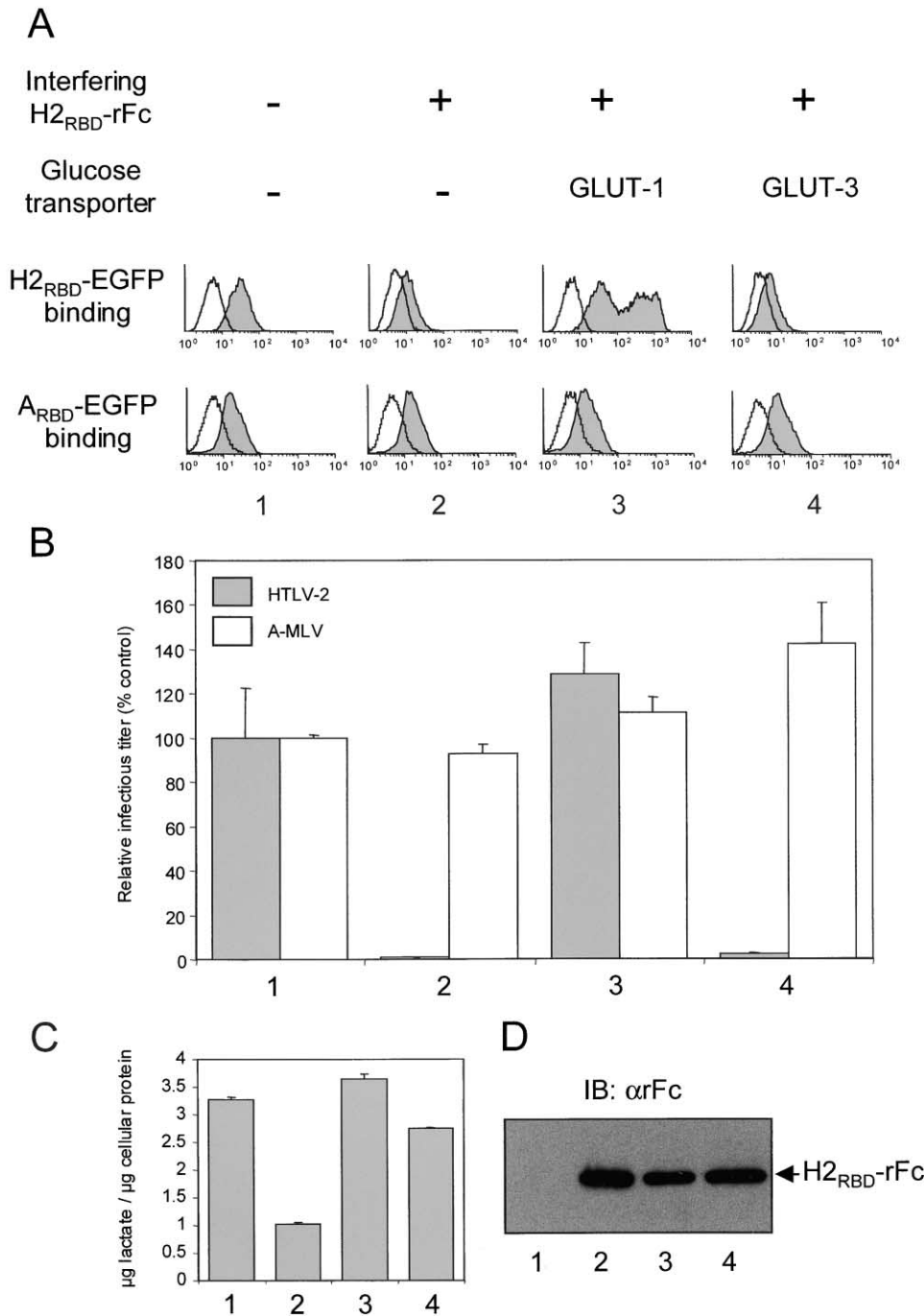


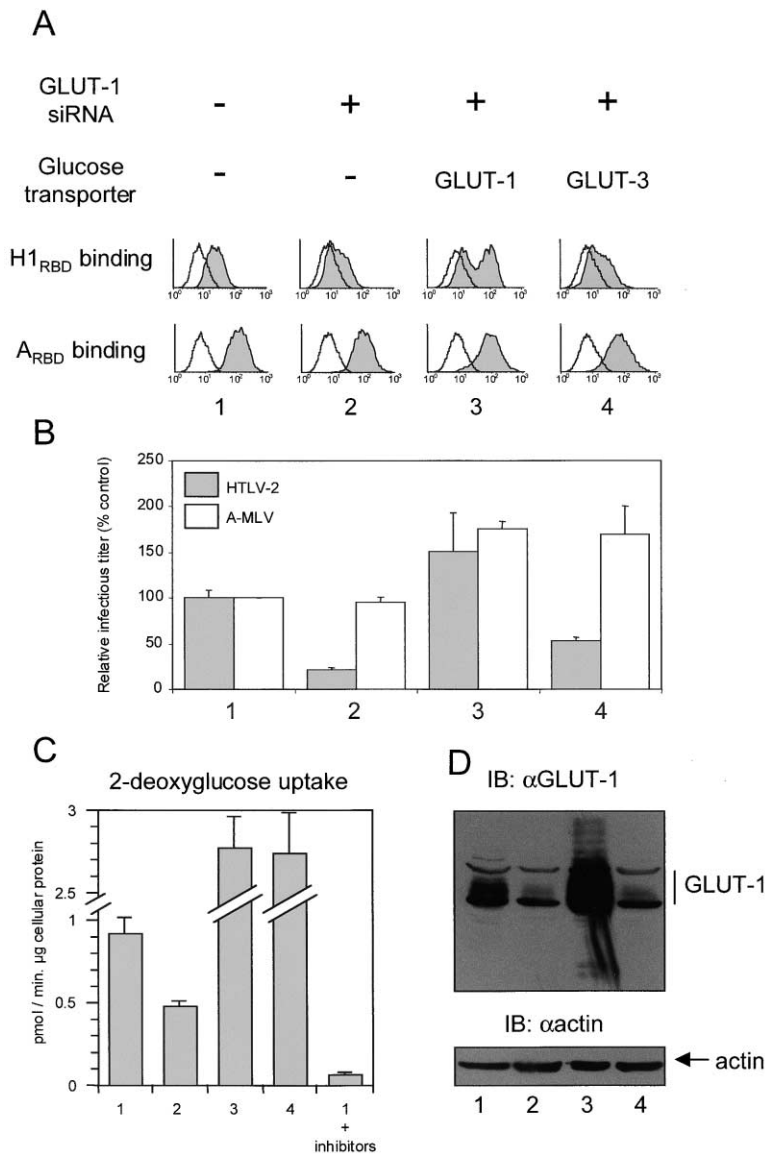
Figure 6. RBD Interference to HTLV Envelope Binding and Infection Is Alleviated by GLUT-1

293T cells were transfected with an irrelevant or interfering HTLV RBD expression vector, alone or together with GLUT-1 or GLUT-3 expression vectors. Transfected cells were assayed for (A) H1<sub>RBD</sub> and A<sub>RBD</sub> binding. Specific binding and intrinsic fluorescence are indicated by filled and solid histograms, respectively. (B) Relative infection titers of virions pseudotyped with either HTLV-2 (shaded) or A-MLV (open) envelopes. Input virions ranged from 50 to 300 PFU per well. (C) Extracellular lactate accumulation in 293T cell culture medium one day after transfection. (D) HTLV RBD (H2<sub>RBD</sub>) expression was monitored by immunoblotting of total protein extracts with an anti-rabbit-IgG antibody.

such as MLV, feline leukemia viruses, porcine endogenous retrovirus, and the gibbon ape leukemia virus (Overbaugh et al., 2001; Taylor et al., 2003), whereas HTLV is a “complex” retrovirus that codes for several additional regulatory proteins. However, we have shown that in contrast to the wide phylogenetic divergence of their

genomes, the HTLV and MLV envelopes share a similar modular organization with highly conserved amino acid motifs in their respective receptor binding domains (Kim et al., 2000, 2003a).

Cell-to-cell contact and oriented cytoskeleton movements appear to be required for HTLV transmission (Iga-



**Figure 7. GLUT-1 Is Required for HTLV Envelope Binding and Infection**

293T cells were transfected with a mixture of three GLUT-1 specific siRNAs or irrelevant siRNAs in combination with either an empty, a GLUT-1, or a GLUT-3 expression vector. Transfected cells were assayed for (A) H1<sub>RBD</sub> and A<sub>RBD</sub> binding. Specific binding and intrinsic fluorescence are indicated by filled and solid-line histograms, respectively. (B) Relative infection titers of virions pseudotyped with either HTLV-2 (shaded) or A-MLV (open) envelopes. Input virions ranged from 50 to 400 PFU per well. (C) 2-deoxy[1-<sup>3</sup>H]glucose uptake. Uptake was measured in cells transfected with the GLUT-1 siRNAs and GLUT-1 and GLUT-3 expression vectors. Cells incubated with cytochalasin B and phloretin inhibitors were used as controls for glucose transport inhibition. (D) Endogenous and ectopic GLUT-1 expression; expression was monitored by immunoblotting total protein extracts with an anti-GLUT-1 antibody. Equivalent protein loading was confirmed by immunoblotting with an anti-actin antibody.

kura et al., 2003). A role for envelope and receptor interactions in these processes has been suggested (Derse and Heidecker, 2003). In agreement with these observations, we found that the HTLV receptor, despite pancellular expression, is specifically concentrated to mobile membrane regions and cell-to-cell contact areas (Supplemental Data). This suggests that the HTLV envelope receptor associates with the cytoskeleton. Indeed, a cytoplasmic binding partner of GLUT-1, GLUT1CBP, which encodes a PDZ domain, has been reported to link GLUT-1 to the cytoskeleton (Bunn et al., 1999). It will therefore be of interest to evaluate the respective impact of the HTLV envelope on membrane organization, its cytoskeleton-associated cellular partners, such as GLUT-1 and GLUT1CBP, and other known or yet to be identified interacting cell surface components.

Because expression of the HTLV receptor is induced upon glucose starvation, transmission of HTLV may be more efficient among cells that are locally starved of glucose and express high levels of GLUT-1, such as

immature thymocytes (Feuer et al., 1996; Yu et al., 2003). The ability of circulating erythrocytes to dock HTLV, as shown here, might provide a means of distributing HTLV to such tissues, provided that they are not readily eliminated by the reticuloendothelial system. Elucidation of GLUT-1 cell surface recycling upon HTLV envelope binding to different cell types, including murine and human erythrocytes, may offer further clues to the *in vivo* distribution of HTLV during infection.

The identification of GLUT-1 as a receptor for HTLV envelopes provides insight into the paradox between the ubiquitous *in vitro* expression of the receptor on cell lines and the apparent restriction of HTLV tropism to T lymphocytes *in vivo*. Early after infection, rapid and dramatic metabolic alterations associated with decreased glucose consumption are likely to occur upon expression of the HTLV envelope. It is tempting to propose that during *in vivo* infection, HTLV initially spreads with a large tropism, but that the vast majority of cells that are dependent on GLUT-1 activity and concomi-

tantly express the HTLV envelope are rapidly eliminated. In contrast, resting T lymphocytes that have a low metabolic rate and as such are much less dependent on glucose uptake, are more likely to tolerate this effect and survive infection. Furthermore, local imbalances in glucose uptake, following HTLV infection may lead to specific physiological alterations (Akaoka et al., 2001). In this regard, the potential relationship between HTLV-associated neuropathologies and the specific dependence of neurons on GLUT-1 mediated glucose consumption (De Vivo et al., 1991; Siegel et al., 1998) becomes particularly relevant.

#### Experimental Procedures

##### Cell Culture and Virus Preparations

Human 293T and HeLa cells were grown in DMEM with high glucose (4.5 g/l) and Jurkat T cells were grown in RPMI with 10% fetal bovine serum (FBS) at 37°C. Freshly isolated hepatocytes were obtained following surgery in accordance with the Hospital Ethics Board as published (Pichard et al., 1990). For glucose starvation experiments, cells were grown in either glucose-free DMEM (Life Technologies) or glucose-free RPMI (Dutscher) with 10% dialyzed FBS (Life Technologies) and glucose (1 g/l) was supplemented when indicated. Replication-defective LacZ retroviral vector was pseudotyped with either HTLV or A-MLV envelopes as previously described (Kim et al., 2003b).

##### Expression Vectors and siRNAs

Full-length envelope expression vectors have been described elsewhere (Denesvre et al., 1995 1996; Rosenberg et al., 1998). The chimeric HTLV/MLV Env, H<sub>183</sub>FEnv, was obtained by replacing the first 269 amino acid residues of the F-MLV Env with the 183 amino-terminal residues of the HTLV-1 Env. H1<sub>RBD</sub>, H2<sub>RBD</sub>, and A<sub>RBD</sub> comprise the first 215 aa, 178 aa, and 397 aa of the HTLV-1, HTLV-2, and A-MLV SU, respectively, fused to either a carboxy-terminal rabbit immunoglobulin Fc (rFc) tag or to the enhanced green fluorescence protein (EGFP) coding sequence. Construction of the chimeric envelope as well as wild-type and mutant truncated envelope expression vectors will be detailed elsewhere. Human *Glut-1* and *Glut-3* cDNA were amplified by PCR from the pLib HeLa cDNA library (Clontech) from the first AUG to the last codon before the termination codon, and individually inserted into pCHIX, a modified version of the pCSI vector (Battini et al., 1999) that includes a C-terminal factor Xa cleavage site, and the hemagglutinin (HA) and histidine tags. *Glut-1* and *Glut-3* cDNA were also inserted into a modified pCSI vector that includes a DsRed2 C-terminal tag. Similarly, human CD147 was amplified from 293T total RNA and inserted into pCHIX. The rat MCT1 expression vector was a gift from J. Mercier and G. Py and the VSVG-tagged human Pit-2 expression vector was a gift from J.-M. Heard (Rodrigues and Heard, 1999). The sequences of the three synthetic annealed siRNAs complementary to the GLUT-1 3'UTR, and which are not present in the GLUT-1 expression vector, are UGAUGUCCAGAAGAAUJUTT, AAUAUUCUUCUGGACAUCATT, and UAUAUAAUACAGACACUAATT (Eurogentec, plus strand indicated).

##### Envelope Expression and Metabolic Measurements

293T cells were transfected with the envelope expression vectors using the calcium phosphate method. After an overnight transfection, cells were washed in phosphate-buffered saline (PBS) and fresh medium was added. Media were harvested, filtered through a 0.45 μm pore-size filter, and lactate and glucose were measured with enzymatic diagnostic kits (Sigma). Values were normalized to cellular protein content using the Bradford assay (Sigma) after solubilization of cells in lysis buffer (Kim et al., 2000) before clarification by centrifugation.

##### Hexose Uptake Assays

The hexose uptake assay was adapted from Harrison et al. (1990) using 2-deoxy-D[1-<sup>3</sup>H]glucose, 3-O-[<sup>14</sup>C]methyl-D-glucose and

D[U-<sup>14</sup>C]fructose (Amersham). After transfection of Env expression vectors, approximately  $2.5 \times 10^5$  293T cells per well were seeded in 24-well plates. The next day, cells were washed in PBS, incubated in serum-free DMEM, washed in serum/glucose-free DMEM, and incubated for 20 min in 500 μl serum/glucose-free DMEM in the presence or absence of inhibitors (20 μM cytochalasin B and 300 μM phloretin; Sigma). Uptake was initiated by adding labeled hexoses to a final concentration of 0.1 mM (2 μCi/ml for 2-deoxy-D[1-<sup>3</sup>H]glucose and 0.2 μCi/ml for D[U-<sup>14</sup>C]fructose and 3-O-[<sup>14</sup>C]methyl-D-glucose) and cells were incubated for an additional five minutes. Cells were then resuspended in 500 μl cold serum/glucose-free DMEM, washed in serum/glucose-free DMEM, and solubilized in 400 μl of 0.1% SDS. Three μl were used for Bradford normalization, and <sup>3</sup>H or <sup>14</sup>C incorporation in the remainder of the samples was counted.

##### Immunoblots

Culture media (10 μl) from 293T cells transfected with HTLV RBD, or lysates from cells overexpressing GLUT-1 or GLUT-3, alone or in combination with HTLV-2 RBD, were electrophoresed in SDS-12.5% acrylamide gels, transferred and probed with either a peroxidase-conjugated anti-rabbit immunoglobulin antiserum or mouse monoclonal antibodies directed against HTLV Env (1C11), HA tag (12CA5), or actin (C4, ICN), or a rabbit polyclonal serum raised against a GLUT-1 carboxy terminal synthetic peptide (generous gift from M. Mueckler), followed by the corresponding peroxidase-conjugated anti-immunoglobulin antiserum. Proteins were visualized using the ECLplus kit (Amersham).

##### Coimmunoprecipitation Assay

293T cells were transfected with the various envelope expression vectors, alone or in combination with the GLUT-1 expression vector. Twenty-four hours following transfection, cells were lysed in Brij buffer (1% Brij97, 150 mM NaCl, 20 mM Tris [pH 7.5], and 5 mM EDTA); 10% of the lysate was used for immunoblots of total protein extracts, while 90% of the lysate was immunoprecipitated with agarose beads coupled to goat anti-rabbit IgG immunoglobulins (Sigma) for 4 hr at 4°C. Beads were washed four times with Brij buffer, denatured in loading buffer, and processed for immunoblotting.

##### Binding Assays

Binding assays were carried out as previously described (Manel et al., 2003). Briefly,  $5 \times 10^5$  cells were washed with PBA (PBS containing 1% bovine serum albumin [BSA] and 0.1% sodium azide), incubated with 300 μl of control, H<sub>1</sub><sub>RBD</sub>, or A<sub>RBD</sub> supernatants for 30 min at 37°C, washed, and labeled for 30 min on ice with a FITC-conjugated sheep anti-rFc antibody (1:500 dilution; Sigma). Cells were immediately analyzed on a FACSCalibur (Becton Dickinson). When indicated, cytochalasin B (20 μM; Sigma) was added to cells for 1 hr prior to binding analyses.

##### Infections

10<sup>5</sup> 293T target cells were treated as follows prior to infection: (1) incubation for 30 min with 20 μM cytochalasin B, (2) transfection with either the interfering H2<sub>RBD</sub> or pCDNA in the presence or absence of GLUT-1 or GLUT-3 expression vectors, or (3) transfection with 20 pmol of an equimolar mixture of three siRNAs complementary to the endogenous GLUT-1 3'UTR in the presence or absence of GLUT-1 or GLUT-3 cDNA expression vectors. Infections were carried out with serial dilutions of LacZ retroviral vectors pseudotyped with either HTLV-2 or A-MLV envelopes. The following day, fresh medium supplemented with fructose (5 g/l) was added. Twenty-four hours later, LacZ expression was monitored by X-Gal staining.

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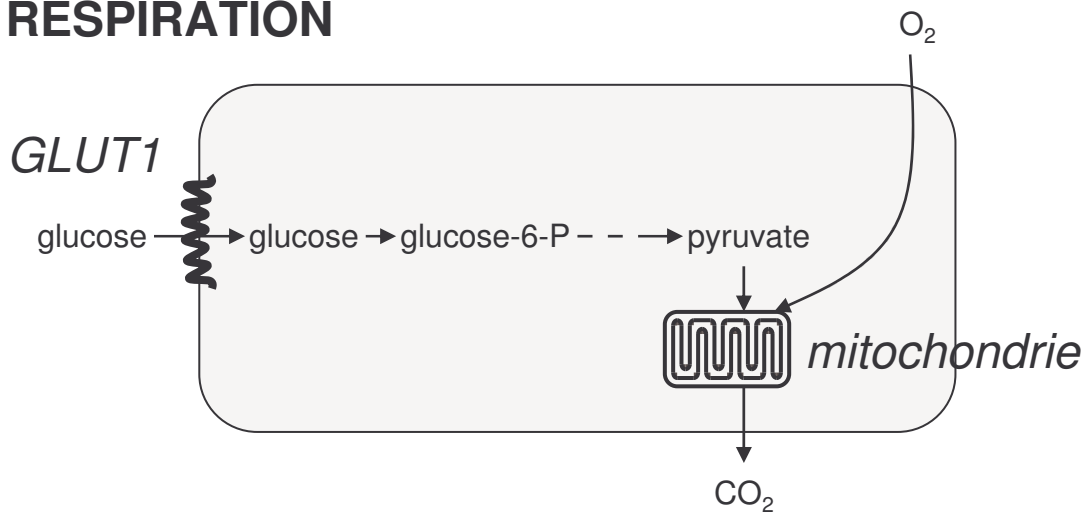
### B.3. Conclusion article 2

L'identification de GLUT1, un transporteur du glucose, comme récepteur HTLV est en accord avec l'homologie structurale observée entre l'Env HTLV et les Env des gammarétrovirus, associée à l'utilisation par ces derniers de transporteurs de nutriments comme récepteurs cellulaires. La capacité de l'Env HTLV à inhiber l'activité de transport de GLUT1 est particulièrement frappante. En effet, alors que GLUT1 est en amont de la glycolyse, toute la glycolyse se trouve affectée par cette inhibition et elle se traduit par un effet macroscopique sur la couleur d'un indicateur de pH du milieu extracellulaire, sans qu'une compensation métabolique immédiate n'ait pris le relais. En conditions normales, le lactate, produit de la fermentation lactique, est co-sécrété dans le milieu extracellulaire avec un proton par un membre de la famille des co-transporteurs de monocarboxylate (*e.g.* MCT1). La fermentation lactique, ubiquitaire dans les systèmes cellulaires *in vitro*, n'est pourtant pas la règle *in vivo*. En effet la plupart des cellules de mammifère produisent leur ATP par respiration, en dégradant dans la mitochondrie le pyruvate issu de la glycolyse, en combinaison avec l'O<sub>2</sub>, sous forme de CO<sub>2</sub> (Figure 7).

Le blocage de toute la glycolyse par l'inhibition de GLUT1 souligne (i) la forte dépendance des lignées utilisées pour le glucose comme substrat de la glycolyse ainsi que (ii) la quasi exclusive dépendance de GLUT1 comme transporteur en amont de la glycolyse, alors qu'il existe au moins 13 autres membres dans cette famille de transporteurs de glucose (Joost and Thorens 2001). Dans un autre système, il a été observé que des cellules déficientes en HIF-1, un facteur impliqué dans l'adaptation au micro-environnement hypoxique des tumeurs, montrent une baisse de l'expression de GLUT1 et sont également moins tumorigéniques que des cellules sauvages (Williams, Telfer et al. 2002). Ces données vont dans le sens d'un avantage procuré par GLUT1 pour la glycolyse et en conséquence la tumorigénèse.

La conséquence sur le long terme de l'inhibition de la glycolyse par l'Env HTLV n'a pas été étudiée, mais il est clair qu'il est crucial de déterminer si elle conduit à la mort

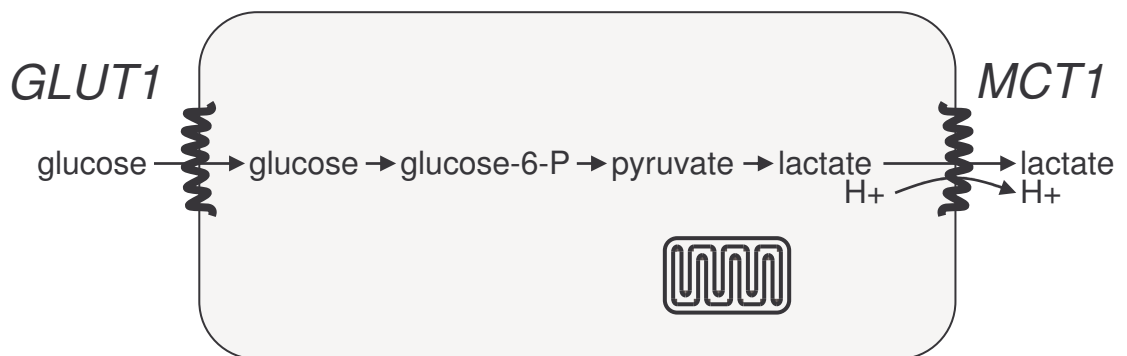
## RESPIRATION



## FERMENTATION LACTIQUE

En absence d' $\text{O}_2$  : glycolyse anaérobie

En présence d' $\text{O}_2$  : glycolyse aérobie (effet Crabtree)



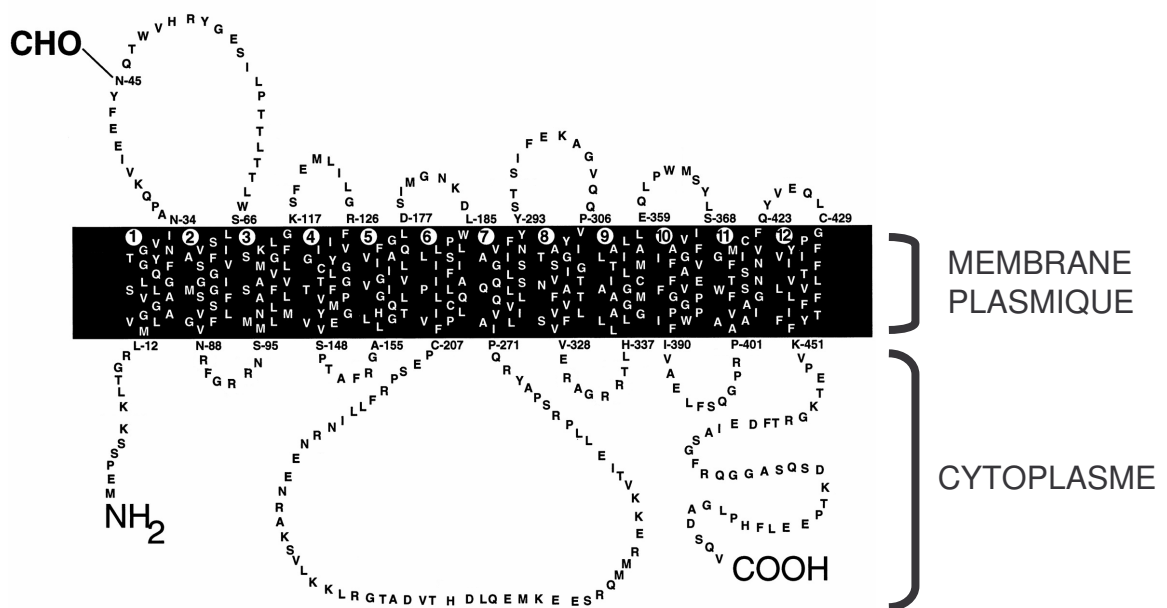
**Figure 7.** Respiration et fermentation lactique des cellules de mammifères.

Au cours de la respiration, le pyruvate issu de la dégradation du glucose est métabolisé en  $\text{CO}_2$  par la mitochondrie à l'aide d' $\text{O}_2$ . En cas de fermentation lactique, le pyruvate est transformé en lactate et secrété dans le milieu extracellulaire par le transporteur MCT1.

cellulaire par nécrose ou par apoptose, ou à l'activation de voies métaboliques compensatoires. A ce titre, il a été montré que des cellules exprimant l'oncogène c-Myc cultivées en carence de glucose, phénotypent de l'inhibition de l'activité de transport de GLUT1, meurent sous 48h d'apoptose, illustrant la stricte dépendance des cellules transformées pour le glucose comme substrat énergétique, à l'opposé des cellules non transformées qui semblent pouvoir utiliser une variété d'autres substrats énergétiques (Shim, Chun et al. 1998).

D'autres part, le caractère ubiquitaire de GLUT1 *in vitro* rend compliquée la modulation de son activité. Idéalement, il aurait été judicieux d'utiliser des cellules n'exprimant pas GLUT1 et d'évaluer leur capacité à être infectées par des virions pseudotypés par l'Env HTLV après introduction de GLUT1. A ce titre, les cellules d'insectes ne semblent pas lier l'Env HTLV (Jassal, Pohler et al. 2001) et leur GLUT1 est divergent du GLUT1 humain (Escher and Rasmuson-Lestander 1999). Toutefois il a été montré que les cellules d'insectes présentent des restrictions post-entrée qui empêchent leur infection par des rétrovirus de mammifères (Dirks and Miller 2001). Les oocytes de xénope, qui n'ont pas de transporteurs de glucose à leur surface, constituent un puissant modèle d'évaluation de l'activité de transport de glucose par GLUT1 (Keller, Strube et al. 1989). Il serait intéressant d'évaluer dans quelle mesure des oocytes de xénope exprimant ou non GLUT1 sont susceptibles à l'entrée de rétrovirus pseudotypés par l'Env HTLV.

GLUT1 possède une structure à multiples domaines transmembranaires avec six domaines extracellulaires distincts associés notamment aux activités de transport de glucose et à l'infection par HTLV (Figure 8). Nous avons donc cherché par la suite à identifier les déterminants de GLUT1 impliqués dans l'infection par HTLV.



**Figure 8.** Topologie de GLUT1.

GLUT1 possède 12 domaines transmembranaires (numérotés de 1 à 12) et un site de N-glycosylation (CHO). Les extrémités N- et C-terminales sont situées du côté cytoplasmique. Adapté de Hruz et al., 2001.

## **C. PROPRIETES DISTINCTES DE LIAISON ET D'INFECTION SUR GLUT1**

*- "Time exists so that not everything happens at once." Paul BOURKE*

### **C.1. Résumé article 3**

GLUT1 possède six domaines extracellulaires susceptibles d'interagir avec l'Env HTLV (Figure 8). Afin de déterminer l'importance relative de ces domaines dans la liaison à l'Env HTLV, nous avons généré une série de constructions chimériques entre GLUT1 et GLUT3. Nous avons démontré que la seule présence des 7 acides aminés de la boucle numéro 6 de GLUT1 suffit pour conférer à GLUT3 une activité de liaison au RBD de l'Env HTLV. Cependant, cette activité n'est pas suffisante pour assurer l'infection des virions pseudotypés par l'Env HTLV. Nous avons donc émis l'hypothèse qu'une autre activité, distincte de l'activité de liaison, était présente sur GLUT1. Afin de définir précisément les déterminants de cette activité sur GLUT1, nous avons développé un système plus résolutif d'infection HTLV par l'intermédiaire de GLUT1, se basant sur l'utilisation de cellules MDBK qui présentent une résistance partielle à l'infection par des virions pseudotypés par l'Env HTLV. L'introduction dans ces cellules d'une chimère GLUT1/GLUT3 portant au moins la combinaison de domaines 1, 5 et 6 de GLUT1 est requise pour permettre l'infection par des virions pseudotypés par l'Env HTLV. GLUT1 possède donc deux propriétés distinctes : une propriété de liaison à l'Env HTLV conférée par la sixième boucle extracellulaire et une propriété permettant l'entrée des particules virales via l'Env HTLV. Ces travaux suggèrent donc l'existence d'une nouvelle activité associée à la notion de récepteur rétroviral.

### **C.2. Article original 3**

# Human T Cell Leukemia Virus Envelope Binding and Virus Entry Are Mediated by Distinct Domains of the Glucose Transporter GLUT1\*

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**The glucose transporter GLUT1, a member of the multimembrane-spanning facilitative nutrient transporter family, serves as a receptor for human T cell leukemia virus (HTLV) infection. Here, we show that the 7 amino acids of the extracellular loop 6 of GLUT1 (ECL6) placed in the context of the related GLUT3 transporter were sufficient for HTLV envelope binding. Glutamate residue 426 in ECL6 was identified as critical for binding. However, binding to ECL6 was not sufficient for HTLV envelope-driven infection. Infection required two additional determinants located in ECL1 and ECL5, which otherwise did not influence HTLV envelope binding. Moreover the single N-glycosylation chain located in ECL1 was not required for HTLV infection. Therefore, binding involves a discrete determinant in the carboxyl terminal ECL6, whereas post-binding events engage extracellular sequences in the amino and carboxyl terminus of GLUT1.**

Interactions of retroviral envelope glycoproteins (Env)<sup>1</sup> with cell surface receptors govern the first steps of retroviral infection. Env mediates both virus binding to the cell surface and fusion of the viral and cellular membranes allowing productive viral entry. Env consists of an extracellular surface component (SU) and an associated transmembrane subunit (TM) harboring an amino-terminal fusion peptide (1). SU and TM are derived from the same polyprotein precursor (1), and it is generally accepted that initial interactions between SU and the receptor are sufficient to induce post-binding conformational changes of Env that unmask the TM fusion peptide (2).

Several Env determinants of gammaretroviruses distinc-

tively involved in binding and post-binding events have been described (3–8). From the context of the cellular Env receptors, little is known concerning potential post-binding determinants and receptor conformational changes that follow SU binding (9, 10). In the case of HIV and other related lentiviruses, it has been shown that post-binding events involve the recruitment of additional molecules, co-receptors, that belong to the seven membrane-spanning chemokine receptor family (11). No such co-receptors have yet been reported for a non-lentiviral Env (12).

Human T cell leukemia virus (HTLV) is a complex deltaretrovirus characterized by several multispliced mRNAs that encode for regulatory proteins. However, despite the large phylogenetic distance (13) between HTLV and simple gammaretroviruses, HTLV and murine leukemia viruses (MLVs) share a common general organization of Env (14–16). Their respective SU are composed of an amino-terminal RBD (14–18) followed by a central proline-rich region (15, 19) and a carboxyl-terminal domain that harbors a conserved (20) and highly reactive CXXC motif (6, 8). Furthermore, the HTLV Env receptor, the glucose transporter 1 (GLUT1) (21), is a multimembrane-spanning molecule, like all identified gammaretrovirus receptors (22).

In this context, we sought to identify GLUT1 determinants that are involved in the different steps of HTLV Env-mediated viral entry. GLUT1, a member of the class I family of glucose transporters, together with GLUT2, GLUT3, and GLUT4 (23) and GLUT14 (24), is a uniport carrier that passively facilitates glucose transport across membranes by switching between two conformational states (25). Biochemical data and modelization indicate that GLUT1 is a type 2 integral membrane protein composed of 12 transmembrane domains that delineate 6 extracellular loops (ECL) (26) (Fig. 1A). GLUT3, the closest isoform of GLUT1, has a similar predicted structure with six ECL but does not allow HTLV Env-mediated binding and infection. All six GLUT1 and GLUT3 ECL sequences present extensive residue differences and their carboxyl-terminal cytoplasmic tails confer different cell trafficking properties to the two transporters (27). Using GLUT1-GLUT3 chimeras and single residue mutants, we identified 7 amino acids in the GLUT1 ECL6 as the sole determinant able to confer HTLV Env binding properties to GLUT3. However, this determinant was not sufficient to mediate viral entry. Indeed, we found that GLUT1 harbors other non-binding determinants whose concomitant presence in GLUT3 was required for viral entry, thereby demonstrating the dual importance of GLUT1 binding and post-binding events for HTLV infection.

## MATERIALS AND METHODS

**Plasmids**—All chimeric and point mutant constructs were derived from pCHIX.GLUT1 and pCHIX.GLUT3, each harboring two COOH-terminal HA tags (21). The fragments encompassing the extracellular

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<sup>1</sup> The abbreviations used are: Env, envelope glycoprotein; SU, surface component; TM, transmembrane subunit; HTLV, human T cell leukemia virus; MLV, murine leukemia virus; RBD, receptor binding domain; ECL, extracellular loop; HA, hemagglutinin; MDBK, Madin-Darby bovine kidney; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; GFP, green fluorescent protein; EGFP, enhanced GFP; VSV-G, vesicular stomatitis virus G protein.

loops were exchanged using the following restriction sites (positions refer to GLUT1 coding sequence): MscI (206), ApaI (461), SacI (781), StuI (1017), and ApaI (1188). The ApaI sites at position 461 in GLUT1 and position 1188 in GLUT3, the SacI site in GLUT1, as well as point mutations N45Q, E426A, Q427A, and L428A in GLUT1 were introduced by the QuikChange site-directed mutagenesis protocol. PCR-amplified regions and ligation boundaries were checked by automated DNA sequencing. pLXSN.NotI was obtained by inserting a NotI linker in place of the HpaI site of pLXSN (28). The GLUT1-HA, GLUT3-HA, and the chimeric constructs were subcloned in pLXSN.NotI using the flanking NotI-BamHI restriction sites.

**Cells**—Human 293T and bovine MDBK cells were grown in DMEM with high glucose (4.5 g/liter) with 10% fetal bovine serum at 37 °C and 5% CO<sub>2</sub>. MDBK cells were provided by P. Mangeat (Centre de Recherche en Biochimie Macromoléculaire, Montpellier, France) and O. Schwartz (Institut Pasteur, Paris). Transient transfection of 293T was performed by the calcium phosphate method.

**Protein Expression and Immunoblots**—293T cells were transfected and lysed as described (21). Unboiled lysates were loaded on a 12.5% SDS-PAGE and transferred onto polyvinylidene difluoride membranes after migration. Membranes were blocked in PBS containing 5% powdered milk and 0.5% Tween 20, probed with a 1:5000 dilution of a 12CA5 anti-HA mouse antibody (Roche Applied Science) for 1 h at room temperature, washed three times with PBS, 0.1% Tween 20, followed by incubation with a 1:20,000 dilution of a horseradish peroxidase-conjugated anti-mouse immunoglobulin for 1 h at room temperature. Immunoblots were subsequently washed three times with PBS, 0.1% Tween 20 and revealed by chemiluminescence (ECL+, Amersham Biosciences).

**Binding Assay**— $5 \times 10^5$  cells were detached with PBS containing 1 mM EDTA and centrifuged at 4 °C, and the cell pellet was resuspended in cold DMEM containing 10% FBS. Cells were further incubated with either control supernatant obtained from pCDNA-transfected 293T cells or H<sub>RBD</sub> supernatant obtained from 293T cells transfected with a vector encoding the H<sub>RBD</sub>-EGFP fusion protein (29). Following incubation at 37 °C for 30 min, cells were harvested by centrifugation, washed one time with 1 ml of PBS, 2% fetal bovine serum, 0.01% sodium azide, and resuspended in 500  $\mu$ l of the same buffer. Cells were analyzed with a FACSCalibur (BD Biosciences).

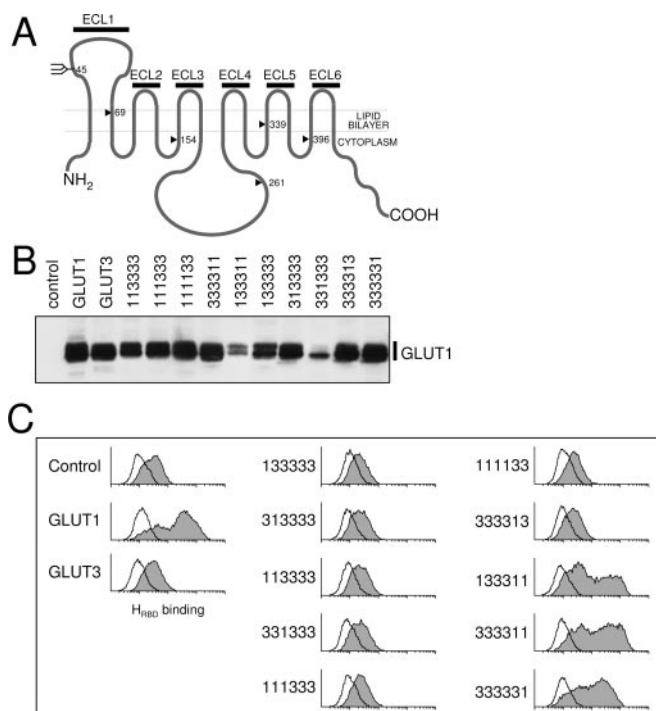
**Infections**—A replication-defective LacZ retroviral vector was pseudotyped with either HTLV-2 or VSV-G envelopes as described previously (30). Stable MDBK cell lines were plated at 75,000 cells per well (12-well plates), and infections were performed in triplicate with two viral dilutions. Viral dilutions ranged between 1/2 and 1/200, depending on the titer of the original viral stock. Fresh medium was added 24 h later, and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside staining was performed at 48 h. Infection foci were counted under conditions where the viral dilution showed at least two foci but not more than 1000 for all wells.

**Glucose Uptake**—The hexose uptake assay was adapted from Manel *et al.* (21) using 2-deoxy-D-[1-<sup>3</sup>H]glucose (Amersham Biosciences). 10<sup>5</sup> MDBK stably expressing the panel of chimeric constructs were seeded in 6-well plates. The next day, cells were washed in PBS, incubated in serum-free DMEM, washed in serum/glucose-free DMEM, and incubated for 20 min in 500  $\mu$ l of serum/glucose-free DMEM. Uptake was initiated by adding labeled deoxyglucose to a final concentration of 0.1 mM (2  $\mu$ Ci/ml), and cells were incubated for an additional 10 min. Cells were then washed three times in PBS and solubilized in 400  $\mu$ l of 0.1% SDS. Three  $\mu$ l were used for Bradford normalization, and <sup>3</sup>H incorporation in the remainder of the samples was counted.

**Transductions**—Retroviral vector particles were obtained by transfecting 293T cells with a VSV-G expression vector (31), a gag-pol expression vector (30), and a control or GLUT1-expressing pLXSN.NotI vector. MDBK cells stably expressing these vectors were obtained by transduction using viral supernatants followed by selection in G418 (1 g/liter).

## RESULTS

**The Sixth Extracellular Loop of GLUT1 Mediates HTLV Env Binding**—GLUT1 and GLUT3 share 63% amino acid sequence identity. Based on secondary structure predictions (26), we delineated six homologous fragments, each containing a single ECL (Fig. 1A), by either natural or introduced restriction sites and generated a series of GLUT1-GLUT3 chimeric molecules. Chimeras referred to by the parental origin of each ECL (1 or 3) were evaluated for HTLV Env binding (Fig. 1C). Most chimeras, with the exception of 133311 and 331333, were expressed



**FIG. 1. The COOH-terminal domain of GLUT1 is responsible for HTLV Env binding.** A, schematic topological representation of GLUT1 (adapted from Hruz *et al.* (26)). Arrowheads indicate the boundaries of the six GLUT fragments as used for allelic exchanges, each containing a single ECL. The single N-glycosylation on residue 45 is indicated. B, expression of the HA-tagged chimeric molecules in transfected 293T cells as revealed by an anti-HA antibody. Cells were transfected with an empty vector (*control*) or a vector encoding HA-tagged parental GLUT1 and GLUT3 or a panel of GLUT1-GLUT3 chimeric molecules. Chimeras are identified according to the presence of parental GLUT1 ("1") or GLUT3 ("3") ECL by a six-letter code corresponding to the six fragments delineated above. C, Binding of the HTLV Env to control and transfected cells was assessed using H<sub>RBD</sub>-EGFP fusion protein. Fluorescence-activated cell sorter analyses showing binding to H<sub>RBD</sub> (solid histograms) or to control supernatant (open histograms) are presented.

at similar levels as parental constructs (Fig. 1B). HTLV Env binding was measured using soluble HTLV Env RBD protein fused to GFP (H<sub>RBD</sub>) (15, 29). An increase in H<sub>RBD</sub> binding, as revealed by the appearance of a new peak of highly fluorescent cells, was only observed upon expression of 333311, 333331, and 133311, to levels similar to that observed in GLUT1-transfected cells (Fig. 1C). These chimeras have the GLUT1 sixth fragment that harbors ECL6 in common. We derived two additional chimeric constructs in which all residues were those of GLUT3 with the exception of either 12 residues (residues 420–431) or the 7 residues (residues 423–429) that constitute the GLUT1 ECL6 (Fig. 2A). Both chimeras, designated 333331 (12) and 333331 (7), respectively, were expressed at similar levels (Fig. 2B) and increased H<sub>RBD</sub> binding (Fig. 2C). Therefore, the 7 amino acids of GLUT1 ECL6 were sufficient to confer HTLV Env binding activity to GLUT3.

Glu<sup>426</sup>, Gln<sup>427</sup>, and Leu<sup>428</sup> are found only in GLUT1 ECL6 (Fig. 3A). Individual mutation of E426A, but not Q427A or L428A, dramatically reduced H<sub>RBD</sub> binding as compared with wild-type GLUT1 (Fig. 3B), despite similar levels of expression of all three mutants (Fig. 3C). These data indicate the crucial role of a single residue, Glu<sup>426</sup>, in HTLV Env binding to GLUT1.

**Determinants of HTLV Env-mediated Infection on GLUT1**—We next sought to determine whether ECL6-dependent HTLV Env binding to GLUT1-GLUT3 chimeras was sufficient to lead to subsequent viral entry and infection. Since most cell lines,



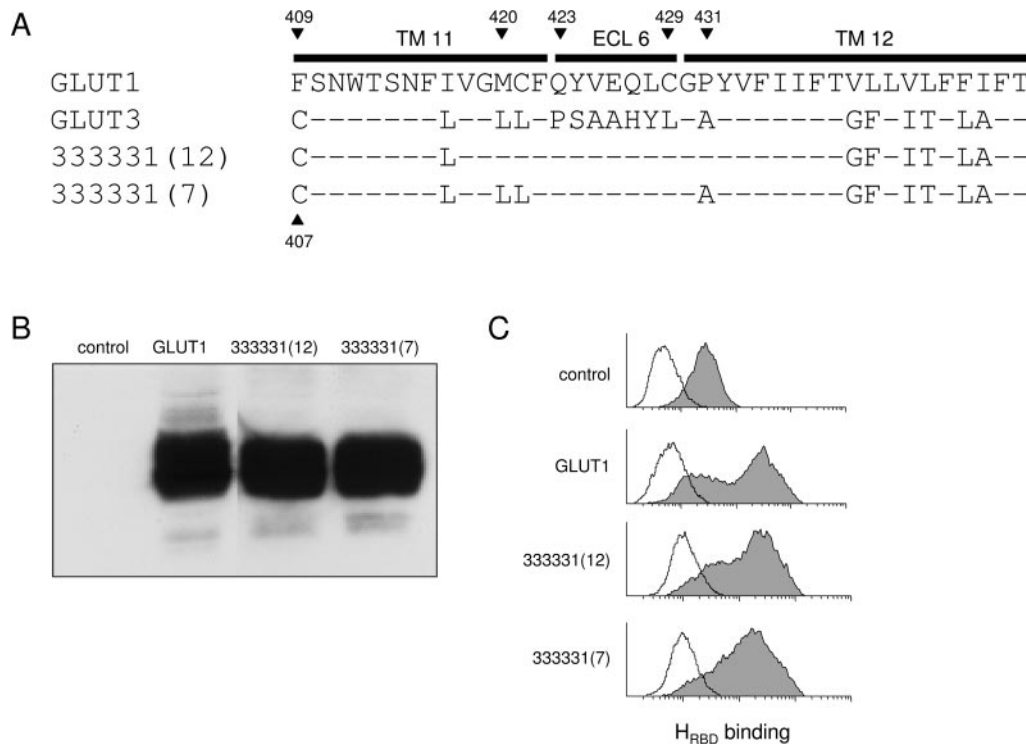


FIG. 2. **Seven amino acids of the GLUT1 ECL6 constitute the HTLV Env binding determinant.** A, alignment between GLUT1 and GLUT3 in a region that includes parts of the transmembrane 11 and 12 domains (TM 11 and TM 12) as well as ECL6. The sequences of the two minimal chimeric molecules tested, 333331 (12) and 333331 (7), harboring 12 and 7 amino acids from ECL6 of GLUT1, respectively, are indicated. Residues that are identical to GLUT1 sequence are indicated by *dashes*. B, expression of the chimeric HA-tagged molecules in total 293T cell extracts using an anti-HA antibody. C, H<sub>RBD</sub> binding to 293T cells transfected with a control vector or vectors encoding GLUT1, 333331 (12) or 333331 (7), was monitored by flow cytometry.

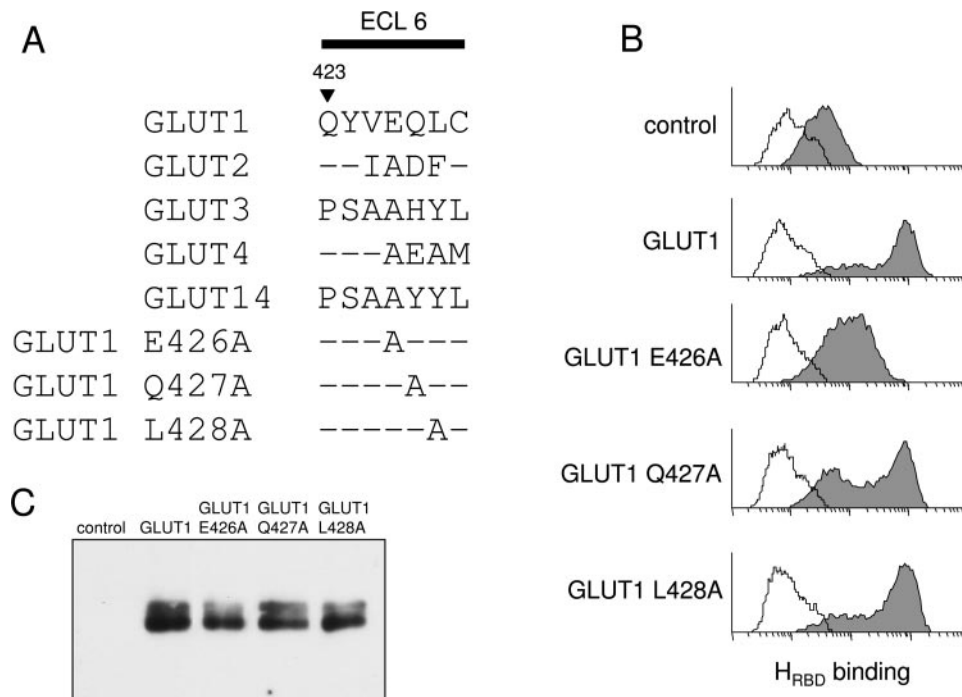


FIG. 3. **Glu<sup>426</sup> in GLUT1 ECL6 is a critical residue for HTLV Env binding.** A, alignment of the extracellular loop 6 (ECL6) from the five members of the class I glucose transporter family and GLUT1 mutants. Residues identical to GLUT1 sequence in other GLUT isoforms are indicated by *dashes*. B, binding of H<sub>RBD</sub> to control 293T or 293T cells overexpressing wild-type GLUT1 or E426A, Q427A, and L428A mutants. C, expression of the wild-type and HA-tagged GLUT1 mutants in total cell extracts as assessed with an anti-HA antibody.

including 293T cells, are readily infectable by HTLV Env-harboring virions, we used bovine MDBK cells that are relatively resistant to HTLV Env-mediated infection (32, 33). Indeed, it has been shown recently that ectopic expression of GLUT1 in MDBK cells significantly increases HTLV Env-me-

diated infection (34). MDBK cells were stably transduced with GLUT1, GLUT3, chimeric retroviral vectors, or a control empty vector. Infection of these cells with HTLV Env-pseudotyped virions consistently demonstrated titers that were 1–2 logs higher on GLUT1-expressing MDBK cells than on control or

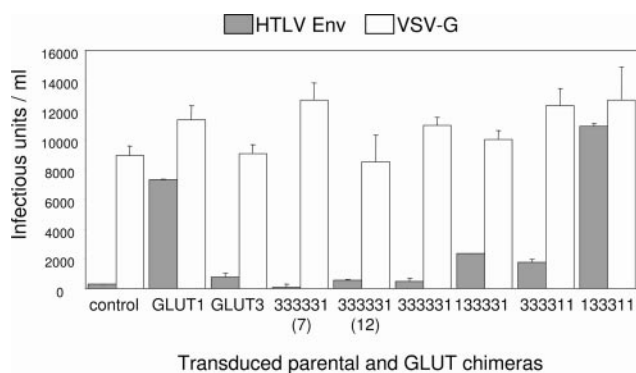


FIG. 4. **Susceptibility of MDBK cells to HTLV Env-mediated infection requires GLUT1 ECL 1, 5, and 6.** Titers of virions pseudotyped with HTLV-2 Env or VSV-G were measured on control MDBK cells or MDBK cells stably expressing a panel of chimeric GLUT molecules after retroviral transduction. Chimeras are the same as described in the legends for Figs. 1 and 2.

GLUT3-expressing cells (Fig. 4). In contrast, control VSV-G-pseudotyped virions had similar titers on all MDBK populations.

Interestingly, MDBK cells expressing a GLUT3 construct harboring the GLUT1 HTLV Env binding domain, namely 333331 and 333331 (12) and 333331 (7), did not show increased susceptibility to HTLV Env-mediated infection (Fig. 4). This indicated that GLUT1 ECL6 was not sufficient to mediate HTLV entry and prompted us to evaluate whether other precise GLUT1 determinants were involved in post-binding events. Indeed, ectopic expression of 333311 and 133331 led to slight increases in HTLV Env-mediated infection ( $p < 0.001$ ), and cells expressing a GLUT chimera that combined fragments 1, 5, and 6 (133311) showed infection levels similar to that mediated by GLUT1 (Fig. 4).

As these data pointed to the role of ECL1 and -5 in post-binding events, it was important to assess whether Env binding was necessary for infection. To this end, ECL6 point mutants (E426A, Q427A, and L428A) were evaluated for HTLV Env-mediated infection. Only E426A, which was not able to bind  $H_{RBD}$ , showed a quasi-complete drop of HTLV Env-mediated infection (Fig. 5A). The Q427A mutation, which did not affect Env binding, led to less important, albeit significant, decreases in HTLV titers (Fig. 5A;  $p < 0.02$ ). The L428A mutation had no effect on HTLV titers. Thus, as binding via Glu<sup>426</sup> is required for infection, residue Gln<sup>427</sup> in ECL6 is likely to be involved in post-binding events.

Irrespective of HTLV Env binding properties, all chimeras increased deoxyglucose uptake. Expression of both 333311 and 333331 (12) chimeras led to an increase in uptake similar to that observed for parental GLUT1 (Table I). The E426A mutant significantly increased glucose uptake but to a lesser extent when compared with wild-type GLUT1. These data confirm correct targeting, folding, and transport function of these chimeras.

Since post-binding events may involve distinct interactions of GLUT1 and GLUT3 with cytoplasmic factors, we further evaluated the contribution of the  $NH_2$ -terminal intracellular tail adjacent to GLUT1 ECL1 in viral entry, as both motifs were present in 133311. Additional chimeric molecules that harbored GLUT1 ECL6 in combination with either the GLUT1 amino-terminal intracellular tail (residues 1–12) or ECL1 alone (residues 34–66) were generated. Increased viral titers were observed only with the latter construct (data not shown), demonstrating that post-binding events mediated by the first fragment of GLUT1 in the GLUT3 context were solely due to the extracellular loop.

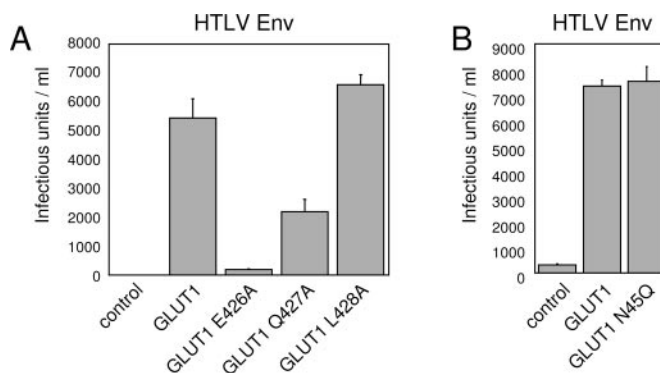


FIG. 5. **The Glu<sup>426</sup> residue in GLUT1, and not N-glycosylation, is required for HTLV Env-mediated infection of MDBK cells.** Titers of virions pseudotyped with HTLV-2 Env were measured on control MDBK cells or MDBK cells stably expressing wild-type GLUT1 or the E426A, Q427A, and L428A mutants (A) or the N45Q N-glycosylation mutant in ECL1 (B).

TABLE I  
Functional properties of GLUT1, GLUT3, GLUT1-GLUT3 chimeras, and GLUT1 mutants

Construct <sup>a</sup>	Mean glucose uptake $\pm$ S.E. <sup>b</sup>	Binding <sup>c</sup>	Infection <sup>d</sup>
Control	100 $\pm$ 4.2	–	–
GLUT1	258.3 $\pm$ 29.2	++	++
GLUT3	230.6 $\pm$ 7	–	–
333331 (7)	159.7 $\pm$ 12.5	++	–
333331 (12)	209.7 $\pm$ 15.3	++	–
333331	130.6 $\pm$ 2.8	++	–
133331	ND <sup>e</sup>	++	+
333311	215.3 $\pm$ 11.1	++	+
133311	168.1 $\pm$ 4.2	++	++
GLUT1 N45Q	ND <sup>e</sup>	++	++
GLUT1 E426A	183.3 $\pm$ 18.1	+	+

<sup>a</sup> Chimeras are identified according to the presence of parental GLUT1 (“1”) or GLUT3 (“3”) ECL. (“7”) and (“12”) indicate the number of residues derived from GLUT1 ECL6 (Fig. 2).

<sup>b</sup> Mean glucose uptake values are expressed as percentage relative to control. S.E., standard error of the mean ( $n = 3$ ).

<sup>c</sup> Binding as observed on transiently transfected 293T cells. –, no binding above background; +, binding  $< 1$  log above background; ++, binding  $> 1$  log above background.

<sup>d</sup> Infectious titers as measured with MLV virions pseudotyped with the HTLV Env on stably transduced MDBK cell populations. –, no titer above background; +, titer  $< 1$  log above background; ++, titer  $> 1$  log above background.

<sup>e</sup> ND, not determined.

**GLUT1 N-Glycosylation Is Not Required for HTLV Env-mediated Infection**—N-Glycosylation of several retroviral receptors has previously been shown to inhibit viral entry (35–39). Since GLUT1 has a N-glycosylation site at Asn<sup>45</sup> in ECL1, we evaluated the influence of this site on HTLV Env-mediated infection. The GLUT1 N45Q mutant had the expected increased electrophoretic migration as compared with wild-type GLUT1, due to suppression of this N-glycosylation site (data not shown). However, neither HTLV Env-mediated binding (data not shown) nor infection were affected (Fig. 5B). Therefore, the requirement of HTLV ECL1 in HTLV Env-mediated infection does not depend on its N-linked glycosylated chain.

#### DISCUSSION

The binding of HTLV-1 and HTLV-2 Env to GLUT1 could be conferred to the heterologous GLUT3 glucose transporter by substitution of 7 amino acids constituting the sixth extracellular loop of GLUT1 (ECL6). However, productive viral entry required the presence of additional determinants located in loops 1 and 5, neither of which appeared to influence binding. This functional dichotomy of GLUT1 with regard to HTLV Env binding and subsequent viral entry steps was further sup-

ported by results obtained with the Q427A mutation in ECL6. Indeed, although GLUT1 Q427A exhibited full binding abilities, this mutant had a significant decrease in HTLV Env-mediated viral entry. Elucidation of the interactions between Gln<sup>427</sup> in ECL6 and ECL5 in addition to those with the more remote amino-terminal ECL1 will likely provide further clues with regard to the conformational changes that contribute to both the glucose transporter and HTLV Env receptor and possibly other cellular functions (40) of GLUT1.

Disruption of GLUT1 single *N*-glycosylation had no effect on HTLV Env binding and infection. Nevertheless, various patterns of GLUT1 glycosylation have been described (41). The impact of these modifications as well as other post-translational modifications of GLUT1 on HTLV Env binding and HTLV Env-mediated infection remains to be determined.

Mutation of Glu<sup>426</sup> in GLUT1 strongly reduced HTLV Env binding and infection; however, it only mildly affected glucose uptake (Table I). Furthermore, disruption of the glucose uptake activity of GLUT1 by introducing the Q161C mutation did not alter HTLV Env binding and infection (data not shown). This indicates that these different activities are mediated by distinct domains on GLUT1. It will thus be of interest to determine the mechanism involved in the inhibition of GLUT1 activity by HTLV Env (21).

The distinct trafficking properties of GLUT1 and GLUT3, previously documented in an epithelial cell line, depend on their COOH-terminal intracellular domains (27). In agreement with this report, we also observed that the COOH-terminal intracellular domain of GLUT1 and GLUT3 was responsible for differences in localization, with GLUT1 accumulating mainly into cytoplasmic vesicles and GLUT3 present predominantly at the plasma membrane (data not shown). However, these differences in overall compartmentation did not appear to influence HTLV Env binding and post-binding events in all constructs we tested. For example, introduction of the GLUT3 cytoplasmic tail in the GLUT1 background did not modify HTLV Env binding (data not shown). Similarly, 133311 and 333331 chimeras, which shared the same major vesicular localization as GLUT1, showed comparable HTLV Env binding due to the presence of the GLUT1 ECL6, while only 133311 allowed HTLV Env-mediated infection.

Mapping of viral entry or binding determinants on gammaretrovirus receptors has also made use of chimeric molecules (10, 42–45), but the presence of receptor determinants that distinctively control Env binding and post-binding viral entry has not been reported. In contrast, binding and post-binding entry determinants located within the viral Env have been characterized for an increasing number of gammaretroviruses (22). In the case of MLV, a disulfide bond-mediated association of SU and TM is isomerized upon binding into an intrasubunit disulfide bond in the SU. This event, which is dependent on a highly conserved CXXC (CWLC) motif in the MLV SU (20), is further controlled in *cis* or in *trans* by the amino-terminal RBD of the SU (3, 4). Interestingly, HTLV-Env also harbors a similar motif in its SU (CIVC). It is therefore tempting to propose that a similar isomerization occurs in HTLV Env, following binding to GLUT1 ECL6. It remains to be determined whether the post-binding events controlled by GLUT1 determinants are involved in the SU isomerization process. In addition, although no co-receptor has been formally identified for GLUT1, it will be of interest

to determine whether these post-binding determinants act through the recruitment of co-receptors or co-factors or alternatively through conformational changes in GLUT1 that favor membrane fusion and productive virion entry.

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### C.3. Conclusion article 3

La nature de l'activité de GLUT1 requise pour l'infection en plus de la liaison du RBD n'est pas connue. Le seul autre cas décrit de récepteur pour gammarétrovirus possédant une telle activité requise en plus de la liaison à l'Env concerne le virus FeLV-B et son récepteur Pit1 (Farrell, Russ et al. 2002). Ainsi Pit1-2A, une construction chimérique de Pit1 dans laquelle la région 550 à 558 a été substituée par celle de Pit2, est toujours capable de lier la SU de FeLV-B, mais des vecteurs rétroviraux pseudotypés par l'Env FeLV-B sont incapables d'infecter des cellules exprimant Pit1-2A. Mais l'activité de Pit1 altérée par l'introduction du fragment de Pit2 n'a pas été établie.

Plusieurs activités post-liaison sont envisageables pour GLUT1. L'hypothèse la plus simple consiste à proposer qu'une conformation de GLUT1, ou son altération, est requise après la liaison afin de permettre la fusion des membranes et donc une entrée virale productive. Il est également possible que GLUT1 recrute une molécule de type co-récepteur, bien qu'aucune molécule de ce type n'ait été identifiée à l'heure actuelle. Enfin, il est clairement établi que l'entrée des gammarétrovirus nécessite, après liaison au récepteur, l'isomérisation d'un pont disulfure entre la SU et la TM de l'Env, au niveau d'un motif CXXC situé dans la SU et caractéristique des disulfide isomérases (Wallin, Ekstrom et al. 2004; Burkhart, D'Agostino et al. 2005). Dans ce contexte, il est concevable qu'après liaison de l'Env HTLV, GLUT1 participe à la réaction d'isomérisation, à la manière par exemple d'un catalyseur chimique de cette réaction. En accord avec cette proposition, une équipe a récemment rapporté que le récepteur au MLV écotrope participe à l'isomérisation de cette Env (Li 2005).

L'activité post-liaison de GLUT1 n'est pas portée par son homologue le plus proche GLUT3. Si cette activité est similaire et conservée chez les autres récepteurs de gammarétrovirus, comme cela pourrait être le cas pour FeLV-B et le MLV écotrope, cela indiquerait que ces récepteurs, qui appartiennent tous à la famille des transporteurs de

nutriments, ont en commun une activité autre que l'activité de transport, et qui pourrait constituer un nouveau critère de classification de ces molécules. Il serait par ailleurs intéressant d'évaluer si cette activité post-liaison présente une signature protéique particulière entre les différents récepteurs.

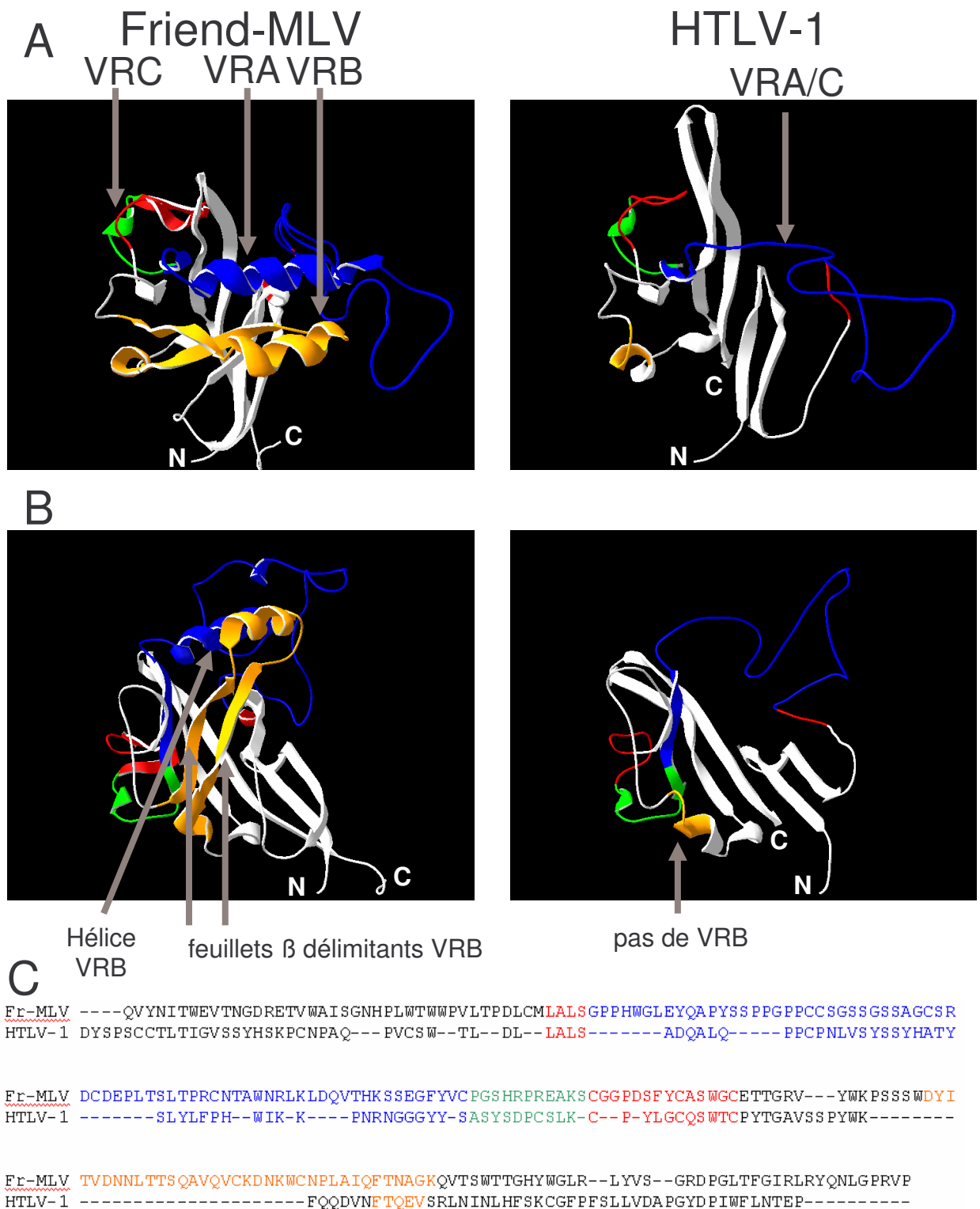
L'identification d'un motif peptidique court conférant l'activité de liaison de l'Env HTLV à GLUT3 appelle à évaluer si ce peptide est capable d'interagir avec l'Env HTLV.

L'invalidation totale du gène codant pour GLUT1 chez la souris est létale (Heilig, Brosius et al. 2003). L'identification de mutants ponctuels de GLUT1 dans la boucle extracellulaire 6 (*i.e.* E426A) qui montrent une baisse significative de leur activité de récepteur HTLV sans altération de l'activité de transport amène à proposer la génération d'un modèle murin de type "knock-in" où seule la séquence codant pour la boucle 6 serait altérée. Une telle lignée de souris serait potentiellement viable tout en étant résistante à l'infection par des virions pseudotypés par l'Env HTLV. Elle permettrait par exemple de déterminer la contribution de l'interaction entre GLUT1 et l'Env HTLV sur le métabolisme de cellules infectées par HTLV (voir Discussion). *In vitro*, des lignées cellulaires correspondantes fournirait un système sans niveau d'infection basal pour décrypter au niveau moléculaire l'entrée de HTLV par GLUT1.

Parallèlement à cette étude des déterminants de GLUT1 impliqués dans l'interaction avec l'Env HTLV, nous avons cherché à visualiser dans l'Env HTLV les motifs de liaison au récepteur décrits pour les MLV (VRA, VRB, VRC), en modélisant le RBD de HTLV-1 à partir de la structure connue du RBD de Friend-MLV, d'un alignement entre ces RBD et du serveur SwissModel ([swissmodel.expasy.org](http://swissmodel.expasy.org)). Cette approche n'était pas possible pour GLUT1 car il n'existe pas de structure tridimensionnelle de GLUT1, ni d'une protéine suffisamment proche pour la modéliser. Le modèle du RBD HTLV obtenu nous donne plusieurs précisions sur l'organisation de ce RBD. Le motif équivalent au VRA est beaucoup plus court chez HTLV que chez Friend-MLV. Le motif VRC semble conservé avec celui des MLV, notamment par la présence du quartet de cystéines C<sub>108</sub>, C<sub>112</sub>, C<sub>117</sub> et C<sub>122</sub>. Le motif

VRB ne semble pas avoir d'équivalent chez HTLV. Le "VRA" de HTLV ne présente pas de structure de type hélice  $\alpha$  comme c'est nettement le cas chez MLV. Chez MLV, le "VRB" est constitué d'une hélice  $\alpha$  parallèle à l'hélice de VRA. Cette hélice est soutenue par 2 feuillets  $\beta$ , conservés chez les MLV. Chez HTLV, l'intégralité de ce domaine structural (feuillelet  $\beta$  - hélice  $\alpha$  de VRB - feuillelet  $\beta$ ) est absent (Figure 9), posant la question de l'avantage de son maintien dans l'enveloppe MLV.





**Figure 9.** Modélisation du RBD de l'Env HTLV-1.

(A) Représentations en rubans des RBD de Friend-MLV (structure PDB 1AOL) et de HTLV-1 (modélisation par SwissModel). (B) Visualisation de VRB chez MLV, qui n'existe pas chez HTLV. (C) Alignement utilisé pour la modélisation. Bleu: VRA. Vert: VRC. Orange: VRB. Rouge: motifs conservés bordant VRA/C.

# DISCUSSION

## A. BIOLOGIE DE L'ENV HTLV

### A.1. Modulation de l'activité de l'Env HTLV

L'activité de fusion des membranes des Env MLV est inhibée par le peptide "R", constituée par les 16 derniers acides aminés de la TM chez Friend-MLV (Green, Shinnick et al. 1981). Le clivage de ce peptide qui intervient au cours de la maturation des virions MLV active l'activité fusogène de l'Env (Ragheb and Anderson 1994; Rein, Mirro et al. 1994). Chez HTLV, un tel peptide n'a pas été formellement identifié. Cependant nous avons constaté que la délétion partielle (8 ou 16 acides aminés délétés) du domaine intracellulaire C-terminal de l'Env HTLV augmente l'activité fusogène de l'Env HTLV dans plusieurs lignées (HeLa, NIH3T3, 293T) (voir "*Human T-cell leukemia virus type 1 envelope-mediated syncytium formation can be activated in resistant Mammalian cell lines by a carboxy-terminal truncation of the envelope cytoplasmic domain*" en annexe). De plus, dans une lignée résistante à l'activité de fusion de l'Env HTLV (NIH3T3 TK-), la délétion du domaine intracellulaire de l'Env HTLV confère une activité de fusion. Cette résistance ne semble pas liée à la disponibilité du récepteur sur ces cellules ou à la capacité fusogène au sens large de ces cellules, et ces cellules sont infectables par des virions pseudotypés par l'Env HTLV. Il semble donc que des propriétés de fusion propres à l'Env HTLV soient altérées dans ces cellules. Il a été récemment montré que la protéine hDlg interagit avec le domaine intracellulaire de l'Env HTLV (Blot, Delamarre et al. 2004). Bien que l'effet d'une modulation de l'expression de hDlg sur l'activité de l'Env HTLV n'ait pas été évalué, il est tentant de proposer que l'activité hDlg est altérée dans les cellules NIH3T3(TK-). Cependant, le même laboratoire qui a identifié hDlg comme partenaire de l'Env suggère qu'une mutation S486L ou la délétion des 4 acides aminés C-terminaux de l'Env HTLV inhibe l'activité fusogène de



l'Env dans des cellules Jurkat (Blot, Delamarre et al. 2004), résultats qui semblent en contradiction avec nos observations. Il apparaît donc que selon les types cellulaires, les interactions entre le domaine intracellulaire de l'enveloppe HTLV et les composants cytoplasmiques varient. L'impact de ces variations sur la capacité infectieuses de virus produits à partir de ces cellules n'est pas connu.

## **A.2. Env HTLV endogènes ?**

Chez la souris, de nombreuses séquences d'Env apparentées aux MLV sont présentes dans le génome. HTLV est caractérisé par une longue coexistence et coévolution avec l'espèce humaine. L'Env HTLV étant apparentée à celle des MLV, on peut supposer qu'il existe des séquences apparentées aux Env HTLV dans le génome humain. Cependant, une recherche exhaustive des motifs caractéristiques de l'Env HTLV dans le génome humain n'a pas pu mettre en évidence de telles séquences (N. Manel, données non publiées). Toutefois, la séquence de la TM de l'Env des rétrovirus endogènes humains de la famille HC2/HERV-T (Kabat, Tristem et al. 1996) semble apparentée à celle des MLV et du HTLV (Benit, Dessen et al. 2001). Dans la SU, certains motifs conservés des Env de type MLV peuvent être identifiés (motifs PIG et LLNLTQ de la région riche en prolines, motif CWLC de la région C-terminale). Chez l'homme, une Env de la famille HC2/HERV-T est exprimée dans la thyroïde, mais la signification de cette expression n'est pas connue (de Parseval, Lazar et al. 2003). Il est possible que ces enveloppes soient des traces d'un intermédiaire évolutif entre les Env MLV et HTLV. Potentiellement, la présence de motifs proches entre les gènes *env* HC2 et HTLV suggère que les séquences de type HC2 pourraient recombinaison avec le gène d'*env* HTLV et conduire à l'émergence de nouveaux virus.

## **B. GLUT1 COMME RECEPTEUR HLTV**

### **B.1. La famille des transporteurs de glucose**

GLUT1 est un membre de la famille des transporteurs passifs de glucose, qui contient 14 membres. Cette famille a été divisée par des critères phylogénétiques en 3 sous-familles (Joost and Thorens 2001). GLUT1 fait partie des transporteurs de type 1, au même titre que GLUT2, GLUT3, GLUT4 et GLUT14. Le transport du glucose est également assuré par la famille SGLT de cotransporteurs  $\text{Na}^+$ /glucose, qui utilisent le gradient de  $\text{Na}^+$  pour transporter le glucose contre son gradient (Wood and Trayhurn 2003).

GLUT1, avec un  $K_M$  d'environ 1 mM pour le glucose, est considéré comme le transporteur ubiquitaire du glucose, et il semble responsable du transport basal de glucose dans la cellule (Berg, Tymoczko et al. 2001). Cependant et comme nous l'avons observé dans le cas des lymphocytes et de lignées cellulaires, l'expression de GLUT1 varie d'un type cellulaire à l'autre, et pour un même type cellulaire d'un état à un autre, et la distribution systématique de GLUT1 reste à décrire.

GLUT2 est exprimé principalement dans le foie et les cellules  $\beta$  du pancréas, et son  $K_M$  pour le glucose, 15–20 mM, est un ordre de magnitude au-dessus de GLUT1. Ainsi, GLUT2 ne va transporter le glucose dans les cellules que lorsque la concentration en glucose sera élevée dans le milieu extracellulaire. Cette propriété a conduit certains auteurs à proposer que GLUT2 soit un "senseur" du glucose extracellulaire, impliqué dans l'ajustement de la sécrétion d'insuline (Efrat, Tal et al. 1994).

GLUT3 possède le même  $K_M$  que GLUT1, mais son expression semble distincte de celle de GLUT1. Ainsi, GLUT3 est fortement détecté dans les neurones, alors que GLUT1 en est absent, et inversement dans le muscle (Shepherd, Gould et al. 1992). Dans les cellules épithéliales, GLUT1 et GLUT3 possèdent également une localisation différente (Inukai, Shewan et al. 2004). Ces différences de distribution ne sont pas encore expliquées. De plus,

de tous les GLUT, seul GLUT3 existe également sous forme de pseudogène (il existe 3 pseudogènes de GLUT3) (Zhang, Harrison et al. 2003).

GLUT4 a un  $K_M$  de 5 mM pour le glucose. GLUT4 est séquestré dans des vésicules intracellulaires, et un traitement à l'insuline induit rapidement le transport à la surface de ce transporteur (Birnbaum 1989). Cette mobilisation est importante pour permettre au muscle et au tissu adipeux d'absorber le glucose. La protéine intracellulaire TUG interagit avec GLUT4 et est impliquée dans le trafic de GLUT4 (Bogan, Hendon et al. 2003).

GLUT14 est un duplicon de GLUT3, présent chez l'homme mais absent chez la souris et est exprimé dans les testicules (Wu and Freeze 2002).

Parmi les autres membres de la famille des transporteurs, GLUT5 n'est pas efficace pour le transport du glucose, mais est responsable du transport du fructose dans l'intestin (Burant, Takeda et al. 1992).

## **B.2. GLUT1 et tropisme HTLV**

Nous avons montré que le récepteur HTLV est indétectable à la surface des lymphocytes T CD4 ou CD8 du sang périphérique de l'adulte par une technique de liaison au RBD de l'Env HTLV. Les mécanismes d'induction de l'expression de GLUT1 au cours de l'activation ne sont pas clairement définis et font probablement intervenir plusieurs aspects. Tout d'abord il est vraisemblable que la transcription de *slc2a1*, le gène codant pour GLUT1, soit induite par l'activation (Chakrabarti, Jung et al. 1994). De plus, il semble qu'un pool de GLUT1 intracellulaire existe dans la cellule T au repos (Sandrina Kinet et Naomi Taylor, observations non publiées) et qui pourraient être recrutée lors de l'activation.

L'absence de détection de GLUT1 à la surface des lymphocytes T incite à penser que les cibles de l'infection ne sont pas les lymphocytes T au repos, mais plutôt des précurseurs lymphocytaires ou des lymphocytes activées. D'une manière plus générale, nous pensons que d'autres types cellulaires peuvent être infectées au cours de la primo infection HTLV. Cependant, ces cellules seraient éliminées, soit par la réponse cytotoxique soit par l'effet

toxique de l'Env HTLV sur l'activité de GLUT1 (voir plus bas), et ne survivraient qu'une fraction des lymphocytes qui échapperaient à ces phénomènes.

GLUT1 est très fortement exprimé à la surface des érythrocytes humains, et la protéine GLUT1 constitue jusqu'à 5% des protéines de la membrane des érythrocytes (Hruz and Mueckler 2001). De plus, il y a 700 fois plus d'érythrocytes que de cellules nucléées dans le sang. Il est donc vraisemblable que des particules HTLV libres relarguées dans le sang circulant seraient immédiatement captées par les érythrocytes. L'entrée de HTLV dans les érythrocytes serait une voie "sans issue" étant donné que les érythrocytes sont dépourvus d'ADN. Alternativement, HTLV pourrait être lié aux érythrocytes circulants sans y pénétrer, et la circulation d'érythrocytes pourrait alors distribuer HTLV à différents tissus. Cette possibilité impliquerait que GLUT1 à la surface des érythrocytes, à la manière de DC-SIGN, ne serait pas capable de permettre l'entrée du HTLV mais seulement sa liaison. Cette idée est confortée par l'observation que la glycosylation, la multimérisation et l'activité de GLUT1 semblent être particulières à la surface des érythrocytes (Cloherty, Heard et al. 1996).

GLUT1 est une protéine très conservée chez les mammifères (86% d'identité entre les GLUT1 de l'homme, du chimpanzé, du lapin, de la souris, du rat, de la vache et l'opossum), ce qui corrèle avec le large tropisme *in vitro* de l'Env HTLV. Notamment, la sixième boucle extracellulaire, impliquée dans la liaison à l'Env HTLV est parfaitement conservée chez ces espèces (QYVEQLC). Par contre, cette boucle présente des variations chez les oiseaux (QYIAQLC chez le poulet) et les poissons (QYVEELC chez la carpe et la truite, QYVEMVC chez la morue). Nous avons pu démontrer que des cellules de poulet sont toujours infectables par des virions MLV pseudotypés avec l'Env HTLV malgré de faibles niveaux de liaison du RBD de l'Env HTLV. Une faible liaison ne semble donc pas empêcher une infection efficace, et cette propriété a été décrite pour d'autres Env rétrovirales (Battini, Danos et al. 1998; Bachrach, Marin et al. 2000; Qian, Donald et al. 2003). Toutefois, nous n'avons pas testé si la surexpression du GLUT1 de poulet dans des cellules hétérologues comme 293T procure le même profil de liaison à l'Env HTLV que le GLUT1 humain, afin d'évaluer si le GLUT1 de

poulet possède des propriétés intrinsèques de faible liaison au RBD comme le suggère sa séquence.

Nous avons montré que GLUT1 est le récepteur de HTLV-1 et HTLV-2, et donc vraisemblablement STLV-1 et SLTV-2. En effet la séquence d'Env des différents isolats STLV-1 ou STLV-2 est très proche de celle de leur équivalent humain (plus de 95% d'identité de séquence). Nous n'avons pas testé si GLUT1 peut également servir de récepteur pour les Env de STLV-3 ou des HTLV-3 et HTLV-4 récemment isolés. Cependant, l'identité de séquence entre les Env STLV-1 et STLV-2, STLV-1 et STLV-3 ou STLV-2 et STLV-3 est de l'ordre de 65% ce qui laisse à penser que GLUT1 est également le récepteur de l'Env STLV-3.

### **B.3. GLUT1 et la famille des récepteurs rétroviraux**

Comme GLUT1 pour HTLV, tous les récepteurs de gammarétrovirus identifiés à ce jour sont des transporteurs de nutriments passifs qui catalysent le transport de type uniport, antiport ou symport de solutés au travers de la membrane plasmique (Feldman, Farrell et al. 2004). La pression de sélection derrière cette convergence reste inconnue, alors qu'il existe une grande variété d'autres systèmes de transports membranaires (canaux, pores, transporteurs actifs, translocateurs de groupe).

Cette convergence de fonction des récepteurs corrèle avec une convergence d'organisation des SU correspondantes à trois modules: RBD, région charnière riche en prolines, et domaine C-terminal possédant un motif de disulfide isomérase. De plus, nous avons indiqué comment l'Env participerait à l'émergence des rétrovirus à partir de rétrotransposons en fournissant à la fois une activité de sortie de la particule hors de la cellule et une activité d'entrée. Il est clairement établi que la fonction d'entrée requiert l'interaction entre l'Env et le récepteur cellulaire. Par contre les mécanismes impliqués dans la sortie de la particule ne sont pas connus. L'hypothèse la plus simple pour expliquer ce mécanisme consiste à proposer que l'interaction Env-récepteur participe également à la sortie des

particules. Cette hypothèse peut paraître contre-intuitive, en effet il est clairement établi que des particules virales peuvent être produites à partir de cellules qui ne possèdent pas le récepteur correspondant. Il semble plus pertinent de placer cette hypothèse dans un contexte évolutif. Le couple Env-récepteur aurait avant tout évolué à partir d'un mécanisme cellulaire impliqué dans le trafic vers la membrane plasmique. Quelle pourrait être l'identité de ce trafic ? Les partenaires impliqués dans le trafic vers la membrane plasmique des récepteurs n'est pas connu. MCT1 (MonoCarboxyalte Transporter 1, gène *slc16a1*) est un transporteur passif de lactate (Garcia, Goldstein et al. 1994), mais n'est pas connu pour fonctionner comme récepteur rétroviral. Il a été établi que la molécule CD147 est requise pour permettre le trafic de MCT1 vers la surface cellulaire (Kirk, Wilson et al. 2000). CD147 agirait comme une chaperone de MCT1. De manière similaire, le transporteur d'acides aminés LAT1 (CD98, gène *slc7a5*) est un hétérodimère composé d'une chaîne légère (un peptide à 12 domaines transmembranaire) et d'un chaîne lourde à un seul domaine transmembranaire (Mastroberardino, Spindler et al. 1998; Verrey, Closs et al. 2004). Alors que la chaîne légère possède une topologie typique de transporteur de nutriment, l'homologie entre la chaîne légère et certaines glycosidases (Mastroberardino, Spindler et al. 1998) l'apparente aux chaperones. Par analogie, nous pouvons donc émettre l'hypothèse qu'un ancêtre des Env rétrovirales pourrait être une chaperone cellulaire analogue à CD147 ou CD98hc impliquée dans le trafic vers la surface cellulaire de transporteurs passifs de nutriments. Récemment, CD98, CD147 et MCT1 ont pu être isolés à la surface des cellules dans des "super-complexes" (Xu and Hemler 2005). Selon le type cellulaire et les conditions d'isolation, ces complexes contiennent également les transporteurs MCT4, MCT8, ASCT2, CAT1. Les expériences préliminaires de ce travail suggèrent que ces transporteurs n'interagissent pas directement, mais via CD147 et la chaîne légère de CD98. Il émerge donc qu'à la surface des cellules un ensemble de chaperones serait impliquées dans la constitution de ces "super-complexes" de transporteurs de nutriments. Cette possibilité soulève plusieurs questions : La gestion des flux de métabolites à la surface cellulaire est-elle gérée dans le cadre de tels complexes ? Quelles sont

les implications structurelles au niveau de la membrane plasmique de l'existence de ces "super-complexes"? Les récepteurs des gammarétrovirus, qui sont des transporteurs de nutriments, font-ils également partie d'un ou plusieurs "super-complexes" ? Les propriétés de ces "super-complexes" permettent-elles d'expliquer la sélection de transporteurs de nutriments comme récepteurs de gammarétrovirus ?

De plus, rappelons que les Env rétrovirales semblent posséder une activité de disulfide isomérase. Cette activité, qui semble être impliquée dans le démasquage de la TM au cours de l'entrée rétrovirale, pourrait encore une fois trouver son origine dans les mécanismes de sortie de la cellule. Le réticulum endoplasmique contient la chaperone PDI (*protéine disulfide isomérase*) impliquée à la fois dans le repliement des protéines et dans leur trafic ultérieur. Il est donc envisageable que les SU rétrovirales aient dérivé à partir de chaperones cellulaires impliquées à la fois dans le repliement des transporteurs passifs de nutriments et dans leur trafic.

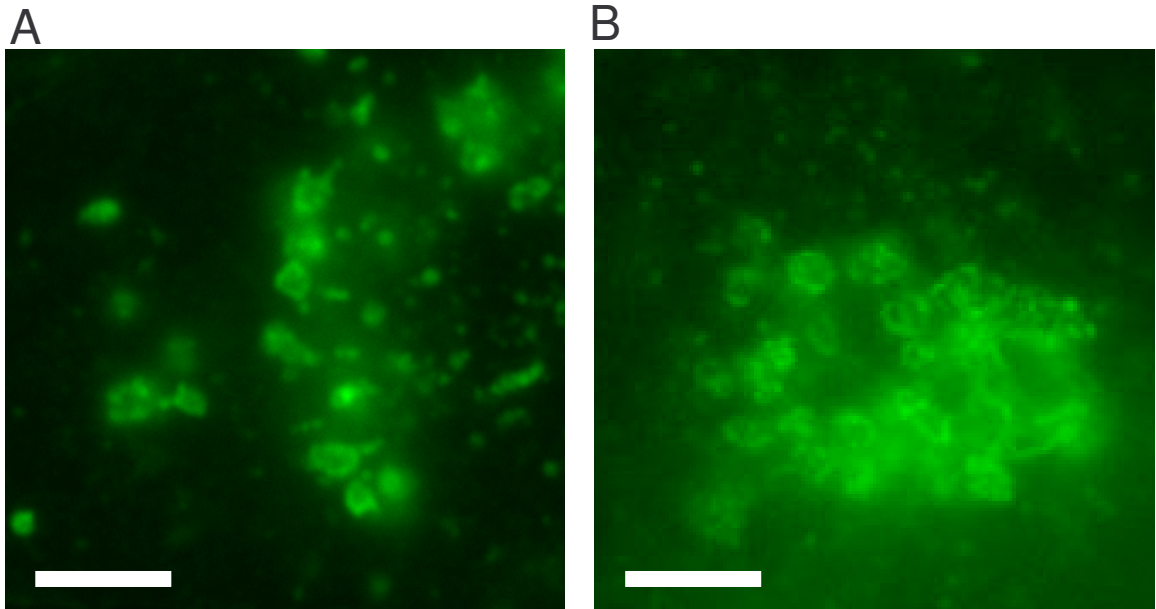
#### **B.4. Transporteurs de glucose, cycle cellulaire et trafic membranaire**

Nous avons pu observer que dans des lignées stablement transduites (MDBK, HeLa et NIH3T3) GLUT1 est principalement concentré dans des vésicules intracellulaires, alors que GLUT3 est exclusivement exprimé à la membrane plasmique. Le déterminant de cette localisation distinctive se situe dans la queue cytoplasmique C-terminale de GLUT3. En effet, l'échange de la queue C-terminale de GLUT1 par celle de GLUT3 confère une localisation de type GLUT3. Ces observations sont en accord avec des travaux démontrant que dans la lignée MDCK, la queue C-terminale de GLUT3 possède un déterminant dominant de localisation apicale (Inukai, Shewan et al. 2004). Il est intéressant de constater que la présence d'un déterminant de localisation apicale dans une lignée polarisée (MDCK) se traduit par une localisation membranaire de surface sans stockage intracellulaire dans une lignée non polarisée (MDBK, HeLa ou NIH3T3).

La composition exacte des vésicules contenant GLUT1-GFP dans les cellules MDBK, mais également HeLa et NIH3T3, n'est pas connue. Cependant, nous avons pu constater que ces vésicules sont sphériques, nombreuses (de l'ordre de la cinquantaine par cellule) de taille importante (environ 4  $\mu\text{m}$  du diamètre cellulaire) et distribuées autour du noyau. Cette localisation est similaire à celle observée pour la protéine  $\beta$ -COP dans des cellules Vero dans le cadre du EMBL/DKFZ Localization Project (<http://gfp-cdna.embl.de/>) (Simpson, Wellenreuther et al. 2000) (Figure 10).  $\beta$ -COP est un composant du complexe COPI, lui-même responsable du transport rétrograde de l'appareil de Golgi vers le réticulum endoplasmique ainsi que de la maintenance de la structure de l'appareil de Golgi (McMahon and Mills 2004).

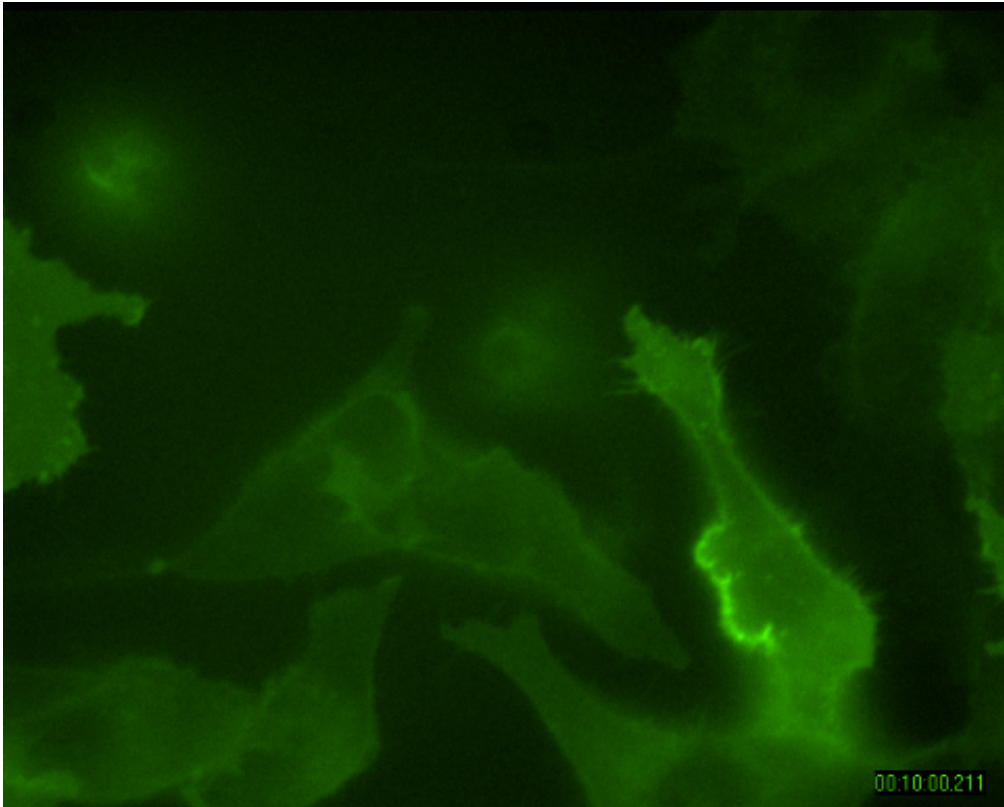
Nous avons réalisé des expériences de vidéomicroscopie de cellules MDBK exprimant GLUT1-GFP ou GLUT3-GFP. La localisation exclusivement membranaire de GLUT3-GFP dans les cellules MDBK nous a permis de suivre nettement la dynamique de surface de GLUT3-GFP, alors que le fort marquage intracellulaire de GLUT1GFP a rendu les observations plus difficiles. Deux phénomènes ont pu être observés avec GLUT3GFP. Tout d'abord, nous avons observé qu'au cours du déplacement cellulaire, GLUT3GFP se concentre dans les zones de croissance membranaire de type lamellipode (Figure 11). De plus, nous avons pu capturé des événements de division cellulaire. Étonnamment, la localisation de GLUT3-GFP évolue rapidement au cours de la scission, et se relocalise majoritairement au niveau de la jonction entre les deux cellules filles (pont cytoplasmique, *midbody*) (Figure 12). L'importance de cette localisation reste cependant inconnue et rejoint les questions soulevées par ailleurs sur la composition biochimique de ces structures (Skop, Liu et al. 2004). En effet, dans ces travaux les auteurs ont analysé le contenu biochimique du pont cytoplasmique par une approche protéomique, et ont pu mettre en évidence la présence de GLUT1CBP, initialement identifié comme GLUT1 par une interprétation erronée de leurs résultats que nous avons corrigé (Skop, Liu et al. 2005). Bien que certaines données de microscopie basées sur l'utilisation d'anticorps anti-GLUT1 suggèrent bien la présence de GLUT1 dans ces





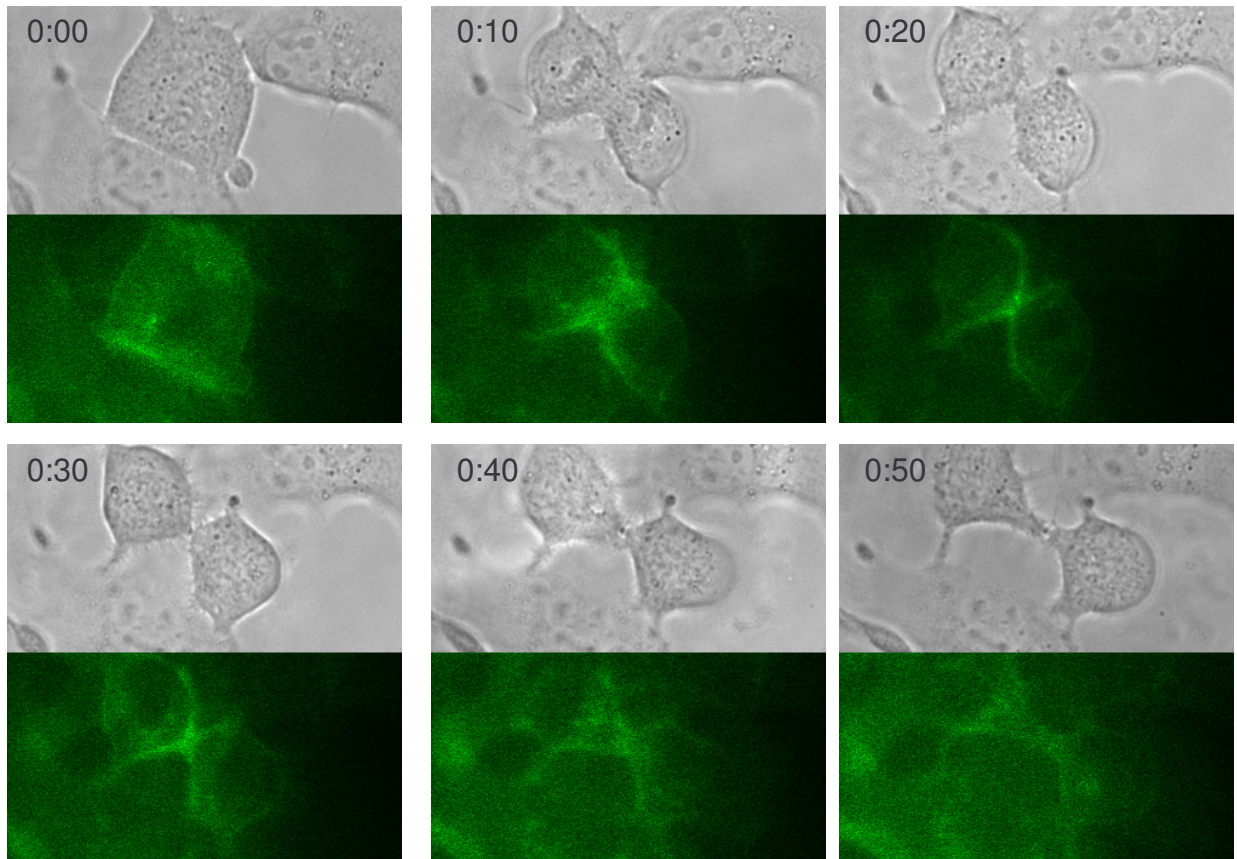
**Figure 10.** Similitude de localisation entre GLUT1GFP et  $\beta$ -COP.

(A) Localisation de GLUT1-GFP dans des cellules MDBK. (B) Localisation de CFP-beta-COP dans des cellules Vero. Image tirée du EMBL/DKFZ Localization Project (<http://gfp-cdna.embl.de/>). Echelle : 6,5  $\mu$ m.



**Figure 11.** Localisation de GLUT3-GFP dans les cellules MDBK observées par vidéomicroscopie.

Vitesse d'acquisition : une image par minute.



**Figure 12.** Localisation de GLUT3GFP dans le "*midbody*" au cours de la division cellulaire.

Cellules MDBK exprimant stablement GLUT3-GFP. Un évènement de fission cellulaire a été capturé sur une minute. Haut : Nomarski. Bas : fluorescence GFP.

structures, la problématique reste ouverte quant à l'implication de transporteurs de glucose dans la mécanistique mitotique.

La transmission de HTLV de cellule à cellule semble impliquer la formation d'une synapse infectieuse. L'Env HTLV serait recrutée au niveau de la synapse infectieuse lors du contact cellule-cellule (Igakura, Stinchcombe et al. 2003). La localisation de l'Env sur les lymphocytes primaires infectés par HTLV, avant formation de la synapse, est sujette à des résultats contradictoires. En effet, alors que l'équipe de Charles Bangham observe une localisation dispersée de l'Env (Igakura, Stinchcombe et al. 2003), l'équipe de Claudine Pique observe une concentration de l'Env dans une zone restreinte (Blot, Delamarre et al. 2004). De plus, cette concentration locale d'Env colocalise avec la protéine hDlg (Blot, Delamarre et al. 2004). La localisation de GLUT1 sur les cellules infectées ou sur les cellules cibles n'a pas été rapportée. Il est possible que GLUT1, sur les cellules cibles, soit recruté dans le voisinage de la synapse par l'Env HTLV exprimée à la surface des cellules infectées (observations non publiées mentionnées dans (Jolly and Sattentau 2004)) par un autre mécanisme, impliquant par exemple les molécules d'adhésion de la synapse immunologique (Derse and Heidecker 2003). En accord avec cette éventualité, des structures membranaires de type radeaux lipidiques sont recrutées au niveau de la synapse immunologique (Bi, Tanaka et al. 2001), et GLUT1 localise dans les radeaux lipidiques de plusieurs lignées cellulaires (Sakyo and Kitagawa 2002; Rubin and Ismail-Beigi 2003; Wielgosz, Rauch et al. 2005)

### **B.5. L'Env HTLV, un marqueur de marqueur**

L'Env HTLV, et notamment son RBD, reconnaît GLUT1 à la surface des cellules. Cette propriété contraste avec l'absence d'anticorps polyclonal ou monoclonal reconnaissant de manière efficace et reproductible la partie extracellulaire (ou domaine exofacial) de GLUT1 (résultats non publiés). Il semble en effet que la surface de GLUT1 soit très peu immunogène car très conservée chez les mammifères. Il est donc envisageable d'utiliser des dérivés de l'Env HTLV comme outil spécifique de marquage de GLUT1 (Jeanteur 2004). Ce

marquage de surface cellulaire est d'autant plus pertinent qu'il représente le GLUT1 fonctionnel, impliqué dans la nutrition cellulaire, et pas le GLUT1 cellulaire global.

GLUT1 est déjà utilisé comme marqueur de nombreuses tumeurs, et une forte expression de GLUT1 est en général un mauvais pronostic (Haber, Rathan et al. 1998). Notamment, la détection de tumeurs cérébrales par PET-SCAN repose sur l'utilisation de fluorodeoxyglucose radioactif qui est exclusivement transporté dans les cellules par GLUT1 (Brown and Wahl 1993; Higashi, Tamaki et al. 1997). Il est donc envisageable d'utiliser le RBD pour détecter l'émergence de microtumeurs métaboliquement actives, par microscopie sur coupe ou par cytométrie de flux, ou encore d'utiliser le RBD pour établir une carte d'activité métabolique indirect des divers tissus pour qui les mesures métaboliques biochimiques sont rarement réalisables. Cette stratégie peut par ailleurs être étendue à l'ensemble des transporteurs de nutriments pour lesquels une Env rétrovirale associée à été identifiée (Jeanteur 2004).

## **B.6. Partenaires de GLUT1**

Alors que de nombreuses molécules ont été identifiées comme participant à la modulation de la localisation et de l'activité de GLUT4, parmi lesquelles TUG apparaît comme le partenaire direct du recrutement de GLUT4 suite à l'action de l'insuline (Bogan, Hendon et al. 2003), aucun partenaire de GLUT1 modulant son activité de manière univoque n'a été caractérisé.

La stomatine, ou bande 7.2b, une protéine transmembranire fortement exprimée dans les érythrocytes, interagit avec GLUT1 (Zhang, Hayashi et al. 1999). L'impact de cette interaction semble réduire l'activité de transport de glucose assurée par GLUT1.

Des expériences de double hybride et de co-immunoprécipitation suggèrent que GLUT1CBP interagit avec GLUT1 (Bunn, Jensen et al. 1999). GLUT1CBP est une protéine intracellulaire à domaine PDZ, capable de multimérisation. GLUT1CBP semble interagir avec plusieurs autres protéines cellulaires, notamment Tax de HTLV (sous le nom TIP-1)

(Reynaud, Fabre et al. 2000), RGS-GAIP (pour *regulator of G protein signaling-Galpa-interacting protein*) (De Vries, Lou et al. 1998), la neuropilin-1 (sous le nom GIPC pour *GAIP interacting protein, C terminus*) (Cai and Reed 1999) et la myosine VI (Bunn, Jensen et al. 1999). La myosine VI est la seule myosine impliquée dans le transport rétrograde le long des filaments d'actine, et elle est impliquée dans la translocation des vésicules d'endocytose en provenance de la périphérie après endocytose (Aschenbrenner, Lee et al. 2003; Aschenbrenner, Naccache et al. 2004). La myosine VI interagit de manière transitoire avec les vésicules, suite au détachement du manteau de clathrine, et se sépare des vésicules avant leur fusion avec les endosomes. La myosine VI constitue donc un candidat intéressant de moteur moléculaire qui pourrait être responsable du trafic rétrograde de GLUT1 dans les cellules. Par extension, un tel trafic pourrait être impliqué dans le transport de virus HTLV endocytés, qui pourrait fusionner non pas à la surface membranaire, mais au niveau de vésicules d'endocytose. Nous avons ainsi montré que liaison au récepteur et entrée virale sont deux activités dissociables sur GLUT1 (voir paragraphe "Propriétés distinctes de liaison et d'infection sur glut1"), et on peut donc imaginer que HTLV lie dans un premier temps GLUT1 à la surface cellulaire, sans qu'il n'y ait entrée virale, et que la fusion proprement dite intervienne plus en aval de l'endocytose. Les vésicules d'endocytoses subissant une acidification progressive, l'éventualité d'une entrée de HTLV après endocytose soulève la question de la sensibilité de l'entrée de HTLV au pH. Bien que la plupart des rétrovirus ne nécessitent pas d'exposition à un pH acide avant liaison au récepteur, il a été récemment montré que pour ALV (*avian leukosis virus*), l'entrée du virus nécessite une exposition à un pH acide après liaison au récepteur (Mothes, Boerger et al. 2000). L'effet d'une variation du pH de vésicules d'endocytose ou extracellulaire au cours de l'entrée de HTLV n'est pas établi, et il est donc possible que l'activité post-liaison de GLUT1 soit en relation avec une variation de pH.

## B.7. Inhibition de l'activité de GLUT1 par l'Env HTLV

Nous avons observé que l'expression de l'Env HTLV, ou de son seul RBD, inhibe l'activité de transport de GLUT1. L'inhibition peut-être due soit à une baisse de l'expression de surface de GLUT1, soit à une inhibition directe de l'activité catalytique de transport. Il est possible de détecter le RBD de HTLV à la surface de cellules transfectées, ce qui suggère que GLUT1 est bien présent à la membrane, et donc que l'inhibition est d'ordre catalytique. De plus, le domaine de liaison à l'Env identifié sur GLUT1 (boucle 6) ne correspond pas aux régions de GLUT1 impliquées dans le transport du glucose (domaines transmembranaires). Enfin, nous avons observé qu'une forme mutante de GLUT1 incapable de transporter le glucose (Q161C C133S C201S C207S C347S) est toujours capable de lier l'Env HTLV et d'assurer l'entrée virale. Ces données suggèrent que l'Env HTLV exerce une inhibition non compétitive de l'activité de GLUT1 à la surface des cellules. Dans le cas du MLV écotrope et de son récepteur le transporteur d'acides aminés cationiques mCAT-1, la liaison de la SU induit la stabilisation d'une des conformations intermédiaires impliquées dans le transport (Tailor, Lavillette et al. 2003). Il est donc probable que de la même manière, la liaison de la SU de l'Env HTLV à GLUT1 stabilise une certaine conformation de GLUT1. L'activité catalytique de transport de glucose de GLUT1 se conforme partiellement au modèle de transporteur passif uniport "E1-E2". Dans ce modèle, GLUT1 bascule constamment entre deux conformations exclusives, E1 ou E2. En conformation E1, le site intracellulaire de liaison au glucose est exposé alors que le site extracellulaire est masquée, et inversement en conformation E2. Certaines mutations ponctuelles de GLUT1 bloquent préférentiellement GLUT1 dans une certaine conformation. Ainsi, la mutation P385I bloque GLUT1 en conformation E1 (*inward*) (Tamori, Hashiramoto et al. 1994), alors que les mutations Y293I (Mori, Hashiramoto et al. 1994), S294A et T296A (Doege, Schurmann et al. 1998) bloquent GLUT1 en conformation E2 (*outward*). L'impact de telles mutations sur la liaison de la SU de l'Env HTLV et l'activité post-binding de GLUT1 n'est pas connu.

## **C. GLUT1 ET LES MODELES DE PATHOGENESE**

*- "La maladie n'est qu'un effet secondaire de la présence du virus. La maladie ne sera prise en compte par la sélection naturelle que lorsqu'elle influera sur la survie du virus." Pierre SONIGO, Ni Dieu Ni Gène.*

### **C.1. Résumé article 4**

L'identification de GLUT1 comme récepteur pour HTLV, et la capacité de l'Env HTLV à inhiber l'activité de GLUT1 soulève de nouvelles problématiques relatives aux deux pathologies induites par HTLV, la leucémie T de l'adulte et la paraparésie spastique tropicale.

### **C.2. Article de revue 4**



## HTLV ENVELOPES AND THEIR RECEPTOR GLUT1, THE UBIQUITOUS GLUCOSE TRANSPORTER: A NEW VISION ON HTLV INFECTION?

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### TABLE OF CONTENTS

1. Abstract
2. Introduction
  - 2.1. Foreword
  - 2.2. Retroviral entry, the viral envelope, and Env, the retroviral envelope glycoprotein
  - 2.3. Cell surface receptors for retroviral envelopes
  - 2.4. HTLV, HTLV Env-mediated tropism, fusion and viral entry: facts and questions
    - 2.4.1. HTLV and STLV, endemic primate retroviruses of the Deltaretrovirus PTLV species
    - 2.4.2. Restricted *in vivo* tissue distribution versus extended *in vitro* tropism
    - 2.4.3. Cell-free and cell-to-cell infection
    - 2.4.4. Env-mediated receptor binding and syncytia formation
    - 2.4.5. pH dependence and receptor requirements
    - 2.4.6. Cellular factors involved in HTLV Env-mediated membrane fusion and viral entry
3. HTLV and STLV envelope glycoproteins: a simple Env in a complex retrovirus
4. HTLV Env receptor expression and T cell activation
5. GLUT1 as a HTLV Env receptor for viral entry
6. Lymphocyte activation and GLUT1-dependent metabolism
7. Impact of HTLV Env-GLUT1 interactions on metabolism
8. Conclusions and perspectives
  - 8.1. Determinants of HTLV Env-GLUT1 interactions
  - 8.2. HTLV Env infection and GLUT1 expression: How to conciliate ubiquity and restricted distribution?
  - 8.3. New insights into HTLV-mediated pathogenesis
  - 8.4. HRBD as a marker for GLUT1 surface expression
  - 8.5. HRBD-GLUT1 interactions as diagnostic and therapeutical tools
9. Acknowledgments
10. References

### 1. ABSTRACT

We identified the ubiquitous glucose transporter GLUT1 as a receptor for Deltaretroviruses HTLV-1 and HTLV-2 envelopes (Env), mediating viral binding and entry. Here, we review the context and key observations that led us to this finding: functional modules of HTLV SU are similar to those of Gammaretrovirus Env which use multimembrane-spanning nutrient transporters as receptors; the HTLV Env receptor is an early marker of T lymphocyte activation; and HTLV Env inhibits glucose transport. We review several molecular, viral, cellular and physiological aspects of HTLV infection in relation to the *in vivo* and *in vitro* properties of GLUT1. Also, we examine the implications of HTLV-1 Env-GLUT1 interactions and altered glucose transport on the two major HTLV-1-induced diseases, adult T cell leukemia (ATL) and neurodegenerative tropical spastic paraparesis/HTLV-associated myelopathy (TSP/HAM). Complementary to the classical models of disease progression, we propose new schemes that emphasize the potential metabolic alterations caused in different cellular compartments. Finally, we review the potential use of HTLV Env-derived constructs as tools for labeling GLUT1 *in vivo* and inhibiting GLUT1 transport

in tumor cells.

### 2. INTRODUCTION

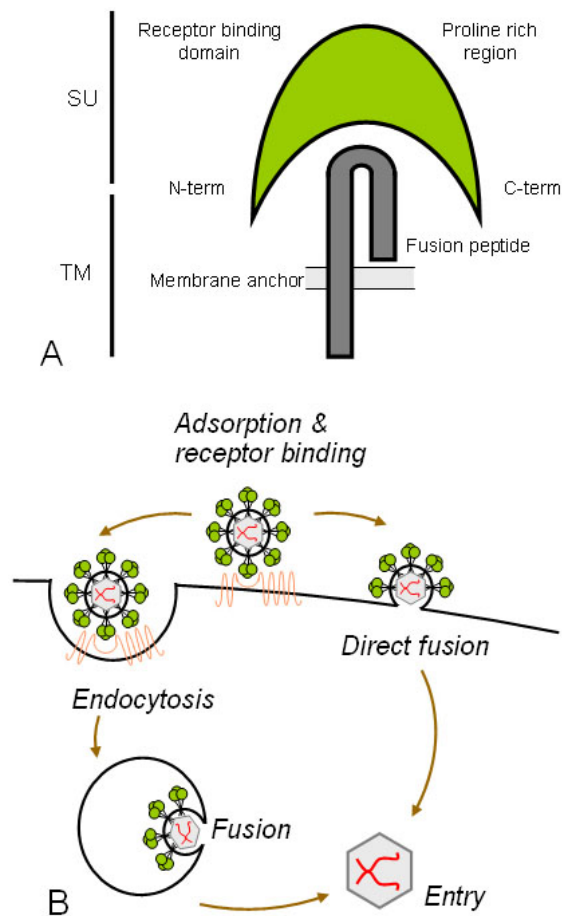
#### 2.1. Foreword

Here, we will not pretend to offer a review of the last twenty years of research on human T-cell leukemia/lymphoma virus (HTLV) and HTLV-related retrovirus envelopes and their cell membrane-associated interacting components. Neither will we try to offer a review of the literature on GLUT1, the ubiquitous glucose transporter that we identified as a receptor for HTLV envelope-mediated viral entry. Rather, we will attempt to provide some insights into the context in which we identified GLUT1 as a receptor for HTLV envelope-mediated infection. We will also highlight some of the questions related to this identification and explore a few issues with regards to HTLV infection and physiopathology that may be advanced by this work.

#### 2.2. Retroviral entry, the viral envelope, and Env, the retroviral envelope glycoprotein

The primary events that condition infection by exogenous retroviruses rely on interactions between the viral

## Glucose transporter GLUT1 as the HTLV receptor



**Figure 1.** Schematic representation of the envelope glycoprotein of Gammaretroviruses. (A) The envelope glycoprotein comprises a surface subunit (SU) and a transmembrane subunit (TM). The former contains receptor-binding properties and stabilizes the fusion peptide present in the amino terminus of the TM which is anchored in the viral envelope or at the cell surface of infected cells. (B) Upon receptor binding, the envelope SU is shed, triggering the release of the fusion peptide required for fusion of the viral and cellular membranes. This fusion process takes place either at the cell surface plasma membrane or after endocytosis, depending on the retrovirus and the target cell. After the formation of a fusion pore, the viral nucleoprotein complex is released into the cytoplasm.

envelope and outer cell surface components. While most interactions between exogenous virions and neighboring cells are likely to lead to the sheer engulfment and catabolism of viral particles via cellular endocytosis (see for instance (1, 2)), viral entry leading to productive infection appears to require interactions between the retrovirus envelope and particular cellular receptors. Successful envelope-receptor interactions lead to membrane fusion and the release of viral capsids into the cytoplasm. Depending on the retrovirus but also on the cell type, fusion is either pH-independent and occurs directly at the cell plasma membrane, or pH-dependent and takes

place after endocytosis in intracellular vesicles (figure 1B).

The retroviral envelope is dragged out of the cell plasma membrane of virion-producing cells and the viral-encoded envelope glycoprotein (Env) is inserted within. The very first steps of infectious retroviral entry are governed by Env. Functional Env molecules of vertebrate retroviruses are synthesized as glycoprotein precursors that are cleaved in the Golgi into two main components: an entirely extracellular and glycosylated surface component (SU) and a transmembrane component (TM) to which SU is associated (figure 1A) (see (3)). All retroviral SU have been shown to harbor the receptor binding determinants, whereas the TM, which harbors a typical fusion peptide at its amino terminus, is directly responsible for the initiation of fusion pores. As worked out in different retroviral models, Env-mediated infectious viral entry appears to include at least the following steps: adsorption of the particles to the cell surface; SU-Env receptor interaction; Env conformational changes that release the TM fusion peptide; formation of TM-mediated fusion pores; and membrane fusion. In the case of oncoretroviruses that belong to the Gammaretrovirus genus, such as murine leukemia viruses (MLV), conformational changes that follow the primary SU-receptor interaction seem to be conditioned by a subsequent interaction between amino and carboxy terminal SU sequences (see for instance (4-6)).

### 2.3. Cell surface receptors for retroviral envelopes

The human immunodeficiency virus (HIV) pandemic has triggered a great deal of effort toward the identification of receptors used by retroviruses to enter and infect cells. Since the identification of CD4 as the HIV Env receptor (7, 8), the list of receptors continues to grow (see table 1). Interestingly, CD4 is one retroviral receptor for which coreceptors, multimembrane-spanning chemokine receptors, have been formally identified. The generally accepted scheme is that an initial interaction of HIV Env with CD4 is followed by the recruitment of chemokine receptors. It is also noteworthy that all receptors identified for mammalian oncoviruses belonging to the Gammaretrovirus group are multimembrane-spanning molecules whose function is to transport different nutrients, such as amino acids, inorganic phosphate and myo-inositol (9-11). In contrast, avian Alpharetroviruses and retroviruses other than mammalian Gammaretroviruses use single membrane spanning or lipid-anchored proteins (10). Despite the fact that HTLV-1 was the first human retrovirus isolated, identification of its Env receptor awaited another twenty years.

### 2.4. HTLV, HTLV Env-mediated tropism, fusion and viral entry: facts and questions

#### 2.4.1. HTLV and STLV, endemic primate retroviruses of the Deltaretrovirus PTLV species

Since the first description of adult T cell leukemias and the isolation of HTLV-1 in endemically infected population in Southern Japan (12) and in a patient with cutaneous T-cell lymphoma (13), the two known types of HTLV, HTLV-1 and the related HTLV-2 type, have been found to be more widespread than initially thought. HTLV is endemic to all populated continents, with the

## Glucose transporter GLUT1 as the HTLV receptor

**Table 1.** Identified retroviral receptors

Retrovirus envelope	Host <sup>1</sup>	Receptor	Topology	Cell function	References
Ecotropic-MLV	Mouse	CAT1	14 transmembrane domains	Basic amino acid transporter	175
Amphotropic-MLV 10A1-MLV <sup>2</sup>	Mouse	PiT2	12 transmembrane domains	Inorganic phosphate transporter	176, 177
Xenotropic Polytropic-MLV	Mouse	XPR	8 transmembrane domains <sup>3</sup>	ND <sup>4</sup>	178-180
M813	Mouse	SMIT1	14 transmembrane domains <sup>3</sup>	Myo-inositol transporter	9
10A1-MLV <sup>2</sup> FeLV-B FeLV-T GaLV	Mouse Cat Cat Monkey	PiT1	12 transmembrane domains	Inorganic phosphate transporter	181
SRV ; BaEV <sup>2</sup> RD114 HERV-W <sup>2,5</sup> SNV ; REV-A	Monkey Cat Human Birds	ASCT1; ATB <sup>0</sup>	10 transmembrane domains	Neutral amino acid transporter	182, 183
FeLV-C	Cat	FLVCR	12 transmembrane domains	Facilitative transporter	183, 184
PERV-A <sup>2</sup>	Pig	PAR1; PAR2	10-11 transmembrane domains <sup>3</sup>	ND	185
HTLV-1; HTLV-2 STLV	Human Monkey	GLUT1	12 transmembrane domains	Facilitative glucose transporter	45
HIV-1 ; HIV-2 SIV-1 ; SIV-2	Human Monkey	CXCR4 <sup>6</sup> ; CCR5 <sup>6</sup>	7 transmembrane domains	G-protein coupled chemokine receptor	186-189
HIV ; SIV	Primate	CD4		TCR signaling	7, 8
MMTV	Mouse	TrfR	Single	Transferrin receptor	190
ALV-A	Birds	TVA	Transmembrane	LDL receptor-like protein	191
ALV-B ; ALV-D ; ALV-E	Birds	TVB		Fas/NFR-like receptor	192, 193
JSRV ENTV	Sheep Goat	HYAL2	GPI anchor	ND	194
FeLV-T	Cat	FeLIX	Soluble	FeLV-B Env origin; Interacts with PiT1	195

<sup>1</sup> Refers to the natural host in which the retrovirus has been isolated, and does not refer to the virus tropism which usually covers a wider species spectrum; <sup>2</sup> Retroviruses that use two different receptors; <sup>3</sup> Debated number of transmembrane domains; <sup>4</sup> ND, not determined; <sup>5</sup> HERV-W is an endogenous Env sequence which is not part of an infectious retrovirus; <sup>6</sup> Only the two main coreceptors identified are indicated. Abbreviations: MLV, murine leukemia virus; FeLV, feline leukemia virus; GaLV, gibbon ape leukemia virus; SRV, simian retrovirus; BaEV, baboon endogenous virus; HERV, human endogenous retrovirus; SNV, spleen necrosis virus; REV, reticuloendotheliosis virus; PERV, porcine endogenous retrovirus; HTLV, human T-cell leukemia virus; STLV, simian T cell leukemia virus; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; MMTV, mouse mammary tumor virus; ALV, avian leukosis virus; JSRV, Jaagsiekte sheep retrovirus; ENTV, enzootic nasal tumour virus.

apparent exception of North Africa and Europe (see (14, 15) and references therein for a review). However, HTLV infections have also been established in these latter areas as sporadic infectious agents, concomitant with the pandemic spread of HIV. A high HTLV incidence has been reported in several indigenous populations of sub-saharan Africa, Middle-East, southwestern Japan, Melanesia, and Americas (15). The endemic presence of HTLV in humans parallels the endemic infection of numerous non-human primate species with closely related simian viruses of several types. Simian T-cell leukemia virus type 1 and 2 (STLV-1 and -2), isolated from several species of monkeys around the world, including new-world spider monkeys, exhibit a close relationship with their human counterparts. On the other hand, a human equivalent for a third STLV type, STLV-3, isolated from different species of monkeys in Eastern and Western Africa (16-18), has yet to be identified. Human HTLV and simian STLV belong to the PTLV (for primate T-cell leukemia virus) retroviral species, which are the main primate members of the Deltaretrovirus genus. Other Deltaretroviruses include the

baboon T cell leukemia virus (BTLV) and bovine leukemia virus. The BTLV Env sequence (accession number U56855) is hardly distinguishable from PTLV-1 (19), whereas BLV Env appears to represent a distinct *bona fide* protein.

### 2.4.2. Restricted *in vivo* tissue distribution versus extended *in vitro* tropism

It has been extensively documented that HTLV-1 infection *in vivo* takes place preferentially in CD4+ T lymphocytes (20), whereas HTLV-2 seems to be more restricted to CD8+ T cells (21). This cellular confinement of HTLV infection *in vivo* is in sharp contrast with the remarkably extended tropism observed for both HTLV types *in vitro*. Thus, HTLV infections *in vitro* have been shown to rapidly propagate in numerous cell types, among which are non-T lymphoid cells, osteosarcoma cells, microglial cells, endothelial cells, placental villous cells and many other cell types (22-27). Moreover, the restricted *in vivo* cell type distribution of HTLV contrasts with the fact that HTLV-1 and HTLV-2 Env-mediated infection,

## Glucose transporter GLUT1 as the HTLV receptor

with replication-defective recombinant viruses, can be established in all tested vertebrate cell lines (28-30). Accordingly, the HTLV-1 Env receptor appears to be present on all tested vertebrate cell lines as assayed by the competitive binding of an isolated HTLV-1 SU (31). In light of these observations, the possibility of a broader *in vivo* tropism for both HTLV types has emerged. Indeed, several reports have shown that in addition to CD4+ and CD8+ T cells, monocytes and B cells can also be infected by HTLV-1 and -2 *in vivo* (32-34).

Because of the intrinsic difficulty in examining patients at the very early stages of infection, the exhaustive investigation of HTLV infection in non-hematopoietic tissues cannot be performed. To circumvent this obstacle, Kazanji *et al.* have used *de novo* HTLV-1 infected squirrel monkeys as a model for the early installment of HTLV-1 infection (35). Although these authors found a marked preferential lymphoid distribution of HTLV-1 proteins, they also reported that numerous non-hematopoietic tissues, including muscle, secretory glands, pancreas and intestine, appear to support HTLV-1 replication (35). The ability of HTLV to infect non-lymphoid tissues in humans is supported by a report describing the infection of breast epithelial cells in a rare case of combined adult T-cell leukemia (ATL) and gynecomasty in a man (36). Thus, while preferential infection of hematopoietic cells, with a marked predilection for T lymphocytes, appears to be the rule for HTLV infections, targeting of HTLV to non-hematopoietic tissues early after infection cannot be excluded and deserves closer examination.

The vast research on HTLV-infected individuals leaves little doubt that T lymphocytes are the major, although not exclusive, virus reservoir *in vivo*. The origin for this T cell-preferred distribution *in vivo* can be due to differences in the availability of Env receptors and co-factors for viral entry (37), as well as to differences at post-entry levels (for a review see for instance (38)). Env-independent cell specific restrictions, taking place at pre and post-integration levels, have been identified for other retroviruses. For example, the murine *Fv-1* and human *Ref-1* genes exert a post-entry restriction on MLV capsids before integration (see (39) and references therein) as well as simian TRIM5a on HIV; also the CEM15/APOBEC-3G cytidine deaminase inhibits infection by targeting the neosynthesized proviral DNA but this effect is neutralized by the HIV Vif protein (see (39) and references therein); and a lack of HIV Tat transcription-enhancing activity in the absence of cyclin T1 results in a cell type-specific restriction (40). Although similar mechanisms have not been documented for HTLV, it is interesting to note that HTLV-1 capsid-containing retroviral cores have been shown to play a role in the low infectivity of HTLV-1 (41). It remains to be determined whether this is due to capsid-directed restrictive cellular factors as described above. Finally, in addition to direct virus-cell interactions, antiviral activities of the host organism, such as the immune response (42) and cell-specific Env-mediated cytotoxic effects (43-45) certainly have a major impact on the *in vivo* retrovirus tissue distribution. In conclusion, the *in vivo* cell type distribution of HTLV infection, as generally evaluated

late after the initial infection, results from a full array of interactions between the virus and the host, and is not likely to precisely match the *in vitro* cellular tropism, *stricto sensu*, of the virus.

### 2.4.3. Cell-free and cell-to-cell infection

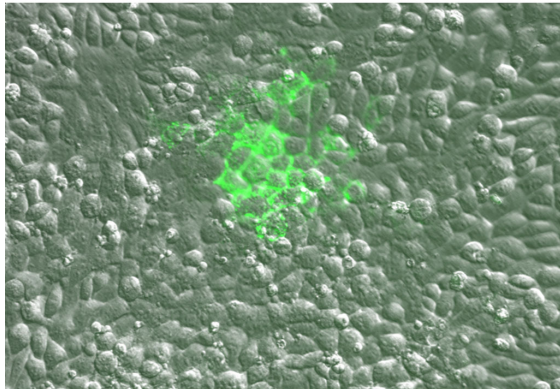
Although achievable, *in vitro* infection with cell-free HTLV-1 virions remains remarkably inefficient when compared to infection by Gammaretroviruses or lentiviruses. Following the earliest attempts to spread HTLV infection *in vitro*, it became clear that infection is remarkably dependent on cell-to-cell contacts (see (46) and references therein). This property has been beautifully illustrated by Igakura *et al.* who used confocal microscopy to visualize the transfer of different virion components from lymphocytes of infected patients to non-infected recipient lymphocytes (46).

The low infectious ability of HTLV-1 virions was also reproduced with replication-defective recombinant vector systems and seems to be due to both the HTLV core (41) and Env proteins (28-30, 47). However, from our own experience, while MLV or HIV cores pseudotyped with HTLV-1 envelope are indeed poorly infectious in cell-free preparations, with infectious titers of approximately  $10^2$  FFU/ml, pseudotyping with HTLV-2 Env leads to titers of  $10^5$  FFU/ml (45). Whether this higher titrating property of the HTLV-2 Env is common to all HTLV-2 strains or is particular to the prototypic Env of the HTLV-2 NRA isolate (48) used in these studies is under investigation. It will also be important to determine whether the low infectious titers of HTLV-1- pseudotyped virions is specific to the Env in the HTLV-1 MT-2 isolate (49). Nevertheless, in the absence of viremia, cell-to-cell infection appears to be the exclusive mode of *in vivo* HTLV transmission (see for instance (50) for a review).

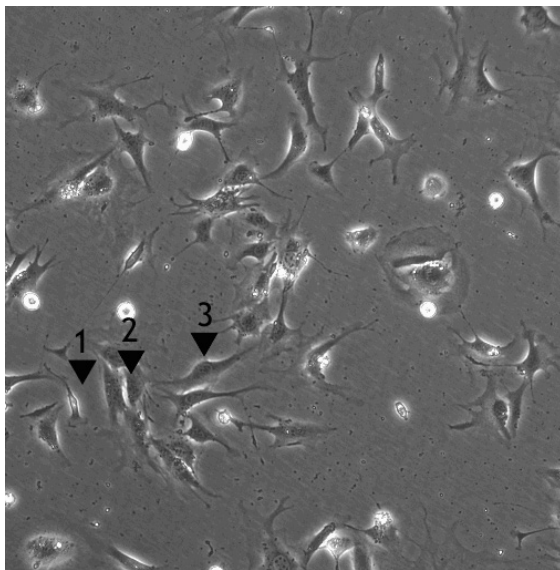
It is important to note that cell-to-cell transmission is also the most efficient mode of transmission for all retroviruses, including HIV and MLV which are generally presented as paradigms of cell-free transmitting retroviruses (see for instance (51)). In the presence of a strong immune response and the absence of viremia, it is likely that cell-to-cell spreading represents the sole mode of *in vivo* transmission for most retroviruses, despite the fact that *in vitro* models of retrovirus replication have favored efficient high-titering viral production at the expense of cell-to-cell transmission. Indeed, when seeding high-titer replication-competent MLV on fully permissive adherent cell monolayers, no rampant viral spreading is observed as monitored by viral antigen expression. Rather, *de novo* infected cells remain confined within restricted foci of infection, even in the absence of any movement-restricting gel which is generally required for plaque assays with cell-free spreading lytic viruses (52) (figure 2). Therefore, as previously put forth by D. Derse, we believe that retroviral spreading arising from cell-to-cell contact between infected and non-infected cells is probably not specific for HTLV but is likely to be « *the rule rather than the exception* » (53). Accordingly, natural transmission of retroviruses requires fluid exchanges that include transfers of significant numbers of infected cells (54).



## Glucose transporter GLUT1 as the HTLV receptor



**Figure 2.** Visualization of a Friend murine leukemia virus (F-MLV) infection focus. As it is the case for HTLV, propagation of replication-competent F-MLV occurs mainly through cell-to-cell contact, resulting in confined foci of infected cells. NIH 3T3 cells were infected at a low multiplicity of infection with F-MLV. A focal immunofluorescence assay [52] was performed 3 days later by staining with an anti F-MLV Env SU monoclonal antibody (H48) followed by a FITC-conjugated secondary antibody.



**Figure 3.** Time-lapse video microscopy of NIH 3T3 cells transfected with a syncytial Env expression vector. Following transfection, images were collected every minute for 16 hours using a wide field microscope. Adherent cells are constantly in motion and establish contacts through filopodiae and lamellipodiae. However, syncytia formation occurs only when cells are expressing fusigenic Env glycoproteins. Three cells that seem to initiate a syncytium are numbered. Cell #1 fuses originally with one daughter of parental cell #3. Immediately after, cell #2 joins the syncytium. The syncytium then expands rapidly, leading to the recruitment, at the center of the syncytium, of more than 20 nuclei that show planar circular movements. DNA-liposome complexes used for transfection are visible as subcellular structures which are rapidly captured and dissolved by cells.

### 2.4.4. Env-mediated receptor binding and syncytia formation

The presence of cellular receptors for Env-mediated viral entry conditions the first steps of retroviral infection and virus cell tropism. The most commonly used assays to study HTLV receptor expression are based on cell-to-cell fusion that take advantage of the highly fusigenic property of HTLV Env, leading to the rapid formation of multinucleated giant syncytia (figure 3). Using adequate target cell types, syncytial properties can be observed for most retrovirus Env (55). In the case of HTLV Env, syncytia formation is spectacularly rampant and develops in almost all vertebrate cell lines tested. However, Env-receptor interactions and HTLV Env-mediated viral entry in the absence of syncytia formation has been observed in a few cell lines (see for instance (29)). Therefore, although syncytia formation appears to be a useful means of monitoring HTLV Env-cell membrane interactions (37, 40), it is not strictly indicative of receptor recognition by HTLV Env. Rather, it is likely to reflect post-binding events required for membrane fusion processes (see (56, 57)).

### 2.4.5. pH dependence and receptor requirements

Retrovirus entry requires the fusion of viral and cell membranes to allow the release of viral cores in the cytoplasm through a fusion pore. This fusion event can take place either at the plasma membrane in a pH-independent manner or after endocytosis when the low pH environment in the endosome triggers Env conformational changes leading to membrane fusion (57). Based on cell-to-cell fusion and vesicular stomatitis virus (VSV) pseudotype infection assays, HTLV-1 was first classified, like most retroviruses, as pH-independent (58-60), consistent with the ability of HTLV to form rampant syncytia. Since we described cells that are specifically resistant to HTLV Env-mediated syncytia formation but remain fully susceptible to HTLV-1 and -2 Env-mediated infection (29), it will be interesting to monitor pH dependence for viral entry in these cells. Indeed, the observation of HTLV particles within endosomes is also consistent with the existence of a pH-sensitive endocytic pathway (61). Interestingly, Trejo *et al.* have reported that HTLV-1 Env-pseudotyped HIV particles infection exhibits intermediate pH-sensitivity on HeLa cells (30), suggesting the use of different routes of infection by HTLV according to the cell type used. Alternatively, acidification may be required at a post-binding level, similar to what has been described for the Env of avian retroviruses (62). Accordingly, it has been suggested that poor HTLV-1 transmission may be due to differences in post-binding events that favor the evolution of a fusion-competent envelope TM protein conformation (37). Thus, low or high abundance of secondary molecules involved in fusion events and different from the primary binding receptor, may influence the tropism of HTLV particles and their mode of entry, as documented for HIV (56, 59). The close evaluation of pH dependence in different physiological settings such as described previously (46) will help bringing new clues to these questions.

### 2.4.6. Cellular factors involved in HTLV Env-mediated membrane fusion and viral entry

The initial search for cell surface components

## Glucose transporter GLUT1 as the HTLV receptor

that are involved in interactions with HTLV Env, leading to viral entry, relied on the use of monoclonal antibodies directed against HTLV Env (63). Thus, cellular components interacting with HLA molecules were suspected to bind HTLV Env at the cell surface because of the cross reactivity reported between HTLV Env and a monoclonal antibody that reacts against the region of an HLA-B locus gene which codes for the extracellular portion of a class I histocompatibility antigen (64). Also, an antibody raised against hIL-2 was found to cross react with Env, suggesting that HTLV receptor could be the hIL-2 receptor (65). On the basis of a relative resistance of murine fibroblasts to HTLV Env-mediated infection and syncytium formation, it was reported that the HTLV-1 and -2 receptor maps to a single locus on human chromosome 17, in 17q21-23, near the thymidine kinase 1 (TK-1) locus (66, 67). However, this hypothesis lost ground before being completely abandoned when mouse fibroblasts were shown to be susceptible to HTLV Env-mediated fusion (68) and infection (28, 30) and the presence of human chromosome 17 in mouse cell hybrids increased neither infection (28) nor HTLV Env binding (31). Interestingly, it was later shown that HTLV could infect numerous cell types in an *in vivo* mouse model (69). Accordingly, mouse cells have been found to bind soluble HTLV-1 Env SU immunoadhesins (31).

Syncytia formation has also widely been used as a read-out for the screening of antibodies directed against Env-receptor interactions. Thus, several monoclonal antibodies found to recognize tetraspanin antigens have been shown to block HTLV Env-mediated syncytia formation and tetraspanins, which are involved in cell adhesion, have been proposed as potential HTLV receptors (70, 71). However, it was later shown that the interaction of CD82 tetraspanin with HTLV envelope inhibits HTLV fusion and cell-to-cell transmission, thus discarding its potential role as a receptor (72).

Several unidentified proteins, ranging from 31 to 70 kDa, have also been suspected as potential receptors based on their recognition by the 34-23 monoclonal antibody which inhibits HTLV-1 virion binding to activated lymphocytes (73). At the time, this was thought to be concordant with the fact that the antigen recognized by 34-23 was found to be expressed from human chromosome 17, at the 17q23.2-17q25.3 position, in the TK-1 locus. It is noteworthy that NIH3T3(TK<sup>-</sup>) cells do not form syncytia upon expression of the HTLV envelope, although these cells possess a functional HTLV receptor (29). Therefore, the syntenic mouse and human locus containing the TK-1 gene may contain a gene coding for a protein involved in a post-receptor binding step.

Peptides derived from the HTLV Env SU were also used to screen for inhibition of syncytia formation in attempts to identify the receptor. Such a peptide (position Asp197-Leu216 of HTLV-1 Env (74) was found to bind the 71-kDa heat shock cognate protein (HSC70) within a beta actin-containing complex (75). Interestingly, HSC70 appeared to be a cell-fusion enhancing factor (76) which interacts with the capsid protein of many enveloped and

non-enveloped viruses (see (77). Against a role for HSC70 as a component of the primary receptor is also the fact that the peptide that binds HSC70 maps to the central proline rich region (PRR) homologue of the HTLV Env SU, while we mapped the receptor binding determinants of the HTLV Env upstream of the PRR homologue (see below) (78).

The finding that HTLV infection is tightly dependent on cell-to-cell contacts has encouraged the evaluation of diverse adhesion molecules in HTLV Env-mediated processes. E-selectin, LFA-I, VLA-4, L-selectin, and CD44 have been shown to be involved in HTLV transmission (79). Moreover, ICAM-1, ICAM-3 and VCAM-1 were also found to modulate syncytia formation, while antibodies directed against integrin beta 2 and beta 7 were shown to inhibit syncytia formation (37, 80). The intervention of non-protein molecules in HTLV receptor functions has also been evaluated. Palmitoyl(16:0)-oleoyl(18:1)-phosphatidylglycerol and lipid rafts were found to be involved in syncytia formation (81, 82). Recently, an implication for heparan sulfate proteoglycans in HTLV Env-mediated fusion and infection has been demonstrated (83).

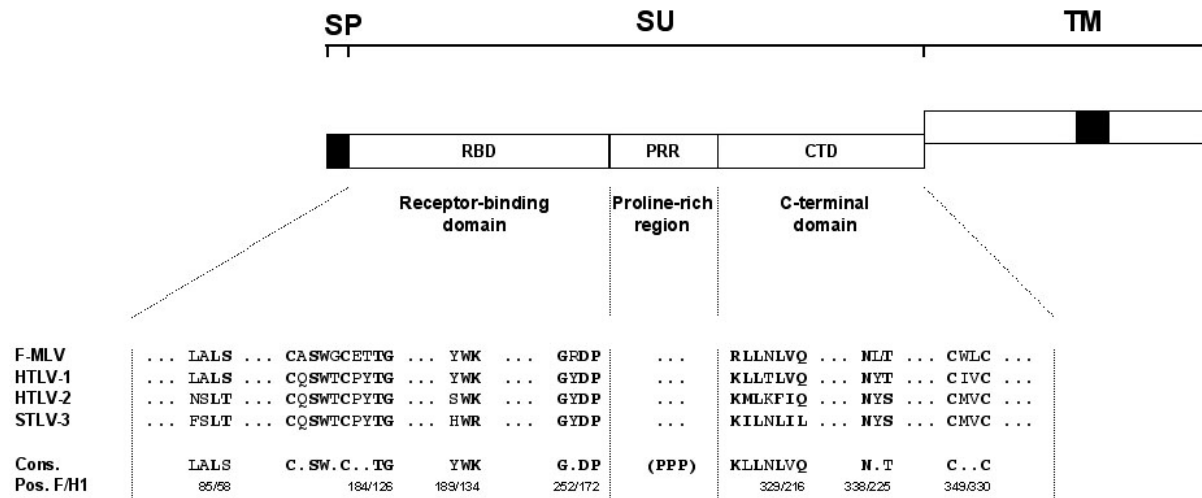
As discussed above, syncytia formation is a multi-step process that depends first on Env-receptor binding but also on post-binding events which bring the fusion pore into syncytial dynamics. The study of HTLV Env-mediated syncytia formation has favored the identification of cellular components involved in post-binding events rather than those implicated in primary receptor binding.

## 3. HTLV AND STLV ENVELOPE GLYCOPROTEINS: A SIMPLE ENV IN A COMPLEX RETROVIRUS

HTLV belong to the Deltaretrovirus genus which is part of the complex retrovirus group that includes the Lentivirus and Spumavirus genera. Complex retroviruses are characterized by the production of several multiple-spliced mRNAs and the production of a whole array of regulatory proteins. Retroviruses which, like MLV, are characterized by single-intron splicing belong to the simple retrovirus group. Phylogenetic analyses based on the conservation of the *pol* gene place HTLV and MLV at the most distant branches of the Retroviridae family phylogenetic tree (84). Therefore, the striking conservation of Env SU motifs between HTLV-1 and the Friend strain of ecotropic MLV (F-MLV) was surprising (44). Those motifs are located upstream of a conserved CxxC motif (85, 86), where x represents hydrophobic residues, and downstream of the MLV SU amino terminal domain that harbors the variable regions sufficient to bind MLV Env receptors (87-89). Based on this SU motif conservation between viruses with phylogenetically distant *pol* sequences and very different overall genetic organizations, we postulated that the MLV and HTLV SU are derived from closely related ancestor genes that were "captured" by ancestral HTLV and MLV retrotransposons (44, 90).

The alignment of the F-MLV SU with the SU of three prototypic HTLV types underscores the presence of a

## Glucose transporter GLUT1 as the HTLV receptor



**Figure 4.** Conservation of motifs in the Env SU of MLV and PTLV. Amino acid sequences of F-MLV (accession number X02794), HTLV-1 (accession number M37747), HTLV-2 (accession number L20734) and STL-3 (accession number CAA61321) SU were aligned using the ClustalW algorithm, and conserved motifs are shown (4 in the receptor binding domain (RBD), and 3 in the carboxy terminal domain). There is no sequence homology in the proline-rich region (PRR) which is very heterogeneous in length (52 residues for F-MLV and 26 for HTLV-1 and -2), as it is the case among Gammaretroviruses. Residues which are either identical between the 4 sequences or belong to the same polarity group are indicated in bold. Other indicated residues in the consensus sequence correspond to shared residues between F-MLV and at least 1 PTLV. The positions of the last residue of each motif are shown for F-MLV and HTLV-1. SP: signal peptide.

PRR homologue as well as several short conserved motifs in the intervariable regions of the MLV receptor binding domain (RBD) and in the carboxy-terminal domain (figure 4). We determined that these conservations reflect a similar functional organization of the HTLV and MLV SU modules. Indeed, a chimeric Env in which the mouse-restricted ecotropic F-MLV RBD is replaced by the HTLV SU amino terminal domain is fusogenic for both mouse and human cells, thus indicating the recognition of an HTLV Env receptor (44).

The fusion triggered by the binding of ecotropic MLV Env to its receptor depends on the interaction of the SU carboxy terminus with a conserved His residue, or any other aromatic residue, at position 8 of the MLV SU (4-6). However, no conserved aromatic residue around this position was found in PTLV SU. It will therefore be interesting to determine whether the HTLV RBD (HRBD) exerts properties similar to those associated with the MLV SU His at position 8. Alternatively, the absence of an equivalent mechanism for triggering fusion may provide additional clues concerning the molecular bases for the low cell-free infectivity of HTLV virions.

#### 4. HTLV ENV RECEPTOR EXPRESSION AND T CELL ACTIVATION

As T cells appear to be the main target for HTLV transformation as well as the major *in vivo* reservoir, we tracked expression of the HTLV receptor on different T cell populations, including CD4 helper T cells, CD8 cytotoxic T cells as well as naive and memory CD4 subsets, using tagged forms of HRBD. As opposed to all cell lines, including transformed T cell lines which express HTLV

receptors, we were surprised that none of these primary T cell populations bound our tagged HRBD. Unlike transformed T cells such as Jurkat, circulating primary T cells are almost entirely in the  $G_0$  phase of the cell cycle and do not express activation markers. The stimulation of primary T cells by antigen occurs *via* the T cell receptor (TCR) and, under normal conditions, promotes an efficient immune response. Upon activation, T cells can undergo 6-8 divisions and secrete various cytokines. This transient phase is associated with the modulation of numerous cell surface markers. Indeed, we and others determined that HTLV receptor expression is transiently induced by TCR activation (45, 91). We also found that receptor expression preceded DNA synthesis and was not strictly dependent on cell division.

Our results indicate that transient expression of the HTLV receptor on T cells following TCR stimulation is likely to be crucial for the constitution of these cells as HTLV reservoirs *in vivo*. In this respect, it is notable that human T cells, including HTLV-infected T cells, can probably survive for more than 20 years in the peripheral circulation. Our data also leads us to speculate that infection of T lymphocytes by the virus preferentially occurs in sites where T cells become activated, such as in inflammatory sites and secondary lymphoid organs. The illustration by Igakura *et al.* of an accumulation of HTLV Gag proteins and genomes at lymphocyte cell-cell junctions and their rapid transfer to uninfected lymphocytes, favors a scenario whereby virus transmission occurs more readily upon extensive contact between cells of the immune system, such as in lymph nodes or inflammatory sites. In a symmetrical scenario, CD4+ T cells directed against HTLV-1 antigens have been shown to be preferentially infected in



## Glucose transporter GLUT1 as the HTLV receptor

HTLV-1 seropositive individuals (92). This may reflect an additional route of transmission from passively infected cells to reactive T lymphocytes. Moreover, when infected with HTLV or transduced with HTLV accessory proteins, T cells express different factors, such as integrins (93, 94), that are likely to enhance the probability of cell contacts.

Breast feeding and accompanying cell transfers that occur between mother and infant have been proposed to be a major route of HTLV transmission (see (54, 95) for a review). The particular "immaturity" of the newborn's immune system may account for this preferential route. Indeed, in contrast to adult peripheral T cells which present a 1:1 ratio of naïve and memory T cells, neonatal T cells are almost exclusively naïve cells, which proliferate and change to a memory phenotype upon encountering with a cognate antigen. Furthermore, the peripheral lymphocytes in newborns have only recently differentiated in the thymus, and as such are designated as recent thymic emigrants (RTE). RTE respond to antigenic and cytokine stimulation differently from adult memory T cells (96-98). RTE enter more rapidly into cycle when activated through the TCR, with 24% of newborn RTE going into G<sub>1</sub> versus 1% for adult T cells, following 15 hours of TCR stimulation (representative data from S. Jaleco and N. Taylor, unpublished results). Moreover, neonatal, and not adult, T cells undergo multiple divisions in response to the IL-7 cytokine (97, 98). Indeed, IL-7 triggered surface expression of the HTLV receptor in CD4<sup>+</sup> lymphocytes isolated from umbilical cord blood, albeit at significantly lower levels than that observed in TCR-activated lymphocytes (99). This expression of the HTLV receptor is not a constitutive characteristic of neonatal T cells but is directly dependent on IL-7 treatment, since HTLV receptor was not observed in freshly isolated quiescent neonatal T cells. Interestingly young HTLV-1 seropositive patients have a decreased proportion of naïve T lymphocytes and display higher levels of plasma IL-7 than non infected individuals (100). Thus, expression of the HTLV receptor by IL-7-stimulated neonatal T cells suggests a possible mechanism *via* which HTLV can be transmitted from an infected mother to the infant, even in the absence of antigenic stimulation.

### 5. GLUT1 AS A HTLV ENV RECEPTOR FOR VIRAL ENTRY

As described above, the HTLV SU RBD appears to share the same organization as the SU of MLV-related retroviruses, and all identified MLV receptors are multimembrane-spanning nutrient transporters (table 1). As such, we focused our search for an HTLV Env receptor on multimembrane-spanning molecules potentially involved in the transport of nutrients.

Transfection of cells with HTLV Env or HRBD constructs specifically blocked milieu acidification. As most of the H<sup>+</sup> proton accumulation in *in vitro* cultured cell lines is due to the activity of H<sup>+</sup>/lactate symporters (101), we first evaluated proteins involved in lactate transport, such as monocarboxylate transporter 1 (MCT1) and its chaperone protein CD147 (102, 103), as potential HTLV

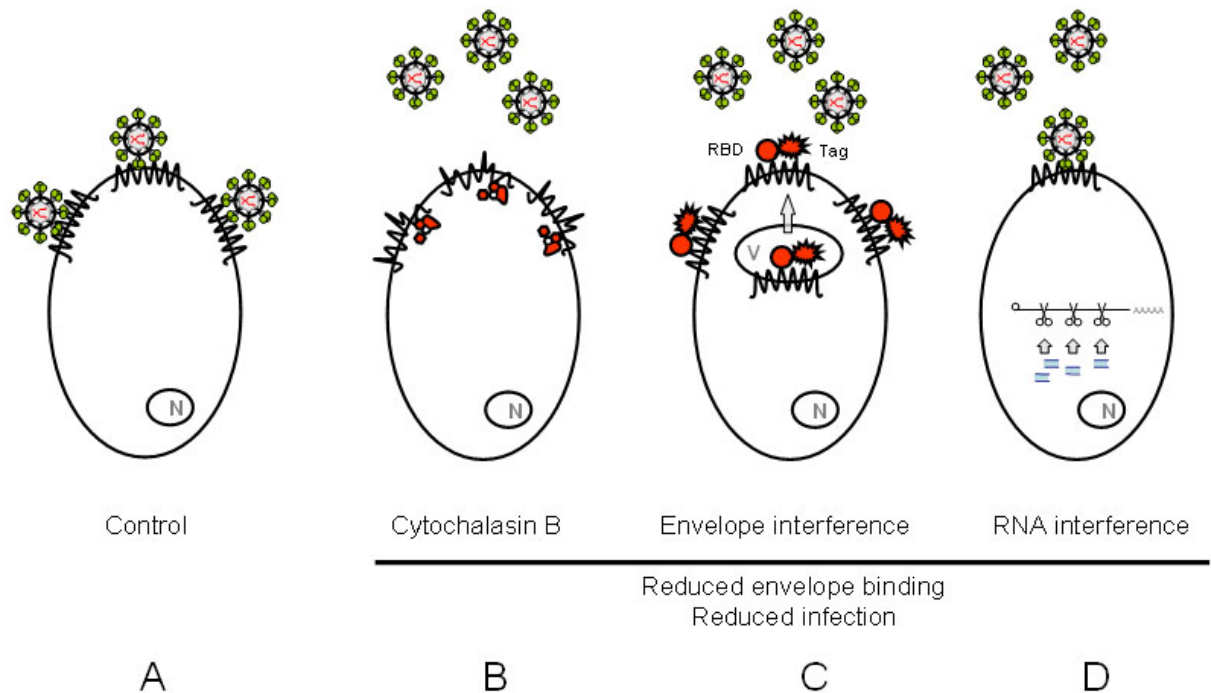
Env receptors (45). Because the HRBD-mediated block of medium acidification was not associated with these candidate receptors and most of the lactate produced in cell cultures is derived mainly from glucose degradation, we next evaluated glucose transporters as HTLV Env receptors.

Vertebrate glucose transporters belong to the GLUT family which comprises 14 members divided into 3 classes that include other hexose transporters, and members of unknown functions (104, 105). Upon treatment with cytochalasin B, an inhibitor of GLUT glucose transporters (106, 107), HRBD binding as well as Env-mediated infection are impaired (figure 5B), in agreement with the involvement of GLUT molecules in HTLV Env-mediated entry process (45). Within this family, we hypothesized that GLUT1 best fit all the known properties of the HTLV receptor. However, because all classical cell culture conditions force cell adaptation to fermentation which tightly relies on glycolysis, all established vertebrate cell lines express GLUT1. As such, they are not suitable for potential receptor screening by classical cDNA transfection strategies. Also, upon transient transfection of GLUT1, or the related GLUT3 isoform cells acquire a round shape and eventually detach from the support, a typical consequence of cell toxicity that can tentatively be attributed to an excess in intracellular glucose levels. Indeed, the selection of stable clones over-expressing GLUT1 was unsuccessful in our hands. For this reason, we based our screening strategy on a transient gain-of-function assay based on glucose transport, HRBD binding and HTLV Env-mediated retroviral infection.

We developed several approaches in order to vary GLUT1 availability at the cell surface. Upon transient transfection of GLUT1 cDNA in several mammalian cell lines, we observed a striking elevation of HTLV Env binding and HTLV Env-mediated infection (45). Based on this observation, we further confirmed the role of GLUT1 as an HTLV Env receptor using two additional assays. First, we evaluated the ability of GLUT1 to specifically restore HTLV Env-mediated infection after blocking the HTLV Env receptor with Env itself (figure 5C). In this interference assay, the HTLV RBD is expressed in cells concomitantly with receptor candidates. Upon expression of the HRBD, the availability of the receptor decreases leading to a drop in viral titers of at least 2 logs. This drop was totally abolished upon cotransfection of GLUT1 but not the GLUT3 isoform, demonstrating a direct effect of GLUT1, independent of its glucose transport activity, in restoring HTLV RBD binding and HTLV Env-mediated infection (45). Furthermore, in a second assay, we used small interfering RNAs to down-modulate endogenous levels of GLUT1 RNA (figure 5D). Following this down-modulation, HTLV Env binding and Env-mediated infection were significantly reduced, while binding and infection mediated by the amphotropic MLV Env were not reduced. In this system as well, introduction of GLUT1, but not the GLUT3 isoform, fully restored HTLV Env binding and infection (45). In these systems, GLUT1 was found to serve as cell surface receptor for both HTLV-1 and -2 infection (45) and unpublished observations).



## Glucose transporter GLUT1 as the HTLV receptor



**Figure 5.** Schematic representation of assays designed to downmodulate GLUT1 transport and expression. Three techniques were used to inhibit GLUT-1 availability or expression in 293T cells. Cells were (A) not treated; or treated with (B) cytochalasin B which inhibits glucose transport activity by interacting with the cytoplasmic face of GLUT1; (C) an interfering HTLV RBD that saturates vesicular or plasma membrane GLUT1 via direct binding to the transporter; and (D) interfering RNAs directed against the 3' untranslated region of the GLUT1 mRNA. In all three of the latter conditions, HTLV Env-mediated binding and infection were significantly reduced as compared to the non treated cells. Moreover, binding and infection could be reestablished by overexpressing GLUT-1, but not the related glucose transporter GLUT-3, in conditions C and D. N: Nucleus; V: Vesicle.

Immunoprecipitation of HTLV RBD with an HTLV Env antibody was accompanied by an efficient pull-down of GLUT1, confirming the close physical interaction between the HTLV RBD and GLUT1 (45). Further analyses of GLUT1-HRBD complexes may lead to the identification of additional partners, such as potential coreceptors. In this regard, it will be interesting to compare pull-down experiments performed with entire SU immunoadhesins (31, 91), and different forms of truncated HTLV SU or chimeric HTLV-MLV Env (44, 45, 78).

GLUT1 physically interacts with an amino terminal RBD fragment of approximately 160 residues that we identified as the minimal receptor-binding domain (78). Furthermore, we identified a conserved tyrosine residue (Y114 for HTLV-1 and Y110 for HTLV-2) as required for receptor binding (45, 78), and our preliminary results suggest that HTLV-1 and -2 RBD interact with the same surface epitope of GLUT1. Thus, the same motifs and probably the same conformations govern the interaction between GLUT1 and the HTLV-1 and HTLV-2 SU. However, this does not preclude differences in their respective affinities for GLUT1 or differences in the recruitment of other membrane-associated cellular factors, including potential coreceptors. In this regard, it is interesting to note that upon transfection of GLUT1, the increased binding of an HTLV-1 HRBD appeared

relatively homogenous whereas HTLV-2 HRBD binding reached higher levels but was more heterogeneous (N. Manel, unpublished observations). Thus, it is possible that other molecules differentially modulate the binding of HTLV-1 and -2 envelopes.

## 6. LYMPHOCYTE ACTIVATION AND GLUT1-DEPENDENT METABOLISM

Using the HRBD, we were not able to detect surface GLUT1 expression on the vast majority of freshly isolated CD4 or CD8 T lymphocytes (99). This is either due to a true absence of surface GLUT1 or to the level of sensitivity of our tagged-HRBD that does not allow detection of low amounts of this protein. Our conclusion that the vast majority of primary human T lymphocytes express low or absent levels of GLUT1 is supported by data wherein GLUT1 expression was not detected in freshly isolated human T cells using a polyclonal rabbit anti-GLUT1 antibody, directed against the intracellular carboxy-terminal domain (108). Nath *et al.* did detect HTLV receptor expression, i.e. GLUT1, on primary human T lymphocytes using an SU immunoadhesin on lymphocytes isolated by counter elutriation, wherein the cells were subjected to a high speed centrifugation for an extended time period, followed by positive selection with CD4 or CD8 antibodies (91). Moreover, when these authors

## Glucose transporter GLUT1 as the HTLV receptor

assessed GLUT1 expression in lymphocytes isolated via negative selection and in the absence of elutriation, only a very low percentage of T cells bound the HTLV SU immunoadhesin (91). In this regard, it is important to note that several recent studies, including work performed in our own laboratory, have shown that the choice of blood coagulant as well as the method used to isolate T lymphocytes can modulate surface expression of receptors pertinent to T lymphocyte function (109).

The observation that glucose metabolism increases upon T lymphocyte activation was reported over 30 years ago (110-113). Soon thereafter, it was also shown that mitogen stimulation increases glucose transport in thymocytes (114). Indeed, in 1978 Whitesell and Regen reported that the thymus contains two populations of cells; quiescent cells where the glucose transport equilibrates with a half-time of 30-50 minutes and "active" cells where the half-time is approximately 1 minute (115). While glucose transport is crucial to the survival, differentiation and proliferation of thymocytes and mature T lymphocytes, the vast majority of studies concerning glucose metabolism in these cells were performed in the late seventies and early eighties, thus precluding studies of GLUT members.

The first glucose transporter (GLUT1) was identified by Mueckler *et al.* in 1985 (116), and in 1994, Mookerjee and colleagues assessed the expression and subcellular distribution of different GLUT isoforms in primary human T lymphocytes (108). They found that GLUT1 was not expressed on quiescent human T lymphocytes but was induced, predominantly at the plasma membrane, by 24 hours post mitogen stimulation. While other GLUT isoforms (GLUT-2 and GLUT-3) were found to be expressed on quiescent T cells, the importance of GLUT1 in T cell activation was strongly suggested by the finding that only its expression was correlated directly with glucose transport activity (108). Later was shown that the serine/threonine kinase Akt induces glucose uptake and cellular glycolysis in 3T3-L1 fibroblasts. This occurs via a translocation of the insulin-responsive GLUT-4 transporter to the plasma membrane and an increase in the synthesis of GLUT1 (117). Studies such as these, performed in several cell systems, were then transposed to T lymphocytes. Indeed, very recently, increased glycolysis due to expression of an activated Akt in murine T cells was shown to result in increased size, resistance to death-by-neglect, and T-cell malignancy (118). Under normal conditions, GLUT1 protein does not appear to be expressed in quiescent murine T cells (119, 120), as is the case for their human counterparts. After the original description that thymocytes can be divided into two groups with regards to glucose transport (115), A. Singer's group explored the molecular basis of this process and showed that there is a large heterogeneity in GLUT1 expression in murine thymocyte subsets (121). Moreover, stimulation of thymocytes with the IL-7 cytokine results in a significant increase in GLUT1 expression and an associated upregulation of the anti-apoptotic Bcl-2 protein (121). Thus, it appears that GLUT1 expression plays a role in the survival, proliferation and transformation of T lymphocytes.

It is important to note that in the vast majority of

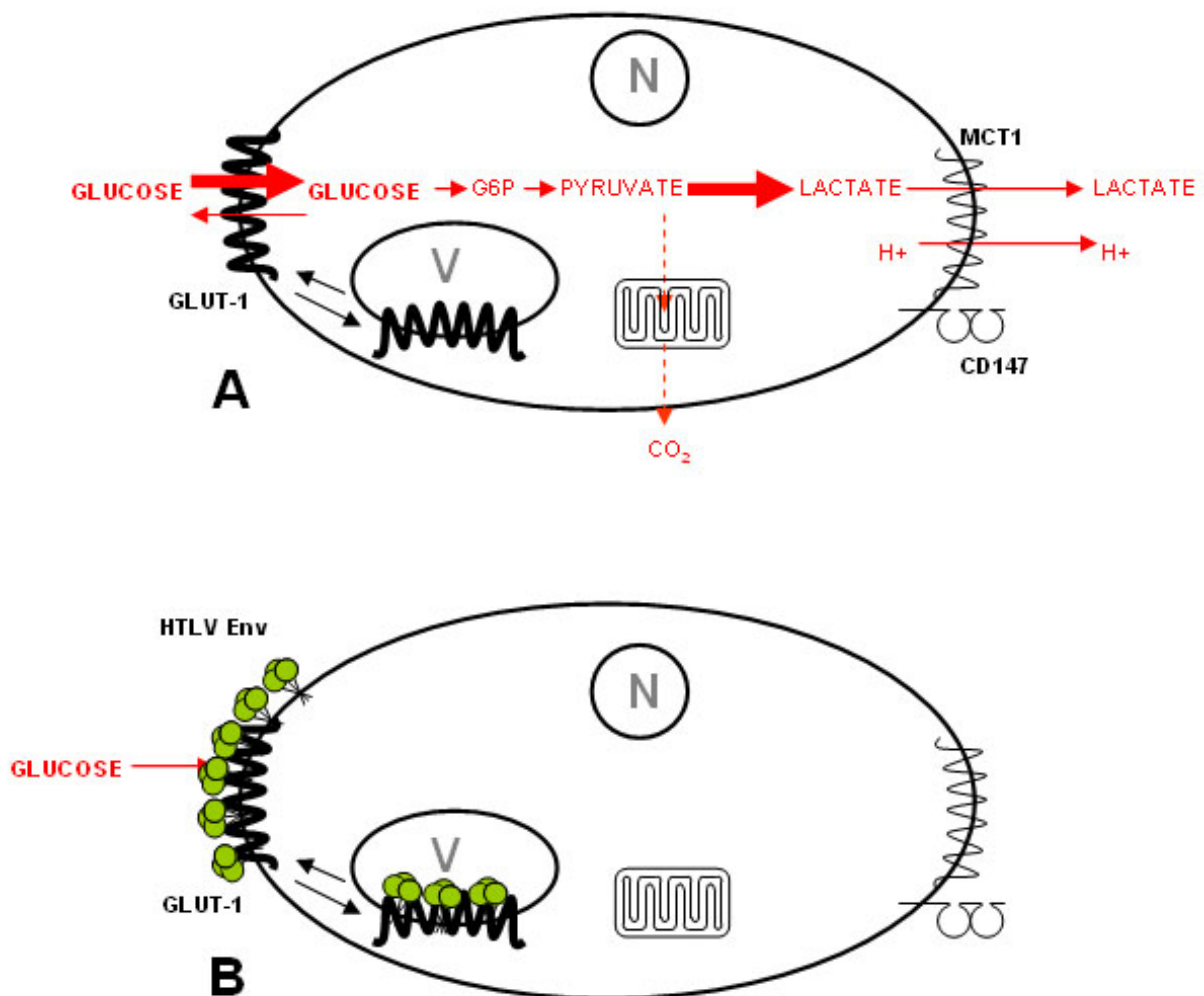
these studies, total cellular GLUT1 expression, and not surface GLUT1, was assessed because of a lack of reagents directed against the extracellular portion of GLUT1. While several commercially-available antibodies have been advertised as recognizing extracellular GLUT1, none of these antibodies were found to be reliable in our hands (unpublished data). Many of our studies, assessing the surface expression of GLUT1 on human T lymphocytes, were performed using tagged HRBD even before our identification of the HTLV receptor as GLUT1. Our data, demonstrating that expression of the HTLV receptor is associated with T cell survival and proliferation, is in complete agreement with that reported for GLUT1. The identification of GLUT1 as the HTLV receptor and the HRBD tools that we derived will help to further explore HTLV infection and the physiological consequences of GLUT1 expression in different human lymphocyte subsets.

## 7. IMPACT OF HTLV ENV-GLUT1 INTERACTIONS ON METABOLISM

Glucose provides a key supply of energy and carbon and glucose transport is a universally conserved property of living organisms. In vertebrates, GLUT1 appears to be the main glucose transporter and its presence at the cell surface as a functional glucose transporter is influenced at many levels. Each GLUT1 molecule harbors two glucose binding sites. The current model of GLUT1 topology (reviewed in (122)) proposes that the two binding sites face opposite sides of the cell membrane and that they are in reverse conformation with regards to glucose binding. In the "in" state of the GLUT1 molecule, it is the cytoplasmic site that binds glucose, whereas in the "out" state glucose binding occurs at the exofacial glucose binding site. A functional transporter is thought to be a GLUT1 dimer, with each monomer in opposite "in" and "out" states, and their opposite states maintained by simultaneous flipping. GLUT1 expression is induced by various treatments (reviewed in (123)), including glucose starvation (124, 125), hypoxia (126), inhibition of oxidative phosphorylation (127) and osmotic stress (128). Under stress, GLUT1 is induced at several levels: increased rate of transcription (129), stabilization of the mRNA (130), and unmasking of glucose binding sites on the GLUT1 molecule (131).

The first level of regulation of GLUT1 depends on its availability at the plasma membrane (132, 133). In *in vitro* models of insulin-induced increase in glucose uptake, GLUT4 massively translocates to the plasma membrane, and although to a lesser extent, translocation of GLUT1 from intracellular pools is also triggered (134, 135). GLUT1 translocation is also accompanied by increased transcription (136) and translation (137). GLUT1 induction by insulin varies with the cell line used and seems to be inversely proportional to the amount of GLUT1 already present at the membrane (138). Another level of modulation is exerted directly on cell surface-associated GLUT1 molecules. GLUT1 binds intracellular ATP (139) in the absence of hydrolysis, which results in inhibition of GLUT1-mediated glucose uptake (140), due to alterations in substrate binding affinity (141).

## Glucose transporter GLUT1 as the HTLV receptor



**Figure 6.** Impact of HTLV Env on glucose metabolism. In control cells grown in *in vitro* culture (A), glucose is taken up by cells and is then converted to pyruvate through glycolysis. Because cells are grown under limited O<sub>2</sub> availability, 95% of the pyruvate is degraded into lactate, which is then secreted together with protons via the monocarboxylate transporter (MCT) leading to acidification of the culture medium. CD147, tightly associated with the main transporter MCT1, is shown. In the presence of HTLV Env (B), glucose transport into cells is strongly decreased, and glycolysis is hampered as observed by the significantly decreased production of lactate and a lack of acidification of the culture medium. Note that although several HTLV Env are depicted as being bound to GLUT1 in this figure, the precise stoichiometry of HTLV Env binding per GLUT1 molecule is not known. N: Nucleus; V: Vesicle.

As indicated earlier, in classical mammalian cell culture systems, cells have adapted from *in vivo* respiration to *in vitro* fermentation, thus relying primarily on glycolysis to maintain their ATP pool (142). Although the selective pressure that drives this switch remains to be fully elucidated, the limited availability of O<sub>2</sub> in culture medium provides a reasonable explanation. This fermentative adaptation to reduced oxygenation has been proposed to be a key step in the *in vivo* development of tumors (143). To fuel fermentation, glucose is the preferential metabolite, and under these conditions, GLUT1 plays a key role as the major glucose transporter (116). Following HTLV Env expression, glucose uptake is reduced due to the binding of HTLV envelope to GLUT1. Reduction of intracellular glucose in cultured cells reduces the glycolysis and thus

production of its end-product, lactate. In culture, most of the lactate is released with protons via the MCT1/CD147 symporter system (102, 103) and figure 6). Therefore, a decrease in lactate production is accompanied by a drop in the acidification of the culture medium (figure 6). Protons co-secreted with lactate from fermentation are thought to account for 95% of the evolution of extracellular medium pH in *ex vivo* cultures (J. Pouyssegur, personal communication). As such, the inhibition of GLUT1 activity by HTLV envelope expression or by GLUT1 siRNA (our unpublished observations) prevents the color change of the pH indicator, phenol red, commonly added to the culture medium.

Interestingly, a switch to galactose as a source of

## Glucose transporter GLUT1 as the HTLV receptor

carbon tends to bias cell metabolism toward respiration (see for instance (144)). When substituting galactose for glucose, we observed that HTLV Env binding increases, thus mimicking glucose starvation (our unpublished observations). Under these culture conditions, the induction of GLUT1 at the surface of 293T or HeLa cells is maintained during several weeks of culture and multiple passages (our unpublished observations).

The exact mechanism by which HRBD inhibits GLUT1 is unclear. To date we have not been able to inhibit glucose transport by adding exogenous HRBD to the cell culture medium. This might be due to insufficient amounts of soluble HRBD molecules produced by HRBD-transfected 293T cells (our main source of soluble HRBD), or because inhibition occurs only upon intracellular production of HRBD. Therefore, it remains to be determined whether binding of HTLV Env and HRBD to GLUT1 inhibits its transport activity, directly by competitive binding with glucose or by inducing conformational changes in GLUT1. Alternatively, GLUT1 inhibition by intracellular HRBD may alter GLUT1 trafficking, resulting in decreased cell surface-associated GLUT1 molecules. The first model is currently supported by our observation that bound tagged HRBD protein can be readily detected at the cell surface of HRBD-producing cells (our unpublished observations) arguing in favor of the presence of significant amounts of HRBD-GLUT1 complex at the cell surface.

Since GLUT1 and glucose transport occupy a central place in cell metabolism, HRBD-GLUT1 interactions also influence the balance of other essential metabolites upstream of lactate production, at all levels of glycolysis. Interestingly, GLUT1 can transport ascorbic acid under certain conditions (see (145) for a review). Potential alteration of this transport by HRBD-harboring HTLV derivatives could exert an influence on *in vivo* cell survival and integrity of the host organism, due to the role of vitamin C (ascorbate) in counteracting oxidative damage of proteins. Also, type 2 adenosine receptor agonists were found to inhibit the cell binding of HTLV virions (146). Although the effect of these compounds on Env-receptor interactions remains to be assessed, the fact that GLUT1 is an adenine nucleotide binding protein under an ATP feedback control regulation (147) may open new alleys for exploring the role of these nucleoside receptors in the early steps of retroviral infection.

In the experimental systems we used, HTLV Env and HRBD proteins were produced by transient transfection. We were unable to obtain stable HTLV Env expression, even when using cell lines that were resistant to syncytia formation, such as the NIH3T3(TK<sup>-</sup>) described above. Attempts to derive such HTLV-expressing cells failed even when the HTLV SU-derived truncated HRBD was used in conjunction with other hexoses than glucose, confirming the essential function of GLUT1 in cell culture. Therefore, the question of the adaptive processes that allow HTLV-producing cell lines like MT-2, to maintain glucose uptake activity and GLUT1 expression remains unanswered. While it is clear that the HTLV envelope is

expressed at the surface of such cell lines, as demonstrated by their ability to form syncytia with other cells, they might be expressed at a sufficiently low level as not to block GLUT1 activity. Alternatively, HTLV-producing cell lines might rely on another glucose transporter.

*In vivo*, only a few cells, among which are muscle cells, rely on anaerobic glycolysis for energy production. Interestingly, when thymocytes undergo proliferation, a transition from aerobic to anaerobic metabolism occurs, despite the presence of O<sub>2</sub>. This phenomenon has been referred to as the *Crabtree effect* (148). At this stage of lymphocyte differentiation, glucose utilization increases 18-fold (148). Furthermore, proliferating thymocytes are strictly dependent on glucose, in contrast to hepatoma cells or human primary fibroblasts, for which uridine can be substituted (149). It is thus tempting to propose that in the course of an HTLV infection, an HTLV Env block of GLUT1 activity in infected thymocytes would provide a strict selective pressure, and potentially promote the emergence of tumorigenic T lymphocyte clones.

## 8. CONCLUSIONS AND PERSPECTIVES

### 8.1. Determinants of HTLV Env-GLUT1 interactions

The identity of the HTLV Env receptor remained elusive for approximately two decades and the search was hampered by the fact that HTLV entry can take place in all established vertebrate cell lines. This long search has been the source of numerous speculations as to the nature of the receptor, including the possibility that a dedicated cellular receptor may not be required for HTLV infection or that many different receptors can be used by HTLV. Based on the elucidation of the modular organization of the receptor binding domain (HRBD) and the generation of new tools which comprised only this region of the HTLV Env (44, 78), we obtained new clues that were key to our finding that GLUT1 is a receptor for HTLV Env. The first clue was based on the MLV-related organization of the HTLV SU (44, 78, 90) and figures 1 and 3). Since all identified Env receptors of MLV and related mammalian retroviruses are transporter-like multimembrane spanning molecules, or their chaperone molecules, we hypothesized that the HTLV receptor would belong to this class of cell surface molecules (table 1). The second clue was that expression of the HTLV Env receptor corresponded to an early marker of T cell activation (99). The third major clue was that transfection of HRBD blocks extracellular acidification and lactate accumulation which, *in vitro*, are both closely dependent on the integrity of the glycolytic pathway. Although GLUT1 appears to a primary HTLV receptor, it is not excluded that other cell surface molecules can be used by HTLV for infection, or alternatively, can potentiate infection.

Experiments of Env cross-interference to superinfection showed that HTLV-1 and -2 and STLV-1 use a common receptor (66, 150). We also observed this phenomenon when using HTLV-1 and -2 HRBD in cross-interference assays based on their cell surface binding (45, 78). Accordingly, the Tyr residue at position 114 of the



## Glucose transporter GLUT1 as the HTLV receptor

HTLV-1 SU, that we identified as a major determinant for receptor-binding (45, 78), is also conserved among all known strains of PTLV-1 and 2. To our knowledge, no Env interference assays have been performed with STLV-2 and STLV-3 strains. However, the high level of conservation of this residue and its surrounding residues in all reported PTLV strains (unpublished observations) augurs for the same receptor usage for all PTLV.

In addition to PTLV, the Deltaretrovirus genus comprises BLV for which the nature of the Env receptor remains uncertain. Given the close relationship between these two viral species (151, 152), the search for a BLV Env receptor that belongs to the multimembrane spanning molecules seems reasonable. It is interesting to note that despite a strikingly different organization of the HIV SU, whose RBD is constituted of a complex array of non contiguous determinants (153), multimembrane spanning molecules (chemokine receptors) serve as HIV Env coreceptors (154). Furthermore, cellular partners of GLUT1 appear to be recruited during HTLV entry as coreceptors (D. Ghez, Y. Lepelletier, C. Pique and O. Hermine, personal communication). It is tempting to speculate that many retroviruses have coreceptors but under conditions where the coreceptor is ubiquitously expressed, they have not been recognized to play this role. In the case of HTLV, further documentation of the interactions between Env, GLUT1 and additional partners will likely benefit from finer mapping of the respective interacting determinants. To this aim, we are currently testing HTLV Env-derived peptides as well as GLUT chimeras, constructed between GLUT1 and other GLUT isoforms, for their abilities to physically interact and modulate infection.

### 8.2. HTLV Env infection and GLUT1 expression: How to conciliate ubiquity and restricted distribution?

One of the recurrent paradoxes in the HTLV field is the restricted tissue distribution of the virus as opposed to the wide range of Env-mediated tropism in cell culture. Indeed, the HTLV Env-mediated tropism *in vitro* is one of the widest of all known retroviruses. Accordingly, GLUT1 is a molecule that is expressed on almost all dividing or metabolically active cells, hence its general expression on established cell lines. Although no definitive explanation can ultimately conciliate this apparent discrepancy, a whole array of observations hint to adequate explanations.

Retroviral tropism depends on viral entry, and for that purpose on Env-receptor interactions. However, a whole sequence of post-entry events leading to particle assembly and release are modulated by the cellular context and can thereby restrict *in vivo* tropism. Whether such restriction(s) play a role in the constitution of the *in vivo* reservoir of HTLV remains to be assessed and may benefit from the use of animal models (35). Another level of explanation comes from a more relative perception of the *in vivo* GLUT1 "ubiquity". Indeed, there are considerable differences in the levels of cell surface GLUT1 expression in different tissues. These differences constitute the basis for the *in vivo* detection of highly metabolically active and glycolysis-dependent cancer cells, by tracking GLUT1 activity with positron emission tomography after injection

of 2-(18F)fluoro-2-deoxy-D-glucose, a non-metabolized glucose analogue (for a review see (155, 156). Precise analyses concerning the number of cell-surface GLUT1 molecules and viral particles that are required to initiate a successful viral entry, as well as the availability of potential coreceptor molecules in different cellular contexts, is likely to provide further information on the *in vivo* distribution of HTLV. It will be of interest to monitor, in experiments such as those described by Igakura *et al.* (46), the respective localizations of cell surface-expressed GLUT1 and Env molecules, as compared to other viral components, during cell-to-cell contacts between different cell types of infected and non-infected cells. Additional explanations for the *in vivo* tropism include the potential elimination of productively infected cells by the immune system (157, 158) and the fact that, like most retroviruses, HTLV infection of T cells requires cell division for integration, replication and further spreading. The ensemble of these restrictions will most likely efficiently limit the *in vivo* expansion of an HTLV infection.

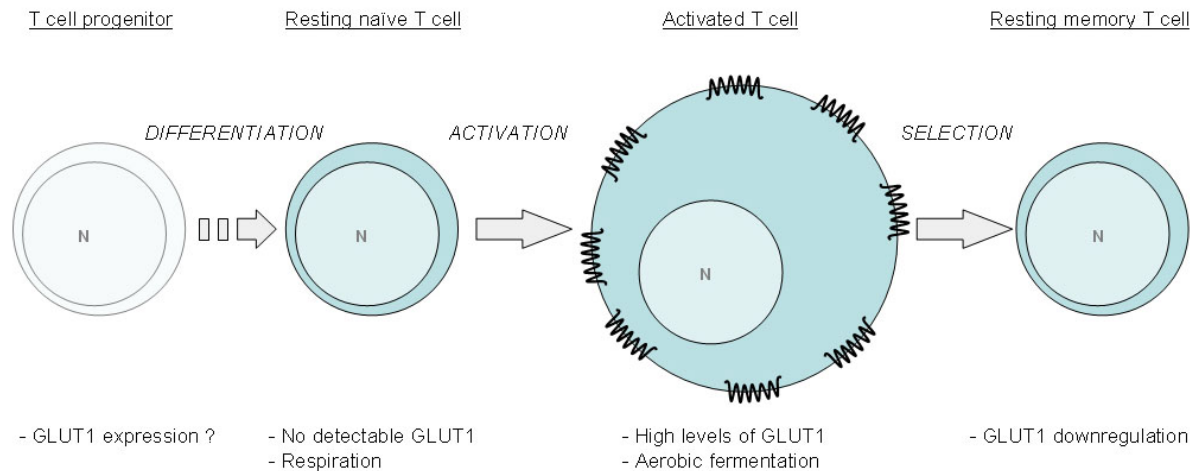
We believe that the interaction of HTLV Env with GLUT1 is likely to be detrimental in certain cells with respect to metabolic function and membrane structures (see for instance (29, 45). This hypothesis is supported by the observations that virion components are extremely difficult to detect in the blood of infected patients and that virion production by T cells from asymptomatic as well as ATL patients is not readily detectable (157, 158) and O. Hermine, personal communication). Thus, productively infected cells may be rapidly eliminated *in vivo*. This would result in an *in vivo* distribution that would be confined to those cells which have by-passed these expected restrictions. We may therefore ask what makes lymphocytes able to sustain HTLV infection for long periods rather than question why non-lymphoid cells are not able to be stably infected with the virus.

### 8.3. New insights into HTLV-mediated pathogenesis

The relatively low incidence of ATL among HTLV-infected patients (<5%) and the long latency observed before disease onset (20-40 years) mirror the multivariable nature of the leukemogenic process (159). As mentioned above, selection pressures are likely to include Env-GLUT1 interactions. In this context, it is conceivable that lymphoid cells are more likely to survive such pressures because of their ability to switch from a GLUT1-dependent status, when activated, to a GLUT1-independent survival state under conditions of quiescence (108) and figure 7). The ultimate emergence of leukemogenic clones may then result from additional effects such as those produced by Tax and other HTLV components (50, 160), and from potential mutations compensating for GLUT1 impairments brought on by recurrent HTLV Env expression. Closer assessment of GLUT1-associated parameters in the lymphocytes of asymptomatic patients and patients at different stages of ATL will allow us and others to determine the validity of this hypothesis.

The identification of GLUT1 as a receptor for HTLV Env and the ability of HTLV Env to block glucose transport may have considerable consequences on neurological

## Glucose transporter GLUT1 as the HTLV receptor



**Figure 7.** Model of GLUT1 expression during *in vivo* T cell activation. The vast majority of peripheral T-cells (>95%) are in the G0 phase of the cell cycle. Resting naïve T cells do not express GLUT1 at their plasma membrane and transport glucose at low basal levels. Upon activation through the T cell receptor (TCR), GLUT1 expression is strongly increased, concomitant with a significantly augmented glucose transport. Activated T cells acquire the phenotype of memory T cells and a small percentage survives and returns to a resting state. These quiescent memory T cells, like their naïve counterparts, do not express significant levels of GLUT1. GLUT1 expression during early stages of T lymphocyte differentiation remains to be determined.

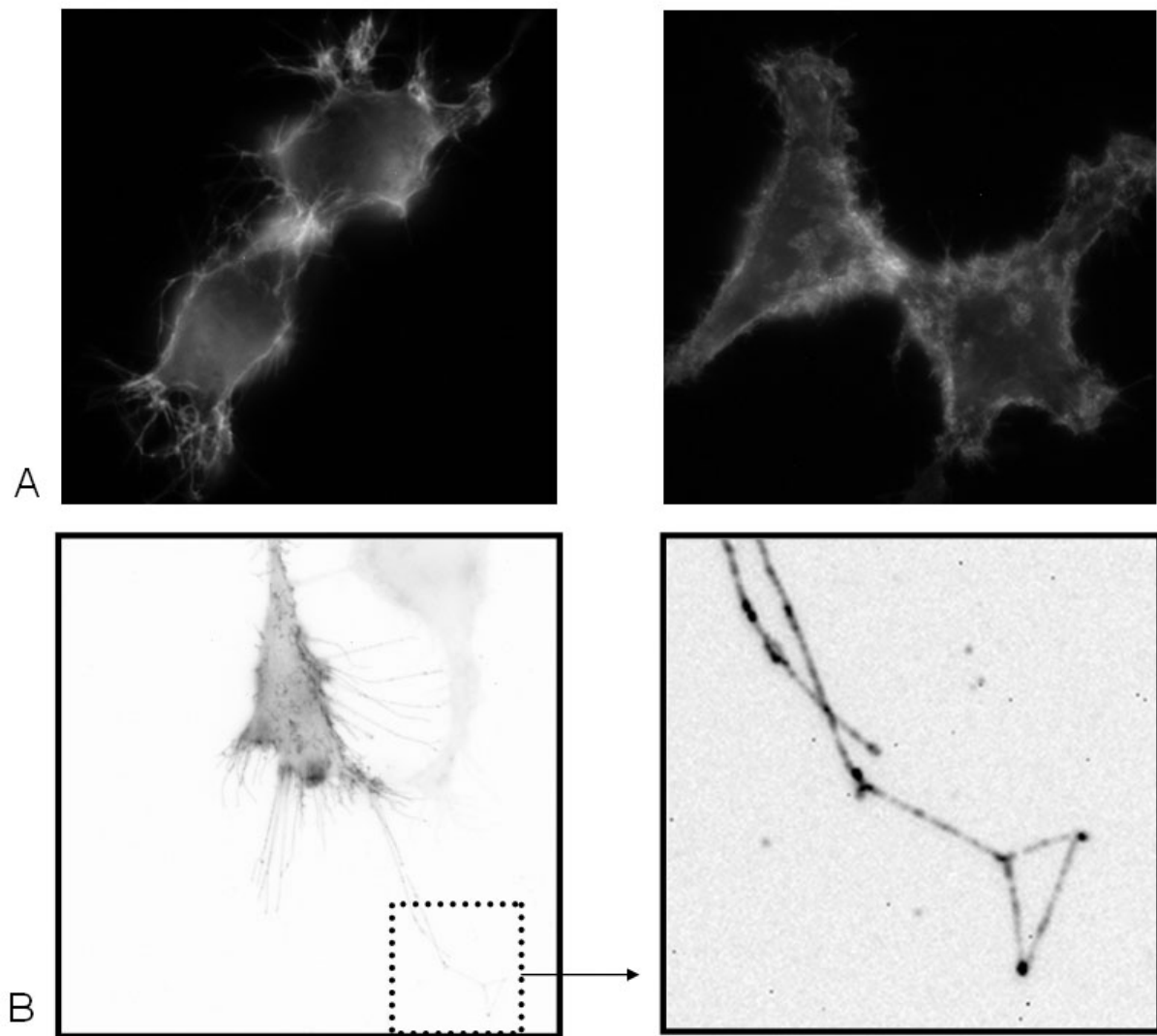
function as exemplified by the neurodegenerescence associated with HTLV infection (157, 161). In this context, it is particularly relevant that the brain is strictly dependent on glucose consumption and moreover, that its usage relies on glycolysis. This process hinges on GLUT1 as shown by the fact that mutations in the GLUT1 gene lead to an impairment of glucose transport that is the cause of a severe cerebral deficiency (162). Moreover, the major clinical symptoms in patients with heterozygous GLUT1 mutations are neurological, with infantile seizures, developmental delay, microcephaly and ataxia (163). Astrocytes are the natural intermediate between brain vessels and neurons (see (164) and a generally accepted scenario of astrocyte-neuron carbohydrate exchanges is that most of the circulating glucose in the brain blood is consumed by glial cells via GLUT1. The glucose is converted into lactate by fermentation, in an aerobic environment, and the secreted lactate constitutes the major energy source for neurons (165-167). Interestingly, HTLV-1 infected lymphocytes have been shown to alter astrocytic functions (for instance see (168). HTLV Env-mediated impairment of glucose transport may therefore impact on neurons through a dysfunction of glucose metabolism in glial cells, such as astrocytes and oligodendrocytes. Finally, since ascorbic acid transport appears to prevent oxidative damage leading to cerebral degenerative processes and GLUT1 relays this transport under certain conditions (169), the presence of HTLV Env in the brain capillary and endothelial cells may favor the triggering of neurodegenerative processes such as those associated with HTLV infection (157). Evaluation of the impact of HTLV Env on ascorbic acid transport in these different cell types should bring additional clues.

Since contact between neuronal cellular components and HTLV-infected cells is likely to occur through the blood flow, the observation that GLUT1 is particularly abundantly expressed on human erythrocytes

may bear some significance to HTLV-mediated neurodegenerescence. Thus, approximately 15,000 GLUT1 molecules can be detected at the surface of a single mature human erythrocyte, corresponding to 5% of the entire cell membrane (123), while only 700 molecules are present on a rat erythrocyte (138). Whether HTLV particles can be titrated out from the circulation by red blood cells opens to new horizons. For instance, it becomes important to determine whether there is a potential link between the relatively rapid development of TSP/HAM after blood contamination (170) and the ability of human erythrocytes to bind HTLV virions. This could potentially occur via viral transfer from lymphoid and red blood cells to endothelial cells of blood vessels. This infection would in turn decrease local glucose uptake by endothelial cells and glial cells, leading to a disturbance of metabolic exchanges such as those described in the "astrocyte-neuron lactate shuttle" (165). In this perspective, to determine whether bound particles enter red cells or remain at their surface and can be passed on to new cells may become of a major significance. To this regard it is important to note that erythrocyte-associated GLUT1 has a different electrophoretic migration pattern than GLUT1 from other cells, due to different glycosylation, as well as different kinetic properties (122, 171). Whether such differences prevent viral entry in erythrocytes and allow red cells to circulate virions to other organs could be addressed *ex vivo*. Consequently, it will be important to assess whether GLUT1 glycosylation modulates HTLV entry.

The abundance of GLUT1 on erythrocytes is a property that humans share with bats (172) and sea mammals (173). This property correlates with, respectively, high glucose metabolism of human in the central nervous system, the ability of bats to undertake sustained flight, and maximization of glucose delivery under hypoxic stress, respectively. In contrast, GLUT1 appears to be expressed at

## Glucose transporter GLUT1 as the HTLV receptor



**Figure 8.** Distribution of GLUT1 on human HeLa cells. The receptor binding domain of HTLV Env was fused to the green fluorescent protein (EGFP) in order to track the distribution of GLUT1. HeLa cells were transfected with this fusion protein. The pattern of fluorescence indicates that GLUT1 is enriched at cell contacts (A). In isolated cells, GLUT1 is enriched at the extremity of membrane extensions (B).

significantly lower levels on non-human primate erythrocytes, as shown by immunoelectronmicroscopy of brain erythrocytes (174). As such, while STLV-1 most likely also recognizes GLUT1 as a receptor (150) and our unpublished observations), viral dynamics and viral dissemination in relation with erythrocyte binding are likely to be fundamentally different between human and monkeys.

### 8.4. HRBD as a marker for GLUT1 surface expression

EGFP-tagged HRBD binds diffusely to fibroblasts with a preferential accumulation at cell-cell contact areas (figure 8A), and filopodia-like structures (figure 8B). Labeling of cells with transfected or externally added tagged-HRBD-containing conditioned media allowed the detailed visualization of filopodia extending tens of microns from the main cell plasma membrane

(figure 8B). These observations illustrate the close association of a major metabolic transporter, such as GLUT1, in the construction of membrane structures involved in cell-cell contact and "explorative" extensions. This likely reflects the adaptive plasticity conferred to the cell by these two synergic architectural and metabolic functions under local changes of resources. The exploration of the association between resource availability and the construction of different types of membrane extensions may provide a new angle at studying cell adaptation and differentiation at a metabolic and an architectural level. The tools derived from HRBD will undoubtedly be useful for these investigations.

### 8.5. HRBD-GLUT1 interactions as diagnostic and therapeutic tools

As mentioned above, glucose uptake via GLUT1

## Glucose transporter GLUT1 as the HTLV receptor

activity is the basis for the *in vivo* detection of tissues with high metabolic activities and tumors (155, 156). Specific detection of cell surface-expressed GLUT1 molecules has been hampered by its high level of conservation, and the lack of reliable reagents recognizing exofacial GLUT1 determinants (M. Mueckler, personal communication). Therefore, conditioned medium containing HRBD-derived molecules constitutes such a convenient source of non-toxic reagents that can be used to easily detect cell surface GLUT1. Thus, these tools can be used to monitor GLUT1 expression in relation to various physiopathological conditions. Finally, as endogenous expression of HRBD will generally exert a toxic effect due to the inhibition of GLUT1, its ectopic expression may help eliminating GLUT1-overexpressing tumor cells (see for instance (155, 156)).

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**Key Words:** HTLV, Envelope, Receptor; GLUT1, T lymphocyte, Glucose, Metabolism, TSP/HAM, ATL, Neuropathogenesis, Leukemia, Review

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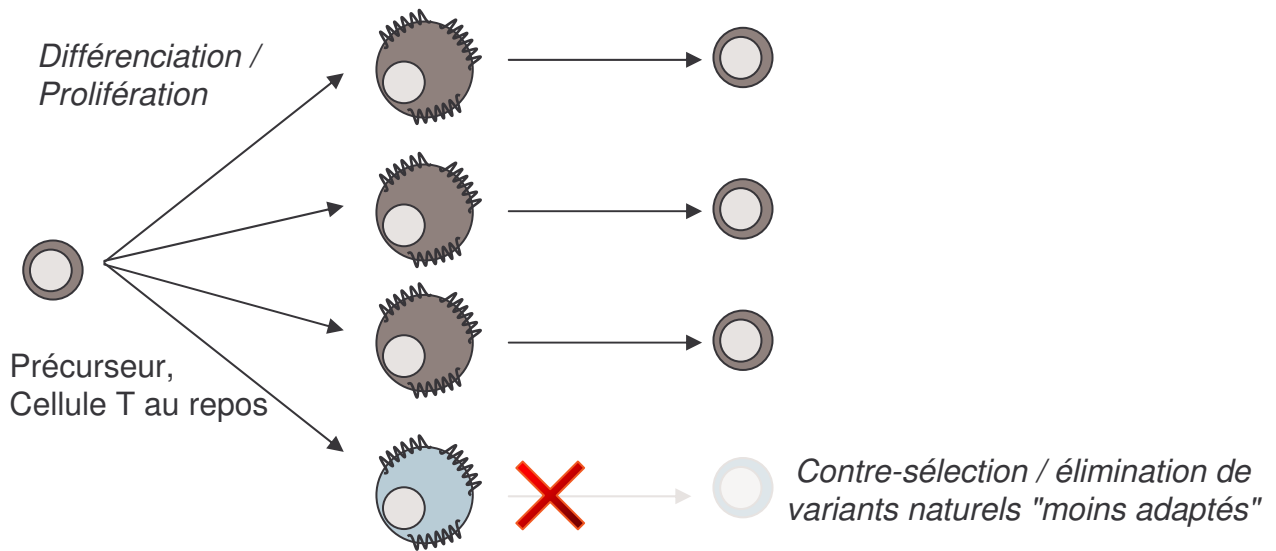


### **C.3. Discussion article 4**

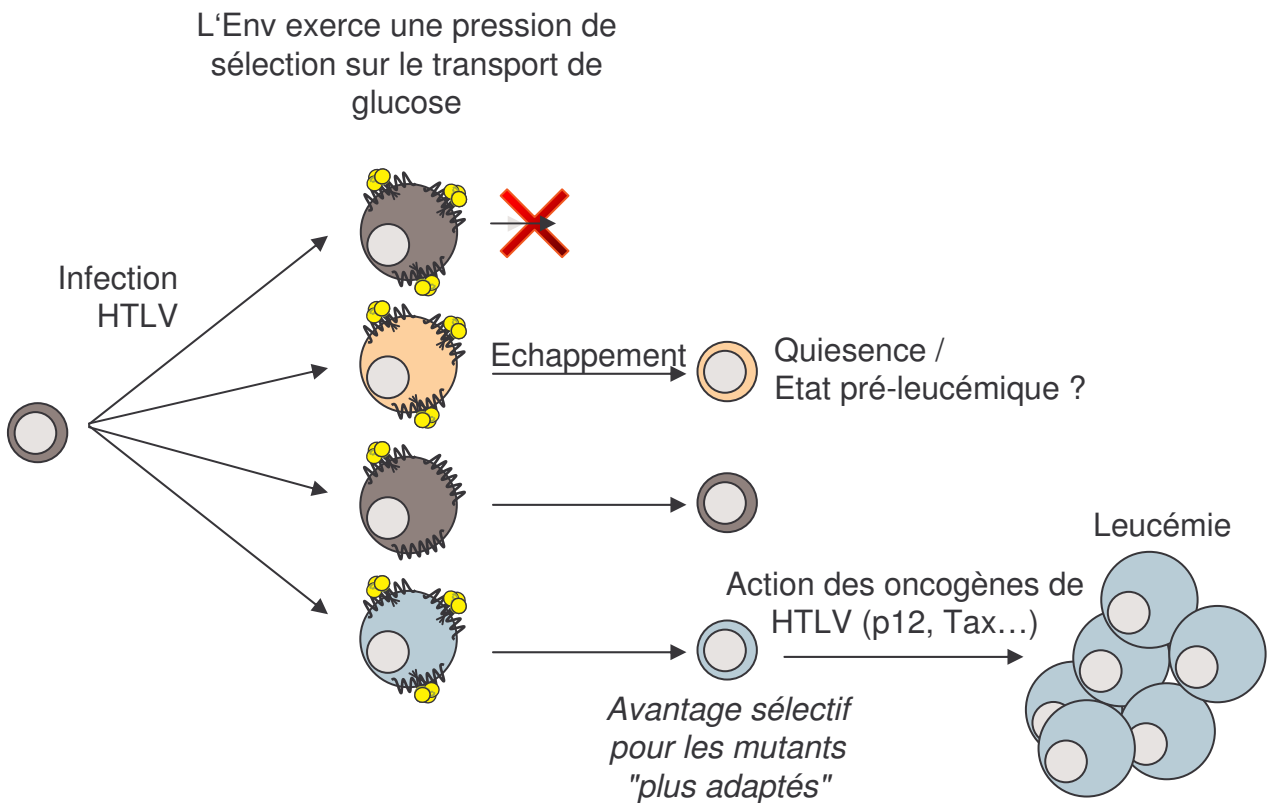
#### C.1.a. La leucémie T de l'adulte

Les modèles actuels de développement de la leucémie T de l'adulte font intervenir principalement l'action de Tax et de p12 (Matsuoka 2003). Tax semble à la fois inhiber et activer l'expression de divers gènes cellulaires via les voies NF-kB (Ballard, Bohnlein et al. 1988; Leung and Nabel 1988; Ruben, Poteat et al. 1988) et CBP/p300 (Suzuki, Uchida-Toita et al. 1999; Mori, Morishita et al. 2001). De plus Tax est capable d'inhiber directement l'activité de certaines protéines cellulaires comme p16Ink4a (Suzuki, Kitao et al. 1996). p12 interfère avec la voie STAT5 et pourrait donc moduler l'expression des cibles de ce facteur de transcription (Nicot, Mulloy et al. 2001). Cependant, la longue période de latence suggère que la leucémogénèse est multi-étapes. Dans ce contexte, l'expression de l'Env HTLV dans les cellules infectées inhiberait initialement l'activité de GLUT1. Ceci pourrait induire une forte pression de sélection, lors de laquelle une substantielle proportion des cellules infectées serait éliminée par carence métabolique. En accord avec une telle sélection, il existe une plus forte diversité des cellules infectées après séro-conversion en comparaison aux porteurs sains de longue durée (Tanaka, Okayama et al. 2005). Suite à des variations d'expression au niveau épigénétique ou de mutations compensatoires, des cellules infectées qui exprimeraient une autre isoforme de GLUT ou qui verraient leur niveau d'expression de GLUT1 altéré seraient alors avantagées (Figure 13B), alors que dans un contexte non infecté, de telles variations seraient contre-sélectionnées (Figure 13A). Un tel scénario de sélection, dépendant de phénomène compensatoire aléatoire, est compatible avec la latence observée. L'action directe de Tax ou p12 pourrait intervenir par la suite dans les étapes ultérieures de leucémogénèse (Figure 13B).

## A - Condition normale



## B- Condition pathologique



**Figure 13.** Modèle du développement de la leucémie T de l'adulte mettant en jeu l'inhibition de l'activité de GLUT1 induite par l'interaction avec l'Env HTLV.

### C.1.d. Considérations métaboliques

Dès 1956, Otto Warburg a démontré qu'une des propriétés fondamentales des cellules cancéreuses est une perte de la capacité des cellules à respirer au profit de la fermentation lactique (Figure 7). Notamment, il explique que :

*"Cancer cells originate from normal body cells in two phases. The first phase is the irreversible injuring of respiration. [It] is followed, as the second phase of cancer formation, by a long struggle for existence by the injured cells to maintain their structure, in which a part of the cells perish from lack of energy, while another part succeed in replacing the irretrievably lost respiration energy by fermentation energy."*

Transposé dans la problématique de l'inhibition de l'activité de GLUT1 par l'Env HTLV, on comprend qu'une pression de sélection sur le transport du glucose ait pour conséquence directe d'inhiber la respiration cellulaire, et correspondrait à la première phase décrite par Otto Warburg. L'utilisation du glucose par fermentation correspondrait donc à un métabolisme de survie en conditions de carence. La seconde phase décrite par Otto Warburg engloberait à la fois les modifications compensatoires de l'inhibition de GLUT1 comme l'action des oncogènes de HTLV.

Il est particulièrement intéressant de noter que les lymphocytes activés ont un métabolisme basé sur la fermentation lactique, alors que ces cellules respirent au repos (Figure 7) (Greiner, Guppy et al. 1994). Or, les lymphocytes activés ne semblent pas se trouver dans des conditions d'hypoxie particulière, la fermentation lactique des lymphocytes a donc lieu en présence d'O<sub>2</sub>, on parle alors d'effet Crabtree (Greiner, Guppy et al. 1994), observé initialement par Otto Warburg (Warburg 1924) et confirmé par Crabtree (Crabtree 1929). Il est possible que cette propriété contribue à la transformation lymphocytaire induite par HTLV en conférant aux cellules infectées un métabolisme "pré-adapté" à la transformation.

D'autres tissus dépendent également de la fermentation lactique en présence d'O<sub>2</sub> : le muscle à l'exercice, les érythrocytes (qui ne possèdent pas de mitochondries), les cellules gliales. Toutefois, le rapport entre fermentation lactique et respiration *in situ* reste étonnamment mal décrit pour la majorité des tissus humains.

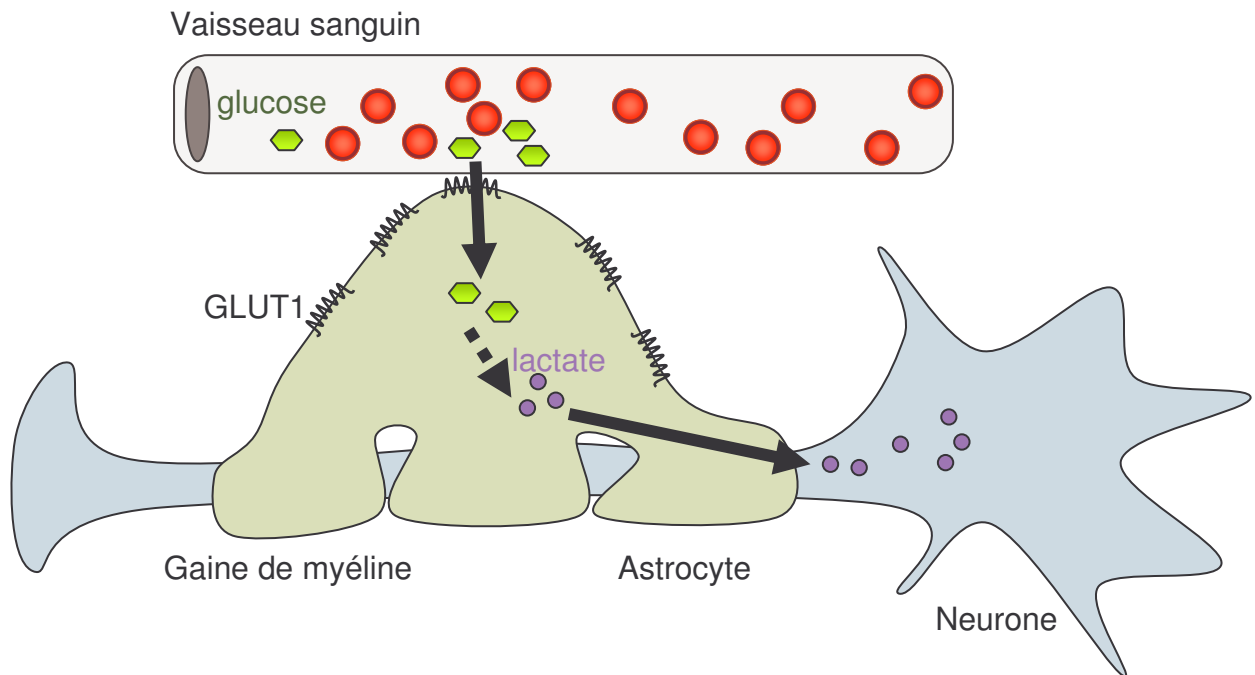
### C.1.c. La paraparésie spastique tropicale

La paraparésie spastique tropicale est une paralysie rapide, létale, caractérisée par une démyélinisation des neurones de la moelle épinière et une inflammation. Les modèles de pathogenèse actuels privilégient un dérèglement de la réponse immunitaire afin d'expliquer l'inflammation locale, qui aurait pour conséquence la démyélinisation (Bangham 2000; Grant, Barmak et al. 2002). Cependant, ces modèles n'apportent pas d'explication quant aux causes initiales de ces effets.

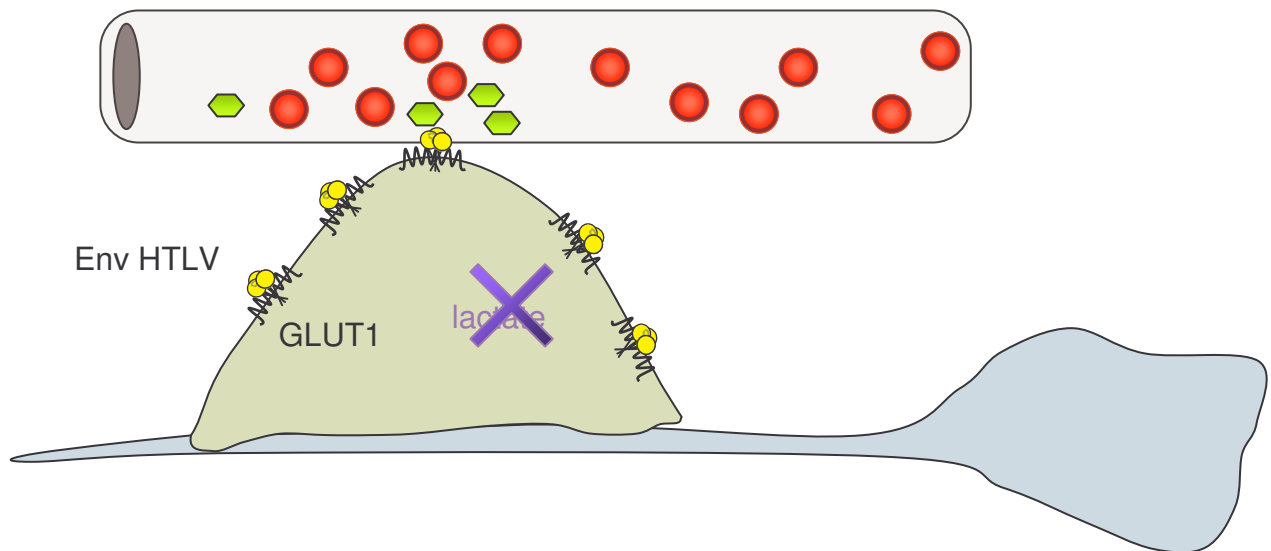
Les neurones présentent la particularité d'utiliser le lactate comme substrat énergétique (Figure 14A). Ce lactate est sécrété par les cellules de la glie (oligodendrocytes dans le système nerveux central ou astrocytes dans le système nerveux périphérique) comme produit de dégradation du glucose (Magistretti, Pellerin et al. 1999). Le métabolisme des cellules gliales est donc de type glycolytique, analogue au métabolisme des cellules cancéreuses et des lymphocytes activés. Ces observations nous conduisent à suggérer que le milieu neuronal est un milieu carencé où la pression de sélection induite par la disponibilité du substrat est particulièrement intense. En accord avec ces observations, GLUT1 est exprimé à la surface des astrocytes et des oligodendrocytes, mais pas à la surface des neurones. Par contre, les neurones expriment GLUT3 : ce transport de glucose neuronal semble pourrait être lié au métabolisme de glycosylation plutôt qu'à la production d'énergie.

Dans ce contexte, l'Env HTLV pourrait inhiber l'activité de GLUT1 à la surface des cellules de la glie. Les conséquences de cette interaction se situeraient à deux niveaux. Tout d'abord, le blocage de la glycolyse dans les astrocytes induirait une diminution de l'énergie disponible, et par conséquent un blocage des processus de myélinisation (Figure 14B). Ensuite, l'absence de production de lactate bloquerait également la production d'énergie dans les neurones, conduisant à leur dégénérescence (Figure 14B). La réaction inflammatoire

## A - Condition normale



## B- Condition pathologique



**Figure 14.** Modèle du développement de la paraparésie spastique tropicale mettant en jeu l'inhibition de l'activité de GLUT1 induite par l'interaction avec l'Env HTLV.

observée pourrait être une conséquence de ces dégénérescences cellulaires. L'Env HTLV pourrait être exprimée directement depuis les astrocytes (Lehky, Fox et al. 1995). Cependant, il semble que le provirus HTLV ne soit pas systématiquement présent dans les astrocytes (Hara, Morita et al. 1994) ce qui exclurait dans ces cellules une expression de l'Env HTLV en *cis*. L'Env HTLV pourrait alors inhiber l'activité de GLUT1 en *trans* à partir des lymphocytes circulants, qui eux portent le provirus et expriment donc l'Env HTLV. Il a notamment été observé que dans un modèle d'interaction glie-lymphocyte T, la présence de lymphocytes T infectés par HTLV induit des changements dans le métabolisme du glutamate, glucose et lactate des astrocytes (Akaoka, Szymocha et al. 2001).

#### C.1.d. Effets directs et indirects de l'Env HTLV sur le transport du glucose

Ces deux modèles reposent sur la capacité de l'Env HTLV à inhiber suffisamment et durablement l'activité de transport de glucose de GLUT1 conduisant à une baisse de la glycolyse et en conséquence une pression de sélection due à une carence de glucose. Il est possible qu'*in vivo* le niveau d'expression de l'Env HTLV à partir de cellules infectées ne soit pas suffisant pour aboutir au même niveau d'inhibition qu'observé *in vitro*.

Chez les eucaryotes, plusieurs molécules de surface sont impliquées dans la détection du glucose extracellulaire, sans activité de transport, notamment Snf3 Rgt2, git3 chez la levure et SLGT3 chez la souris. D'autres senseurs possèdent une activité de transport de nutriments (*e.g.* l'ammonium perméase Mep2, l'acide aminé perméase Gap1, plusieurs phosphates perméases chez la levure). De telles propriétés de senseurs ont également été décrites pour GLUT1. Ainsi la concentration de glucose extracellulaire module l'activité de ERK1/2 via GLUT1 (Bandyopadhyay, Sajan et al. 2000). Par ailleurs il a été montré que l'expression d'Akt, une sérine/thréonine kinase impliquée dans la transformation cellulaire, stimule la glycolyse aérobie des cellules cancéreuses. En présence d'Akt, les cellules deviennent strictement dépendante du glucose comme source d'énergie, et meurent

rapidement en carence de glucose, alors que des cellules témoins sont capables d'exploiter d'autres substrats énergétiques (Elstrom, Bauer et al. 2004). Une expression modeste de l'Env HTLV, insuffisante pour bloquer directement la glycolyse, pourrait par contre altérer l'activité des kinases ERK et Akt à un niveau suffisant pour déréguler le métabolisme cellulaire du glucose. Ceci constituerait une pression de sélection propice à l'émergence de pathologies dans des conditions d'expression de l'Env HTLV limitées.

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# ANNEXES

## **Annexe 1 – Autres articles**

### **Article original 5**

Kim FJ, **Manel N**, Boublik Y, Battini JL, Sitbon M. Human T-cell leukemia virus type 1 envelope-mediated syncytium formation can be activated in resistant Mammalian cell lines by a carboxy-terminal truncation of the envelope cytoplasmic domain. *Journal of Virology*. 2003 77:963.

### **Article original 6**

Kim FJ, **Manel N**, Garrido EN, Valle C, Sitbon M, Battini JL. HTLV-1 and -2 envelope SU subdomains and critical determinants in receptor binding. *Retrovirology*. 2004 1:41.

### **Article de revue 7**

Kim FJ, Battini JL, **Manel N**, Sitbon M. Emergence of vertebrate retroviruses and envelope capture. *Virology*. 2004 318:183.

### **Article de revue 8**

Kim FJ, **Manel N**, Battini JL, Sitbon M. La capture d'enveloppe par les rétrovirus. *Médecine Sciences*. 2004 20:876.

### **Article de revue 9**

**Manel N**, Kinet S, Kim FJ, Taylor N, Sitbon M, Battini JL. GLUT-1 est le récepteur des retrovirus humains HTLV. *Médecine Sciences*. 2004 20:277.

## **Article de revue 10**

**Manel N**, Battini JL, Sitbon M. HTLV-1 tropism and envelope receptor. *Oncogene*. 2005, sous presse.

# Human T-Cell Leukemia Virus Type 1 Envelope-Mediated Syncytium Formation Can Be Activated in Resistant Mammalian Cell Lines by a Carboxy-Terminal Truncation of the Envelope Cytoplasmic Domain

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**Human T-cell leukemia virus (HTLV) envelope (Env) glycoproteins induce fusion, leading to rampant syncytium formation in a broad range of cell lines. Here, we identified murine, hamster, canine, and porcine cell lines that are resistant to HTLV-1 Env-induced syncytium formation. This resistance was not due to the absence of functional receptors for HTLV Env, as these cells were susceptible to infection with HTLV Env-pseudotyped virions. As murine leukemia virus (MLV) Env and HTLV Env present close structural homologies (F. J. Kim, I. Seiliez, C. Denesvre, D. Lavillette, F. L. Cosset, and M. Sitbon, *J. Biol. Chem.* 275:23417–23420, 2000), and because activation of syncytium formation by MLV Env generally requires cleavage of the R peptide in the cytoplasmic domain of the Env transmembrane (TM) component, we assessed whether truncation of the cytoplasmic domain of HTLV Env would alleviate this resistance. Indeed, in all resistant cell lines, truncation of the last 8 amino acids of the HTLV Env cytoplasmic domain (HdC8) was sufficient to overcome resistance to HTLV Env-induced syncytium formation. Furthermore, HdC8-mediated cell-to-cell infection titers varied according to the target cell lines and could be significantly higher than that observed with HTLV Env on HeLa cells. These data indicate that a determinant located within the 8 carboxy-terminal cytoplasmic amino acids of TM plays a distinct role in HTLV Env-mediated cell-to-cell infection and syncytium formation.**

Current understanding of fusion mediated by retroviral envelope (Env) glycoproteins suggests that interaction of the Env surface (SU) component with a receptor(s) leads to conformational changes that unmask fusion determinants of the transmembrane (TM) subunit (29). The TM ectodomain is highly conserved among retroviral Envs, whereas the carboxy-terminal cytoplasmic domain is significantly more variable and has been shown to modulate Env-mediated cell fusion and infection in various retroviruses (2, 10, 11, 13, 20–22, 25, 30), including human T-cell leukemia virus (HTLV) (19).

HTLV infects and induces fusion with rampant syncytium formation in a broad range of cell lines *in vitro*. However, cell-free virus titers are generally poor, and infection by HTLV occurs mostly by transmission through cell-to-cell contact (7, 18, 26). The factors and mechanisms underlying the apparently paradoxical characteristics of highly fusogenic Env and poorly infectious virions remain unclear. We describe several mammalian cell lines that are resistant to HTLV Env-mediated syncytium formation, including the murine NIH 3T3 cell line lacking thymidine kinase (TK<sup>-</sup>) originally derived from NIH 3T3 cells (28). Despite the lack of syncytium formation, these syncytium formation-resistant cells were susceptible to HTLV Env-mediated infection. Also, partial truncations in the HTLV Env TM cytoplasmic domain overcame resistance to HTLV

Env-mediated syncytium formation in all of the resistant cell lines tested.

## MATERIALS AND METHODS

**Construction of HTLV Env cytoplasmic tail truncation mutants.** The parental HTLV-1 and Friend murine leukemia virus (MLV) Env expression vectors pCEL/H and pCEL/F were based on the MT2 strain of HTLV-1 (GenBank M37747 and AAA46185) and the 57 strain of Friend MLV (GenBank X02794), respectively. Three allelic restriction sites have been introduced in the two Envs, at the SU/TM cleavage site, the amino terminus of the membrane-spanning domain of the TM, and immediately after the Env stop codon, to facilitate domain swapping (8, 9, 14). HTLV Env lacking the entire cytoplasmic domain, HdC, and the Friend MLV R peptide truncation mutant pCEL/FΔR have been described previously (9, 14).

HTLV-1 TM-truncated mutants were obtained in pCEL/H after insertion of a TAATATT sequence that combined an in-frame TAA stop codon with an overlapping AATATT *Ssp*I site. Stop codons were located immediately after codon 480 (HdC8) and 472 (HdC16). The 167-bp *Nsi*I-*Pst*I cassettes containing the mutation were reintroduced into the parental pCEL/H Env vector and verified by using ABI Prism sequencers.

**Cell lines.** We used the following cell lines: NIH 3T3 murine fibroblasts, NIH 3T3(TK<sup>-</sup>) murine fibroblasts, derived from NIH 3T3 murine fibroblasts after selection with bromodeoxyuridine (28), A23 hamster fibroblasts, XC rat fibroblasts, D17 canine osteosarcoma cells, PK15 porcine kidney fibroblasts, COS African green monkey kidney fibroblasts, HeLa human cervical carcinoma cells, and 293T human fetal kidney cells expressing the simian virus 40 T antigen. The NIH 3T3(Hyg<sup>r</sup>), NIH 3T3(TK<sup>-</sup>)(Hyg<sup>r</sup>), and HeLa(Hyg<sup>r</sup>) hygromycin B-resistant cell lines used as target cells in the coculture infection assay were generated after stable transfection of the pSV/hygro plasmid and selection in 0.3 mg of hygromycin B (Invitrogen) per ml. All cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Gibco), 2 mM L-glutamine, and penicillin-streptomycin at 37°C and 5% CO<sub>2</sub> in a humidified incubator.

**Syncytium assay.** Twenty-four hours prior to transfection, 2 × 10<sup>5</sup> NIH 3T3, NIH 3T3(TK<sup>-</sup>), and other cells, when indicated, were seeded onto six-well tissue culture plates (Nunc). Approximately 75% confluent monolayers were transfected with 0.1 to 6 μg of pCEL/Env by using Lipofectamine Plus (Invitrogen). Immediately after transfection, cells were detached, reseeded in 12-well or 6-well

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tissue culture plates, and cultured for an additional 24 to 48 h. Separate duplicate wells were used for immunoblot analyses and for methanol fixation before May-Grunwald-Giemsa staining. When indicated, cocultures were performed as described previously (14), except May-Grunwald-Giemsa staining was performed. Syncytia were quantified on a stereomicroscope from nine randomly selected fields on a premarked grid and are represented as the mean number of syncytia per field ( $\pm$  standard deviation). Mean number of nuclei per syncytium ( $\pm$  standard deviation) was calculated by counting nuclei in five random syncytia in each of the nine fields.

**Cell-to-cell infection assay by coculture and hygromycin selection.** Virions consisting of MLV cores pseudotyped with HTLV-1 Env were produced after transfection of  $10^6$  293T cells by a modified version of the calcium phosphate method, with 1.5  $\mu$ g of pCEL/Env, 1.5  $\mu$ g of pCL/Gag-Pol, and 3  $\mu$ g of pCLMFG-LacZ, providing a packageable RNA coding for the *lacZ* gene marker (16). The Moloney MLV-based pCL/Gag-Pol expression vector was derived from pCL-Eco (16) by excising an *env* gene fragment between the *ScaI* and *ClaI* sites.

Following overnight transfection,  $2 \times 10^5$  293T virion-producing cells were used as infectious centers and cocultivated for 24 to 48 h with either  $2 \times 10^5$  HeLa(Hyg<sup>r</sup>), NIH 3T3(Hyg<sup>r</sup>), or NIH 3T3(TK<sup>-</sup>)(Hyg<sup>r</sup>) target cells in six-well tissue culture plates. Most of the 293T virion-producing cells were then detached by several cold washes with a phosphate-buffered saline solution, and the cocultivated cells were subsequently trypsinized and reseeded at 1:4, 1:8, and 1:12 dilutions on new 35-mm culture dishes (Nunc) in medium containing 0.3 mg of hygromycin B (Invitrogen) per ml to eliminate 293T producer cells. Cells were washed, and hygromycin-containing selection medium was changed every 2 days for approximately another 5 to 7 days, until the indicator target cells reached 90 to 100% confluence.

Target cells were fixed with 0.5% (wt/vol) glutaraldehyde in phosphate-buffered saline and stained with a 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) solution to reveal  $\beta$ -galactosidase activity. Blue CFU, resulting from the infection of hygromycin B-resistant target cells with MLV(HTLV-1) virions produced by the 293T cells, were counted, and titers are expressed as CFU per  $2 \times 10^5$  virion-producing cells, taking into account the respective dilutions of the producer and target cells. Statistical significance of differences was determined by using a pairwise Student's *t* test. Data are represented as mean interference  $\pm$  standard deviation.

**Envelope expression and incorporation into virions.** Twenty-four hours prior to transfection,  $2 \times 10^6$  to  $5 \times 10^6$  293T cells were seeded in 100-mm culture dishes. Transfections were performed as described above with 4  $\mu$ g of pCL/Gag-Pol, 8  $\mu$ g of pCLMFG-LacZ, and 4  $\mu$ g of the following pCEL/Env constructs: the parental HTLV Env (pCEL/H), the HdC8, HdC16 and H $\Delta$ C TM truncation mutants, or the pcDNA3.1 (Invitrogen) control vector with no *env* gene. Virion-producing cell extracts were collected 48 h posttransfection in 1 ml of cell lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1.0% Nonidet P-40, 0.5% deoxycholate, and a cocktail of mammalian protease inhibitors [Sigma]) and clarified by two successive centrifugations at 13,000 rpm for 10 min at 4°C in a microcentrifuge.

Approximately 20  $\mu$ l of each extract, adjusted after normalization for protein concentration by using the Bradford assay (Sigma), were subjected to electrophoresis on SDS-15% acrylamide gels, followed by transfer onto nitrocellulose (Protran; Schleicher & Schuell). Membranes were blocked in phosphate-buffered saline containing 5% powdered milk and 0.5% Tween 20, probed with a 1:100 dilution of the 5a rat anti-HTLV-1 TM monoclonal antibody (3) (a kind gift of C. Carrington and T. Schulz), washed three times with phosphate-buffered saline-0.1% Tween 20, and probed with the corresponding horseradish peroxidase-conjugated anti-rat immunoglobulin before three additional washes and detection by chemiluminescence were performed.

For pelleted virion preparations, HTLV Env-pseudotyped virions [MLV(H)] were generated as described for the cell-to-cell infection assay. Three milliliters of cell culture medium was recovered 48 h posttransfection, filtered through a 0.45- $\mu$ m-pore-size filter, and ultracentrifuged on a 2-ml 20% sucrose cushion (in Tris [pH 7.4], 100 mM NaCl, and 10 mM EDTA) at 24,000 rpm for 2 h in an SW41 rotor (Beckman). The pellet was lysed in 50  $\mu$ l of the cell lysis buffer and electrophoresed in SDS-15% polyacrylamide (anti-TM immunoblots) and SDS-12.5% polyacrylamide gels (anti-SU and anti-CA immunoblots) before transfer. Immunoblot analyses were performed as described above, with the additional detection of the MLV capsid (CA) protein with a 1:200 dilution of the rat R187 anti-CA monoclonal antibody (a kind gift of B. Chesebro) as described previously (1). Detection of mature SU and Env precursor was performed with a 1:100 dilution of the 1C11 mouse anti-HTLV-1 SU monoclonal antibody (Epitope).

## RESULTS

**Resistance of several cell lines to HTLV Env-mediated syncytium formation.** While most vertebrate cell lines are sensitive to HTLV Env syncytium formation, we found several mammalian adherent cell lines, including hamster A23, canine D17, porcine PK15, and murine NIH 3T3(TK<sup>-</sup>) cells, to be resistant to HTLV Env-mediated syncytium formation. We compared in more detail the NIH 3T3(TK<sup>-</sup>) cell line with the parental NIH 3T3 cells. The two cell lines were equally sensitive to syncytium formation by the Friend MLV ecotropic Env lacking the R peptide (F $\Delta$ R) (Fig. 1A). A fusion-active form of the amphotropic MLV Env (A $\Delta$ R) and the vesicular stomatitis virus G glycoprotein also induced equivalent numbers of large syncytia in NIH 3T3(TK<sup>-</sup>) and NIH 3T3 cells (not shown).

Thus, although NIH 3T3(TK<sup>-</sup>) cells were not intrinsically resistant to syncytium formation, no syncytia were observed upon transfection with as much as 6  $\mu$ g of HTLV Env expression vector (Fig. 1A). In contrast, introduction of only 1  $\mu$ g of the same vector in NIH 3T3 cells resulted in the formation of several hundred clearly detectable syncytia per well (Fig. 1A and Table 1). Since the NIH 3T3(TK<sup>-</sup>) as well as the nonmurine A23, D17, and PK15 resistant cell lines were sensitive to syncytium formation induced by the A $\Delta$ R Env and vesicular stomatitis virus G proteins (not shown), resistance of these four cell lines to syncytium formation was specific to the HTLV Env.

We verified that lack of syncytium formation in NIH 3T3(TK<sup>-</sup>) cells was not due to lower levels of HTLV Env expression (not shown) or to expression of nonfunctional Env, since NIH 3T3(TK<sup>-</sup>) cells transfected with HTLV Env induced significant syncytium formation with cocultured HeLa cells (Fig. 1B). This coculture led to smaller fusion foci compared to the rampant formation of large syncytia observed with homogeneously susceptible cell cocultures (14). Furthermore, the lack of syncytium formation in NIH 3T3(TK<sup>-</sup>) cells was not due to the absence of functional HTLV receptors at the surface of these cells, since a soluble form of HTLV Env receptor-binding domain bound NIH 3T3(TK<sup>-</sup>) cells at least as efficiently as NIH 3T3 cells (F. J. Kim, E. N. Garrido, N. Manel, M. Sitbon, and J.-L. Battini, unpublished data). This was further confirmed by the finding that both cell-free virion titers (not shown) and cell-to-cell infection titers (Table 2) of MLV(H) virion pseudotypes were similar for NIH 3T3 and NIH 3T3(TK<sup>-</sup>) cells. Similarly, the A23, D17, and PK15 syncytium-resistant cell lines were readily susceptible to infection by MLV(H) virion pseudotypes (not shown).

**Partial truncation of the HTLV Env cytoplasmic tail allows syncytium formation in resistant cell lines.** Since HTLV and MLV Env have a common SU modular organization (14; F. J. Kim, E. N. Garrido, N. Manel, M. Sitbon, and J.-L. Battini, unpublished data), and since the carboxy-terminal R peptide of the MLV TM cytoplasmic tail inhibits syncytium formation (20, 21), we evaluated whether partial cleavage of the HTLV Env cytoplasmic domain would reveal an effect similar to that observed after cleavage of the MLV R peptide. Because no consensus R peptide cleavage sequence could be identified in the HTLV TM, the HdC8 and HdC16 cytoplasmic domain truncation mutants were empirically generated by eliminating the carboxy-terminal 8 and 16 amino acids, respectively, of the



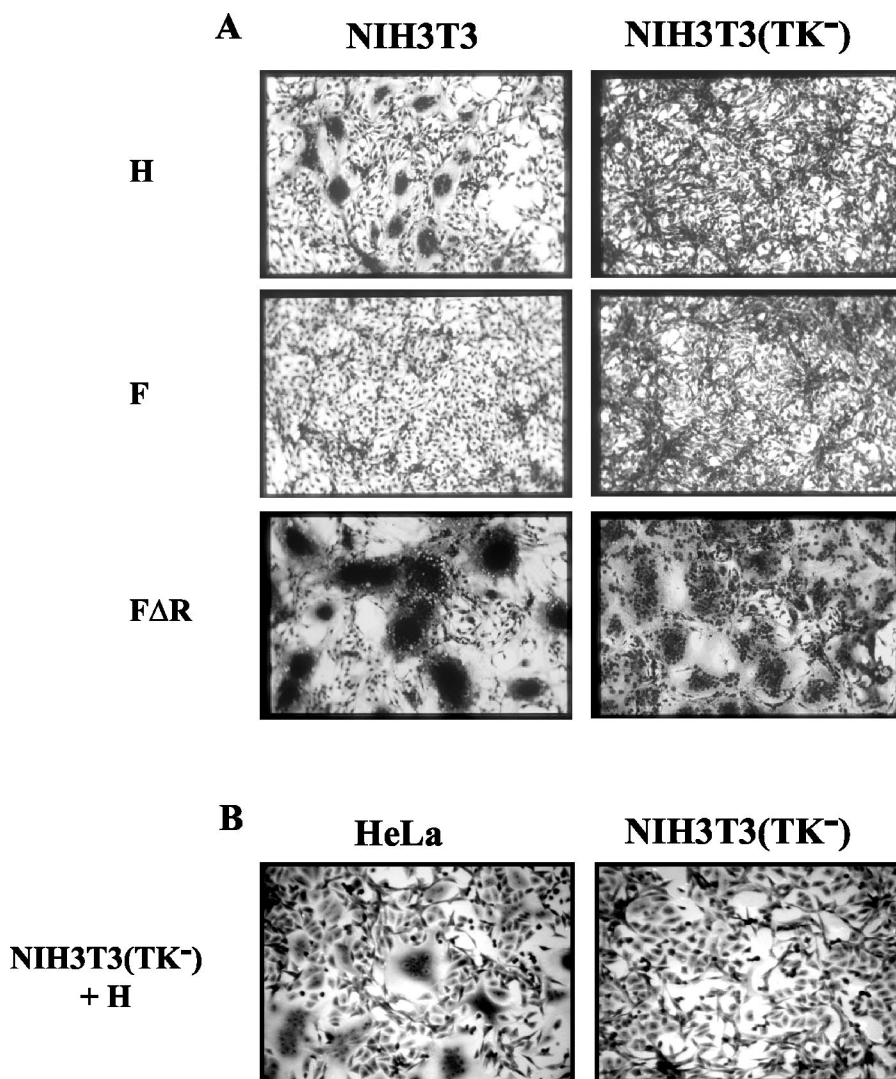


FIG. 1. Distinct susceptibility of NIH 3T3 and NIH 3T3(TK<sup>-</sup>) cells to HTLV Env-induced syncytium formation. (A) NIH 3T3 and NIH 3T3(TK<sup>-</sup>) cells were tested for their susceptibility to HTLV-1 Env (H)-induced syncytium formation. Cells were transfected with up to 6 μg of envelope expression vector. As controls, both cell lines were transfected with vectors encoding either the parental, full-length Friend MLV Env (F) or the fusogenic Friend MLV Env lacking the R peptide (FΔR). Syncytia are observed as dark plaques. (B) NIH 3T3(TK<sup>-</sup>) cells transfected with the HTLV-1 Env, NIH 3T3(TK<sup>-</sup>)+H, were tested for their ability to trigger syncytium formation with overlaid HeLa (left panel) and NIH 3T3(TK<sup>-</sup>) target cells (right panel).

cytoplasmic domain, immediately carboxy terminal of serines 480 and 472 of the parental HTLV Env (Fig. 2A).

Both TM truncations led to significantly increased numbers of syncytia in NIH 3T3 cells. Furthermore, transfection of HdC8 and HdC16 resulted in the formation of syncytia in the initially resistant NIH 3T3(TK<sup>-</sup>) cells (Fig. 2B), albeit to a lesser extent than observed in parental NIH 3T3 cells (Table 1). Despite the more compact appearance of the NIH 3T3(TK<sup>-</sup>) syncytia (Fig. 2B), no significant differences were observed in the number of nuclei per NIH 3T3 and NIH 3T3(TK<sup>-</sup>) syncytium formed by the parental, HdC8, and HdC16 HTLV Envs. In all cases, the mean number of nuclei per syncytium ranged from 11 to 18 (± 6 to 10). As expected from a previous study using NIH 3T3 cells (9), the HΔC mutant, which lacked the entire cytoplasmic domain of the TM, was also unable to induce syncytium formation in NIH

TABLE 1. HTLV Env truncation mutants overcome resistance of NIH 3T3(TK<sup>-</sup>) cells to HTLV Env-mediated syncytium formation<sup>a</sup>

Env	Mean no. of syncytia <sup>b</sup> ± SD	
	NIH 3T3	NIH 3T3(TK <sup>-</sup> )
Mock	<1	<1
H	276 ± 15	<1
HdC8	457 ± 7	83 ± 5
HdC16	453 ± 32	32 ± 5
HΔC	<1	<1
F	<1	<1
FΔR	650 ± 74	678 ± 35

<sup>a</sup> NIH 3T3 and NIH 3T3(TK<sup>-</sup>) cells were transfected with 4 μg of pCEL/Env. Data are from three experiments performed in duplicate and are represented as mean ± standard deviation.

<sup>b</sup> Syncytia containing at least four nuclei were counted in nine fields for each experiment.

TABLE 2. Cell-to-cell transmission of HTLV-1 Env-pseudotyped virions into NIH 3T3, NIH 3T3(TK<sup>-</sup>), and HeLa target cells<sup>a</sup>

Pseudotyping Env <sup>b</sup>	No. of CFU in hygromycin-resistant target cells		
	NIH 3T3	NIH 3T3(TK <sup>-</sup> )	HeLa
None	<10	<10	<10
H	784–1,082	608–888	2,720–7,080
HdC8	353–576	292–384	6,752–13,128
HdC16	304–360	128–196	2,368–7,680
HΔC	<10	<10	<10

<sup>a</sup> Cell-to-cell transmission was evaluated by the number of LacZ-positive blue CFU of the specified hygromycin-resistant target cells obtained after coculture with  $2 \times 10^5$  293T cells producing Env-pseudotyped *lacZ* virions. Data are presented as the range of titers obtained in three independent experiments.

<sup>b</sup> Pseudotyped MLV cores were produced by 293T cells transiently transfected with control plasmid DNA (none) or with parental (H) or HdC8, HdC16, or HΔC HTLV-derived Env truncation mutants (diagrammed in Fig. 2A).

3T3(TK<sup>-</sup>) cells. The ability of the HdC8 and HdC16 truncation mutants to induce syncytium formation was also observed in the resistant A23, D17, and PK15 cell lines (not shown).

**Cell-to-cell transmission of MLV virions pseudotyped with parental and truncated HTLV Env.** Infection by HTLV or HTLV Env-pseudotyped virions has been shown to be significantly more efficient upon cell-to-cell contact than via cell-free viral supernatants (7, 26). We therefore assayed transmission of MLV virions pseudotyped with either the parental HTLV-1 Env, MLV(H), the HdC8 mutant MLV(HdC8), or the HdC16 mutant MLV(HdC16), using a cell-to-cell transmission assay. Infection of hygromycin B-resistant target cells was assessed following coculture with pseudotyped virion-producing 293T cells. Input 293T cells were progressively eliminated by hygromycin treatment, and CFU were quantified in the resistant target cells. Although slightly more efficient on NIH 3T3(Hyg<sup>r</sup>) cells than on NIH 3T3(TK<sup>-</sup>)(Hyg<sup>r</sup>) target cells, cell-to-cell transmission of MLV(H) virions was similar ( $P > 0.15$ ) in the two cell types, with titers ranging from 784 to 1,082 CFU (mean  $\pm$  standard deviation,  $947 \pm 151$  CFU) and 608 to 888 CFU (mean,  $712 \pm 153$  CFU) per  $2 \times 10^5$  virion-producing cells, respectively (Table 2). This similar susceptibility to infection was in striking contrast to the aforementioned distinct susceptibilities of the two cell lines to HTLV Env-mediated syncytium formation (Fig. 1 and Table 1).

Cell-to-cell transmission was undetectable with the HΔC pseudotypes, and despite their increased fusogenic properties, the partially truncated HdC8 and HdC16 TM mutants demonstrated decreased cell-to-cell transmission compared to parental HTLV Env-pseudotyped virions (Table 2). Lower infectious titers of the HdC8 and HdC16 Env mutants were observed on both NIH 3T3 and NIH 3T3(TK<sup>-</sup>) target cells, with titers ranging between 353 and 576 CFU (mean,  $448 \pm 115$  CFU) and 304 and 360 CFU (mean,  $326 \pm 30$  CFU) in NIH 3T3 cells and between 292 and 384 CFU (mean,  $345 \pm 48$  CFU) and 128 and 196 CFU (mean,  $164 \pm 34$  CFU) in NIH 3T3(TK<sup>-</sup>) cells, respectively ( $P < 0.03$  in all cases, compared to parental HTLV) (Table 2).

Coculture controls, as described elsewhere (F. J. Kim, E. N. Garrido, N. Manel, M. Sitbon, and J.-L. Battini, unpublished data), were performed in parallel and confirmed that LacZ-positive blue colonies were indeed due to infection by

MLV(Env) virions and not to either Env-induced cell-to-cell fusion or spontaneous transduction of target cells with the pCLMFG-LacZ vector. Blue colonies above background were not observed in the absence of virions or Env. Furthermore, no blue colonies were observed in control experiments where human HeLa target cells were cocultured with virions pseudotyped with ecotropic MLV Env (not shown).

Interestingly, when HeLa(Hyg<sup>r</sup>) cells were used as target cells in the cell-to-cell virion transmission assay, the highest titers were reproducibly obtained with MLV(HdC8) virion pseudotypes, with titers (per  $2 \times 10^5$  virion-producing cells) ranging from 6,752 to 13,128 CFU, versus 2,720 to 7,080 and 2,368 to 7,680 CFU with MLV(H) and MLV(HdC16) virion pseudotypes, respectively ( $P < 0.02$  compared to parental HTLV and HdC16) (Table 2). As these data are in contrast to those observed with NIH 3T3 and NIH 3T3(TK<sup>-</sup>) cells, it appears that distinct properties of target cells modulate the impact of the TM cytoplasmic domain truncation on cell-to-cell infection.

**Expression of parental and truncated HTLV Env and their incorporation into viral particles.** To assess whether infection with the various HTLV Env-pseudotyped virions was modulated by distinct incorporation of the mutant Env into virions, the amount of H, HdC8, and HdC16 present in the MLV particles was monitored by immunoblotting with an anti-TM monoclonal antibody. This allowed detection of incorporated Env irrespective of the level of SU shedding. Under these conditions, we detected significant amounts of the mature parental and truncated TM incorporated in all virion preparations with the exception of the HΔC TM mutant (Fig. 3, center panel), which appeared to be expressed at a lower level than the other Env constructs (Fig. 3, left panel). Accumulation of slower-migrating uncleaved precursors in all virion preparations was assessed by dual anti-TM and anti-SU recognition. Whether these forms were cell membrane- or virion-associated Env precursor intermediates remains unclear. It is noteworthy that low levels of cleaved SU were detected in all virion preparations (Fig. 3, right panel), indicative of a labile SU/TM association which may account for the low cell-free infectivity of HTLV Env-harboring particles.

## DISCUSSION

The HTLV Env induces the formation of syncytia and mediates infection of a broad range of vertebrate cell lines (26, 27), including murine NIH 3T3 cells (9, 26). Here, we report several mammalian adherent cell lines that are specifically resistant to HTLV Env-mediated syncytium formation. This resistance was overcome by partial truncation of the TM cytoplasmic domain carboxy terminus (Fig. 2). Cytoplasmic domain truncation did not lead to increased production of mature Env in the resistant NIH 3T3(TK<sup>-</sup>) cell line, as monitored with anti-TM antibody (not shown). Among other mechanisms, the TM carboxy-terminal amino acids may influence the kinetics of Env maturation, cell membrane interactions, and stability of the SU-TM association.

We have previously proposed that lability of the SU-TM association might explain the increased fusion ability of proline-rich region mutants of the MLV Env SU (15). A weaker SU-TM association would also favor infection by cell-to-cell

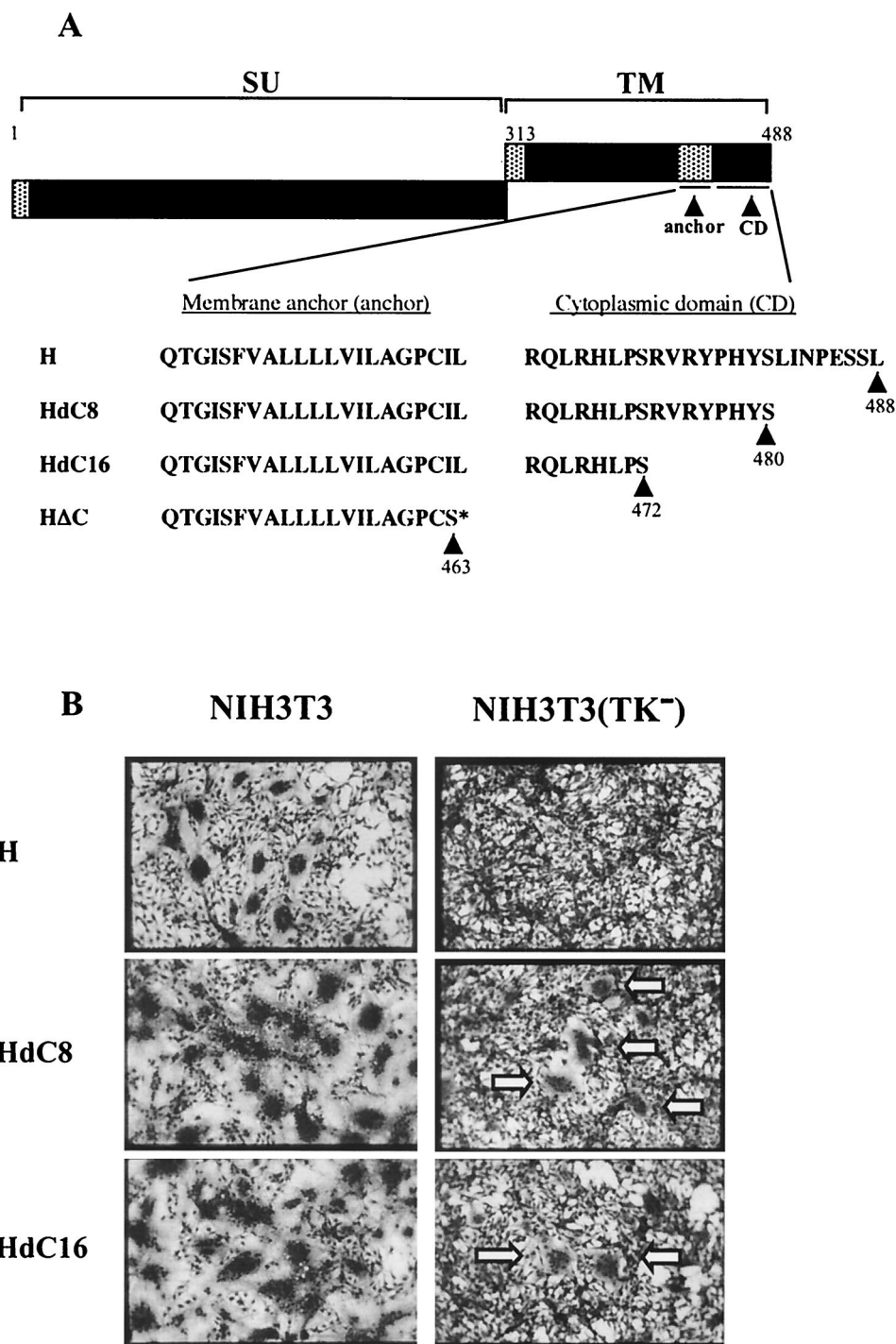


FIG. 2. Truncation of HTLV-1 Env TM induces syncytium formation in NIH 3T3(TK<sup>-</sup>) cells. (A) Schematic representation of HTLV Env. The SU and TM subunits are indicated. The HTLV-1 Env signal peptide, fusion peptide, and TM membrane anchor are shown as dotted boxes, from the amino terminus to the carboxy terminus, respectively. Amino acid residues of the membrane anchor (anchor) and cytoplasmic domain (CD) are shown, and the position of the last carboxy-terminal residue of each mutant is indicated with an arrowhead. Amino acid residue numbering starts from the first signal peptide methionine of the HTLV-1 Env precursor. (B) Parental HTLV-1 Env (H) and truncation mutants lacking the 8 (HdC8) or 16 (HdC16) carboxy-terminal amino acids of the TM were tested for their ability to form syncytia after transfection in NIH 3T3 or NIH 3T3(TK<sup>-</sup>) cells. Open arrows in the panels on the right point to syncytia.

transmission due to the continuous production of enveloped virions in close proximity to target cells, whereas infection by cell-free virions lacking SU would be considerably reduced. Truncation of the MLV R peptide has been shown to be

essential for MLV Env function (20, 21). However, it remains to be determined whether equivalent cleavage occurs in bona fide HTLV virions. Indeed, in the absence of a defined R peptide consensus cleavage site, our truncation mutations were



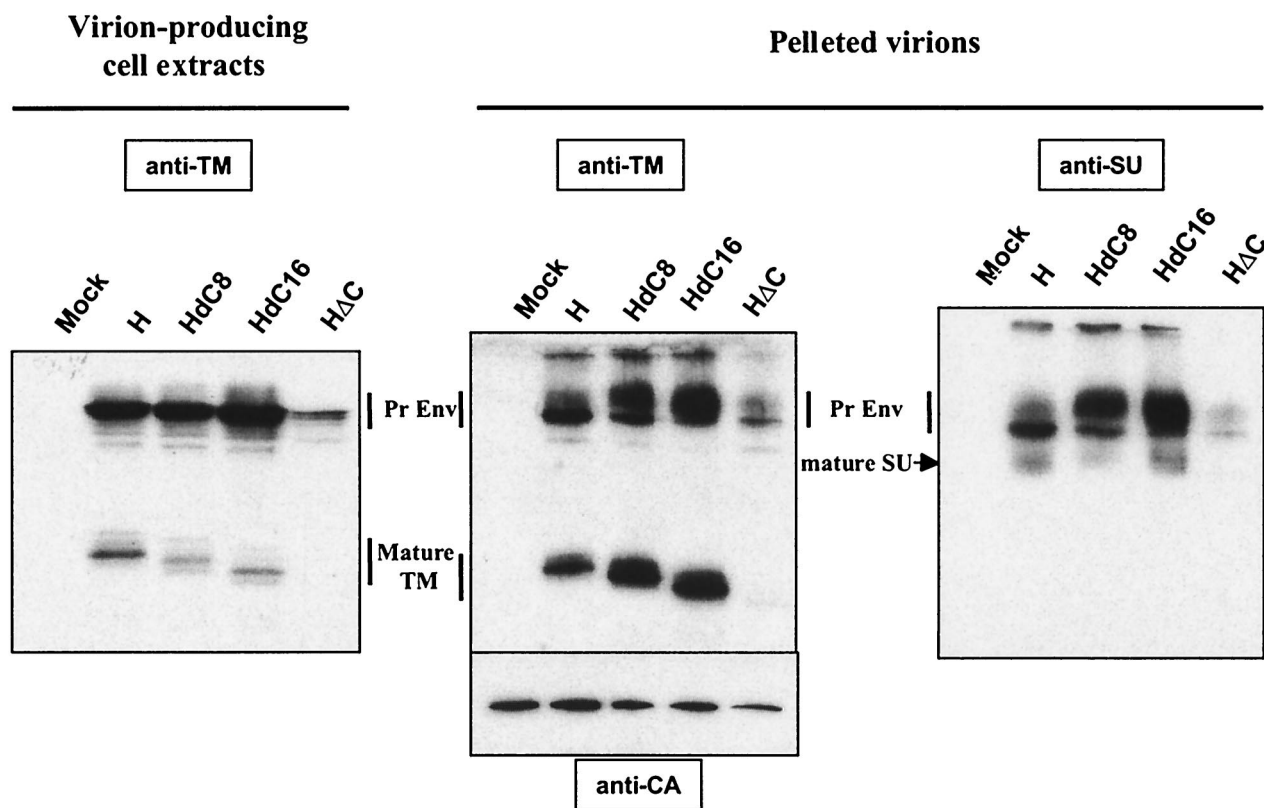


FIG. 3. Expression of HTLV-1 Env TM truncation mutants and their incorporation into virions. Equal amounts of cell extracts were obtained from  $2 \times 10^6$  to  $5 \times 10^6$  293T cells cotransfected with 8  $\mu$ g of an MLV-based *lacZ* reporter gene vector, 4  $\mu$ g of the MLV p/CLGag-Pol expression vector, and 4  $\mu$ g of either an HTLV Env-derived expression vector (H, HdC8, HdC16, or H $\Delta$ C) or a control vector (Mock). The different HTLV Env TM mutants are diagrammed in Fig. 2. Cell extracts were immunoblotted with the 5a anti-HTLV TM monoclonal antibody (3) (left panel). Pelleted virions were obtained from supernatants of 293T transfectants after concentration by ultracentrifugation on a 20% sucrose cushion. Resulting pellets were immunoblotted with either the 5a anti-HTLV TM monoclonal antibody (anti-TM, center panel) or the 1C11 anti-HTLV SU monoclonal antibody (anti-SU, right panel). Respective levels of virion expression were controlled by immunoblotting with the R187 anti-MLV capsid monoclonal antibody (a kind gift of B. Chesebro) (anti-CA, center panel). The positions of the HTLV-1 Env precursors (Pr Env), mature TM, and mature SU envelope proteins are indicated.

introduced at arbitrary positions in the HTLV Env cytoplasmic domain.

Among the syncytium-resistant cell lines, we extensively studied the NIH 3T3(TK<sup>-</sup>) cell line, which was selected for resistance to bromodeoxyuridine and lack of thymidine kinase activity (28). However, neither bromodeoxyuridine resistance nor lack of TK activity appeared to directly correlate with resistance to HTLV Env-mediated syncytium formation. Indeed, other bromodeoxyuridine-resistant cell lines recently derived in our laboratory to address this point were readily susceptible to HTLV Env-induced syncytium formation (not shown). Furthermore, introduction of the herpes simplex virus type 1 *tk* gene in NIH 3T3(TK<sup>-</sup>) cells did not lead to HTLV Env syncytium susceptibility (not shown).

Resistance to syncytium formation appeared to be limited to HTLV Env, since NIH 3T3(TK<sup>-</sup>) (Fig. 1) and the other resistant cell lines were fully susceptible to several other fusogenic Envs, such as F $\Delta$ R (Fig. 1), A $\Delta$ R, and the vesicular stomatitis virus G glycoprotein (not shown). Also, resistance was due neither to lower expression levels of mature Env by NIH 3T3(TK<sup>-</sup>) cells (not shown) nor to a lack of functional HTLV Env expression in these cells, as coculture with HeLa or NIH

3T3 target cells resulted in readily detectable syncytium formation (Fig. 1B and data not shown). Furthermore, resistance to syncytium formation was not due to the absence of HTLV receptors, as NIH 3T3(TK<sup>-</sup>) cells bound a soluble form of the HTLV Env receptor-binding domain (F. J. Kim, E. N. Garrido, N. Manel, M. Sitbon, and J.-L. Battini, unpublished data) and were infected by MLV(H) virions to levels similar to those observed with NIH 3T3 cells (Table 2).

The two HTLV Env truncation mutants that overcame resistance to syncytium formation were infectious for all cell lines, as determined by our cell-to-cell transmission assay with MLV-pseudotyped virions (Table 2 and data not shown). It is noteworthy that both mutations resulted in either partial (HdC8) or total (HdC16) deletion of the YSLI motif, which has been described previously as being required for cell-to-cell transmission of HTLV virions (6). This apparent discrepancy between our results and the reported importance of the YSLI motif in cell-to-cell transmission might be due to different experimental parameters, including assay read-outs, use of MLV versus HTLV cores, and the precise location of the TM truncations.

The HTLV TM cytoplasmic domain truncation mutants de-

scribed here had distinct effects on cell-to-cell infection of NIH and HeLa target cells. In HeLa target cells, MLV(HdC8) but not MLV(HdC16) virion pseudotypes demonstrated consistently increased coculture infection titers compared to parental HTLV Env (Table 2). In contrast, virions that were pseudotyped with either mutant had decreased titers in NIH 3T3 cells. These results most likely illustrate the fact that cell membrane components, including adhesion molecules (4, 5, 12), cytoskeleton-associated proteins (24), lipids (23), and lipid raft components (17), have distinct influences on HTLV Env-mediated cell-to-cell fusion and virus transmission.

Our observations that NIH 3T3(TK<sup>-</sup>) cells are resistant to HTLV Env-mediated syncytium formation but not to infection reinforces the hypothesis that different factors are involved in these two processes. In this regard, it is interesting that, as monitored by time-lapse video microscopy, NIH 3T3(TK<sup>-</sup>) cells were readily distinguishable from standard NIH 3T3 cells in at least two aspects: (i) the NIH 3T3(TK<sup>-</sup>) cells were considerably more mobile in culture and (ii) they exhibited a relatively compact morphology, with more compact lamellipodia and increased filopodium formation (not shown). Our data suggest that syncytium formation is not strictly dependent upon receptor availability, but is likely to involve post-receptor-binding membrane dynamics. We showed that a determinant located within the 8 carboxy-terminal residues of the TM cytoplasmic domain plays a major role in these dynamics.

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Research

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## HTLV-I and -2 envelope SU subdomains and critical determinants in receptor binding

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### Abstract

**Background:** Human T-cell leukemia virus (HTLV) -1 and -2 are deltaretroviruses that infect a wide range of cells. Glut1, the major vertebrate glucose transporter, has been shown to be the HTLV Env receptor. While it is well established that the extracellular surface component (SU) of the HTLV envelope glycoprotein (Env) harbors all of the determinants of interaction with the receptor, identification of SU subdomains that are necessary and sufficient for interaction with the receptor, as well as critical amino acids therein, remain to be precisely defined. Although highly divergent in the rest of their genomes, HTLV and murine leukemia virus (MLV) Env appear to be related and based on homologous motifs between the HTLV and MLV SU, we derived chimeric HTLV/MLV Env and soluble HTLV-I and -2 truncated amino terminal SU subdomains.

**Results:** Using these SU constructs, we found that the 183 and 178 amino terminal residues of the HTLV-I and -2 Env, respectively, were sufficient to efficiently bind target cells of different species. Binding resulted from *bona fide* interaction with the HTLV receptor as isolated SU subdomains specifically interfered with HTLV Env-mediated binding, cell fusion, and cell-free as well as cell-to-cell infection. Therefore, the HTLV receptor-binding domain (RBD) lies in the amino terminus of the SU, immediately upstream of a central immunodominant proline rich region (Env residues 180 to 205), that we show to be dispensable for receptor-binding and interference. Moreover, we identified a highly conserved tyrosine residue at position 114 of HTLV-I Env, Tyr<sub>114</sub>, as critical for receptor-binding and subsequent interference to cell-to-cell fusion and infection. Finally, we observed that residues in the vicinity of Tyr<sub>114</sub> have lesser impact on receptor binding and had various efficiency in interference to post-binding events.

**Conclusions:** The first 160 residues of the HTLV-I and -2 mature cleaved SU fold as autonomous domains that contain all the determinants required for binding the HTLV receptor.

## Background

Human T-cell leukemia virus type 1 (HTLV-1) has been found primarily in CD4+ and CD8+ T-lymphocytes *in vivo* [1-3], whereas CD8+ T-lymphocytes are thought to be the *in vivo* reservoir of HTLV-2 [4]. However, the *in vitro* tropism of HTLV-1 and -2, as determined using HTLV envelope-pseudotyped virions or envelope-induced cell fusion assays, appears to be ubiquitous [5-7]. Indeed, we recently showed that Glut1, the ubiquitous vertebrate glucose transporter, serves as a receptor for HTLV-1 and -2 envelope glycoprotein (Env) [8]. While the precise organization and properties of the receptor-interacting Env domains has not been reported, we found that the amino terminal two-thirds of the HTLV-1 extracellular surface component (SU) are sufficient to confer HTLV-1 tropism to an ecotropic Friend murine leukemia virus (F-MLV) Env [9]. A cell fusion interference assay performed with this HTLV/F-MLV Env chimera and the parental Env confirmed that this 215 amino acid Env domain, harbors HTLV-1 receptor-binding determinants [9].

The corresponding domain in MLV Env SU – located upstream of a conserved K/R L L T/N L V Q motif in the SU of the HTLV-1 and F-MLV Env [9,10] – is well characterized and comprises two main functional regions: an amino terminal sequence harboring the receptor-binding determinants, VRA, VRB and VRC [11-13], and a proline-rich region (PRR), starting at the first proline residue of the GPRVPIGP sequence [11,14] and flanked by two highly conserved GXDP [15] and CXXC [16] motifs (Figure 1). In the ecotropic and amphotropic (Ampho) MLV Env, the PRR is a putative hinge region implicated in conformational changes, triggered after receptor binding, and subsequent fusion [17,18]. In the central region of the HTLV SU, a short sequence (Env residues 180 to 205) harbors high proline content and could be a homologue of the MLV PRR.

Several studies using synthetic peptides and neutralizing antibodies against the HTLV Env have shown that determinants within this proline rich region homologue (PRRH) are involved in interference to Env-mediated syncytium formation [19-21]. The PRRH had been thought to encode the receptor-binding domain, as based on cell-to-cell fusion assays [19,22-24]. However, although PRRH synthetic peptides can block HTLV Env-mediated syncytia formation, they have no effect on HTLV SU binding [25] and infection [26]. Indeed, we and others have shown that Env receptor binding *per se*, as well as interference to receptor-binding, cell-to-cell fusion, syncytium formation, and infection involve several distinct cell surface-associated parameters [27-29]. In the present report, we produced soluble forms of wild-type and mutant HTLV-1 and 2 SU amino terminal subdomains and tested their receptor-binding abilities. We also tested their ability to

specifically interfere with HTLV Env cell surface binding, Env-mediated cell-to-cell fusion, and retroviral infection. By testing these essential parameters of Env-mediated dissemination, we delineated the Env receptor-binding domain (RBD) to the first 160 residues of the mature HTLV-1 and -2 SU, excluding the PRRH, and we identified a conserved tyrosine residue at position 114 of HTLV-1 Env as a critical determinant for HTLV Env receptor binding.

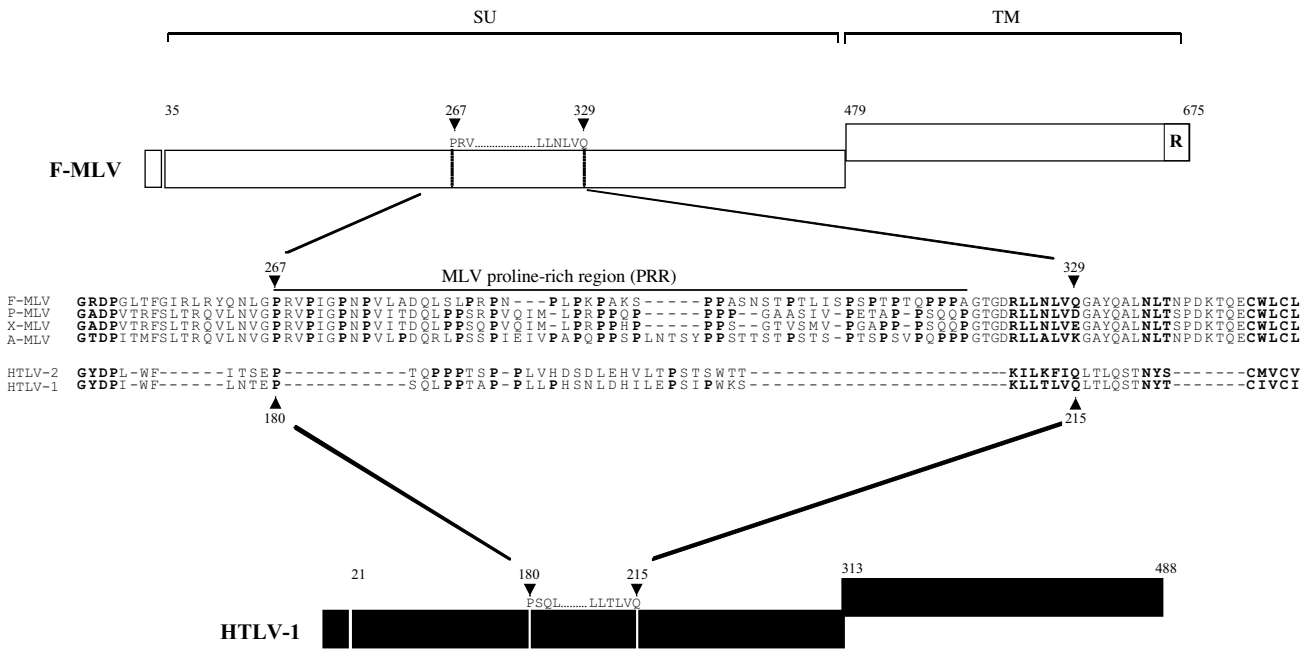
## Results

### **Motif conservation and similar modular organization of HTLV and MLV SU, and identification of a proline-rich region homologue (PRRH) in the HTLV SU**

As shown in Figure 1, our alignment of the MLV and HTLV SU reveals several notable motif conservations outlining a similar modular organization of the MLV SU and HTLV SU. A (K/R)LL(T/N)LVQ motif, highly conserved between the F-MLV and HTLV-1 SU, is located immediately downstream of the PRR and its PRRH counterpart, respectively. Another highly conserved motif between MLV and HTLV, GXDP, is found immediately upstream of the PRR/PRRH (Figure 1). These two motifs compelled us to notice the PRRH, between the PSQ and KLLTLVQ sequences in HTLV-1, and between the PTQ and KILKFIQ sequences in HTLV-2 (Figure 1). As counted from the first and last proline in the delineated sequence, the PRRH has a proline content of 30.8% and 30.4% for HTLV-1 and -2, respectively. This is slightly lower than the 35.3%, 36%, 36%, and 35.6% proline content for the ecotropic, polytropic, xenotropic, and amphotropic MLV Env, respectively (Figure 1). The presence of a PRRH in the HTLV SU appeared to be characteristic of their MLV-like modular organization, since HTLV SU average proline content outside of the PRRH does not exceed 11%.

### **Functional, soluble HTLV Env-receptor binding determinants**

MLV SU receptor binding determinants are all located upstream of the PRR [11,30]. To test whether the HTLV Env receptor binding determinants are also located upstream of the potential PRRH, we constructed a chimeric Env and several soluble HTLV-1 and -2 SU amino terminal subdomains. The chimeric HTLV/MLV Env, H1<sub>183</sub>FEnv, comprises the 183 amino terminal residues of the HTLV-1 SU ending with the PSQL residues fused to the PIGP sequence of the F-MLV PRR (Figure 2A). In this Env chimera the receptor-binding domain (first 269 residues) of the F-MLV Env was replaced with the potentially corresponding domain of the HTLV-1 Env SU (Figure 2A). The chimeric H1<sub>183</sub>FEnv construct – which lacks the HTLV PRRH but has the MLV PRR – was properly expressed in transfected cells and was revealed on immunoblots with an anti-MLV SU polyclonal antibody (Figure 3A). Accordingly, an anti-HTLV-1 monoclonal antibody raised

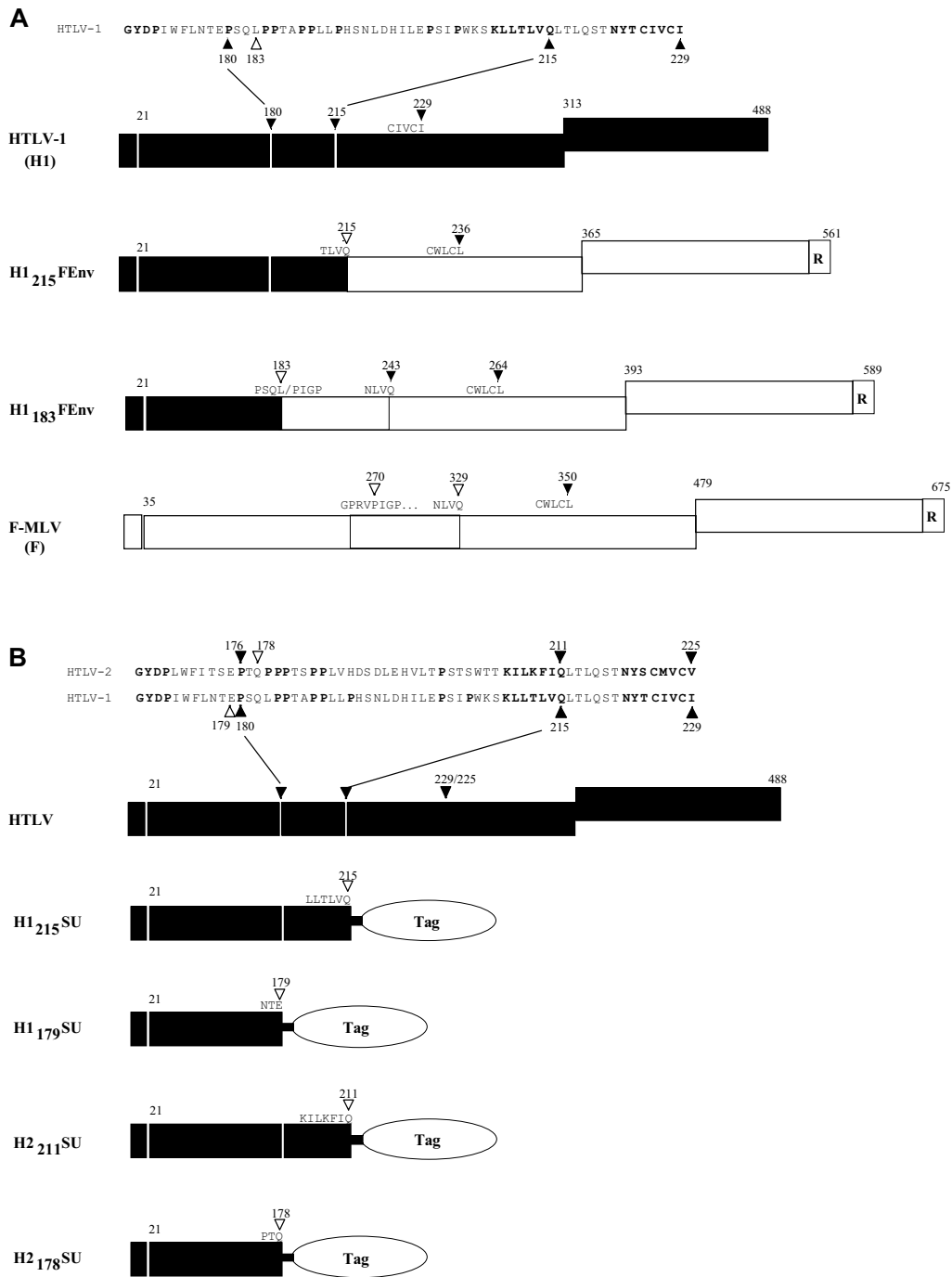


**Figure 1**  
**Homologous modular domains in HTLV and MLV envelopes.** Friend-MLV (F-MLV) Env and HTLV-I Env are schematically represented as open and solid boxes, respectively. Boxes represent, from left to right, the signal peptide which comprises the first 34 and 20 amino acid residues of F-MLV and HTLV Env, respectively, the extracellular surface component (SU) and the transmembrane component (TM) including the carboxy terminal R peptide in F-MLV, which is cleaved in the mature Env glycoprotein [64, 65]. Env landmark positions are indicated and the MLV proline-rich regions (PRR) and the HTLV SU proline-rich region (PRRH) are delineated by vertical lines within the SU at the positions indicated by solid arrowheads. The PRR and PRRH start at the first proline (P) residue downstream of the conserved GXDP motif. Env sequences represented in the figure are obtained from F-MLV strain 57 (accession number CAA26561); P-MLV, F-MCF polytropic MLV (AAA46483); X-MLV, NZB xenotropic MLV (AAA46531); A-MLV, amphotropic MLV strain 4070A (AAA46515); HTLV-2 (NP\_041006); and HTLV-1, MT2 strain (VCLJMT). Residue numbering starts from the first methionine of the Env signal peptides. Proline residues and homologue motifs are noted in bold. Amino acid sequence alignments were performed using the Clustal program in the Megalign alignment software package (DNASar) with manual adjustments.

against a PRRH epitope did not bind this chimeric Env (data not shown).

HTLV-1 and -2 SU amino terminal subdomains with or without their respective PRRH were constructed as fusion proteins with either an influenza hemagglutinin (HA) or rabbit immunoglobulin Fc (rFc) carboxy terminal tag (Figure 2B). The H1<sub>215</sub>SU and H2<sub>211</sub>SU subdomains comprise the first 215 and 211 residues, counting from the first methionine in the signal peptide through the KLLTLVQ of HTLV-1 and KILKFIQ of HTLV-2 Env, respectively (Figure 2B). The H1<sub>179</sub>SU and H2<sub>178</sub>SU, comprising the amino terminal 179 and 178 amino acids of the HTLV-1 and -2 Env, respectively, exclude the PRRH sequence (Figure 2B).

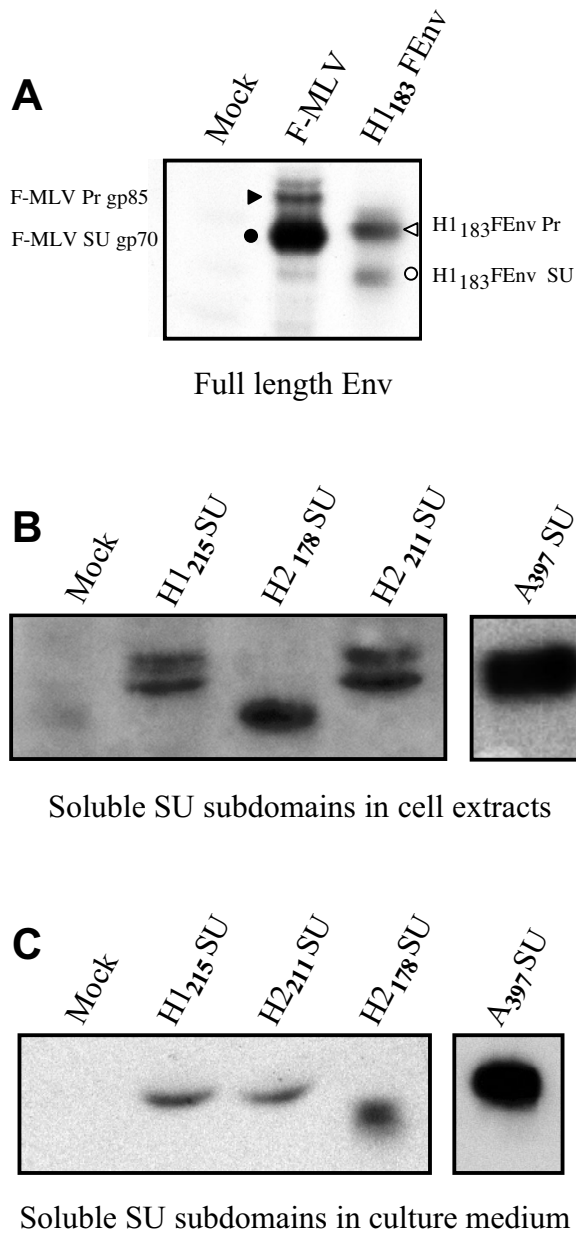
Cell lysates and cell culture supernatants were analyzed to evaluate intracellular expression and secretion of functional SU amino terminal domains in transfected-cell cultures, respectively. H1<sub>215</sub>SU and H2<sub>211</sub>SU, containing the PRRH sequence, and H2<sub>178</sub>SU lacking this PRRH were all efficiently expressed in transfected cells (Figure 3B). It is noteworthy, however, that recovery of tagged H1<sub>179</sub>SU molecules was largely inefficient because the vast majority of this protein was cleaved (data not shown). In contrast, no significant cleavage was observed with the other soluble domains released in the medium (not shown) (Figure 3C). As expected for immunoadhesins, H1<sub>215</sub>SU, H2<sub>211</sub>SU, and H2<sub>178</sub>SU rFc-tagged domains were detected as dimers under non-reducing conditions (not shown). Immunoblots of cell extracts revealed two forms of



**Figure 2**

**Schematic representation of HTLV/MLV Env chimeras and HTLV SU amino terminal subdomains.** Env landmark positions are indicated and SU landmark sequences and positions are indicated by arrowheads. Open arrowheads indicate the position of construct borders. (A) HTLV/MLV Env chimeras. The H1<sub>215</sub>FEnv and H1<sub>183</sub>FEnv HTLV/MLV Env chimeras were obtained by replacing the 329 and 269 amino terminal residues of the F-MLV Env (open boxes) with the amino terminal 215 and 183 amino acid residues of the HTLV-1 Env (solid boxes), respectively. The H1<sub>215</sub>FEnv chimera, previously described and formerly designated HHproFc [9], has been renamed here for sake of nomenclature homogeneity. (B) Soluble HTLV-1 (H1) and HTLV-2 (H2) SU amino terminal subdomains, H1<sub>215</sub>SU, H2<sub>211</sub>SU, H1<sub>179</sub>SU, and H2<sub>178</sub>SU were constructed as fusion proteins with a carboxy terminal hemagglutinin (HA) or rabbit immunoglobulin Fc (rFc) tag. All amino acid residue numbering starts from the first methionine of the HTLV-1 or -2 Env signal peptide, the amino terminal 20 and 21 aa residues, respectively.





**Figure 3**  
**Intracellular expression of HTLV-I Env chimeras and soluble SU subdomains.** Cell extracts (A, B) or culture supernatants (C) were prepared from 293T cells transfected with either full length Env (A) or soluble SU subdomains (B, C) expression vectors as depicted in figure 2. Membranes were probed with either (A) an anti-MLV SU antiserum to detect F-MLV and H1<sub>183</sub>FEnv uncleaved Env precursor proteins (F-MLV Prgp85 and H1<sub>183</sub>Fenv Pr, respectively) indicated by arrowheads, and cleaved SU (F-MLV SUgp70 and H1<sub>183</sub>FEnv SU, respectively) indicated by circles, or (B, C) an anti-rabbit IgG antiserum to detect carboxy terminal rFc-tagged soluble subdomains, including the Ampho-MLV SU subdomain (A<sub>397</sub>SU).

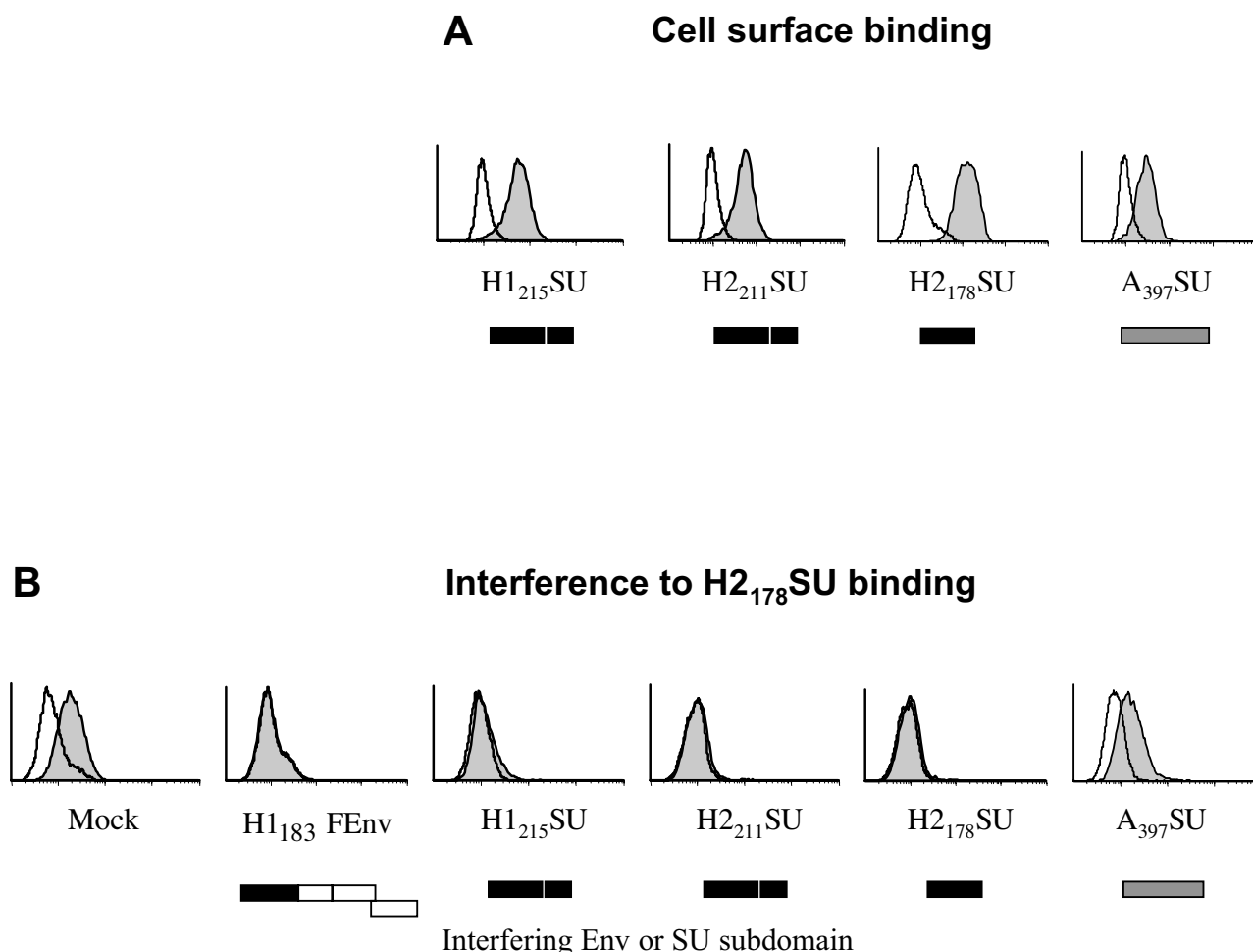
intracellular H1<sub>215</sub>SU and H2<sub>211</sub>SU (Figure 3B); this was likely due to variable glycosylation of these subdomains. However, a single secreted, soluble form of each of these amino terminal subdomains was detected in cell culture supernatants (Figure 3C).

A truncated Ampho-MLV SU-rFc fusion protein that comprises the amino terminal 397 residues of the Ampho-MLV Env fused to a carboxy terminal rFc tag was constructed (A<sub>397</sub>SU) and used as a heterologous control. A single form of this truncated SU was efficiently expressed in transfected cells (Figure 3B), and abundantly secreted in cell culture medium (Figure 3C).

**HTLV-1 and -2 SU subdomains with HTLV receptor binding properties**

The amino terminal subdomains were tested for their ability to bind to HTLV receptor-presenting cells by flow cytometry. Using this cell surface binding assay, all of the soluble HTLV SU subdomains bound to the A23 hamster fibroblast cell line (Figure 4) as well as to all other cell lines tested, including 293T (human kidney fibroblasts), NIH3T3 and NIH3T3TK<sup>-</sup> (murine fibroblasts) [29], HeLa (human ovarian carcinoma cells), D17 (canine fibroblast), Jurkat (suspension human T cell line), activated primary human T cells, and numerous other cell lines and primary cell types that are thought to express the HTLV receptor. As expected from our previous work [31], none of these soluble HTLV SU subdomains showed detectable binding on resting T lymphocytes. Notably, binding of the HTLV SU to these cells occurred whether they formed or not syncytia in the presence of HTLV Env [29] and data not shown). Binding by H2<sub>178</sub>SU was similar to H2<sub>211</sub>SU, demonstrating that the first 158 residues of the mature HTLV-2 SU, without the 20 amino acids of the amino terminal signal peptide, are sufficient for cell surface binding, and therefore that the PRRH is not required for receptor binding (Figure 4A).

To determine whether cell surface binding of these soluble SU domains corresponded to *bona fide* binding to the HTLV receptor, we performed an Env-specific binding interference assay. In this assay, transfection of the above described chimeric Env and SU subdomains into 293T cells resulted in interference to cell surface binding by the soluble HA-tagged H2<sub>178</sub>SU subdomain (Figure 4B). Indeed, nearly complete interference was observed when cells were transfected with the amino terminal subdomain constructs, in the presence and absence of PRRH sequences (H1<sub>215</sub>SU and H2<sub>211</sub>SU versus H1<sub>183</sub>FEnv and H2<sub>178</sub>SU) (Figure 4B). This effect was specific as HTLV SU binding was not inhibited by a heterologous A<sub>397</sub>SU domain (Figure 4B). Therefore, we showed that the first 163 and 158 residues, with a cleaved signal peptide, of the mature HTLV-1 and HTLV-2 SU, respectively, contained



**Figure 4**  
**HTLV-1 and -2 SU subdomains interfere with HTLV Env SU cell surface binding.** (A) Conditioned medium from control 293T cells (open histograms) or from 293T cells expressing soluble rFc-tagged HTLV-1 H1<sub>215</sub>SU, HTLV-2 H2<sub>211</sub>SU and H2<sub>178</sub>SU, or Ampho-MLV A<sub>397</sub>SU subdomains (filled histograms), were incubated with A23 hamster cells for 30' at 37°C and binding was assessed by flow cytometry following addition of a secondary FITC-conjugated anti rabbit IgG antibody. Similar results were obtained in binding assays performed using all cell lines described in the text. (B) To assess binding interference, target 293T cells were transfected with the indicated Env construct and subsequently incubated with the HA-tagged H2<sub>178</sub>SU domain (filled histograms). Binding was detected by FACS following incubation with an anti HA 12CA5 mouse mAb and a FITC-conjugated anti mouse IgG antibody. Open histograms represent background levels of fluorescence. SU constructs are schematically represented below each graph by solid (HTLV), open (F-MLV) or grey (Ampho-MLV) boxes.

the entire HTLV Env RBD. These data also showed that HTLV-1 and 2 cross-interfered, consistent with the fact that they recognize the same cell surface receptor for infection [8,32].

**Interference to HTLV Env-mediated cell-to-cell fusion by HTLV SU amino terminal subdomains**

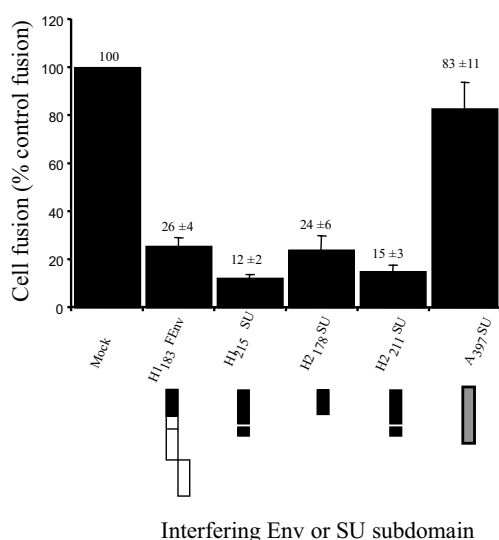
Viral envelope interference occurs when cell surface receptors are occupied by receptor-interacting Env components [33-35]. Since interference to the different Env-mediated

functions involves distinct components [27-29], we also tested the abilities of the H1<sub>183</sub>FEnv and the HTLV SU amino terminal subdomains to interfere with HTLV Env-mediated cell fusion. Interference to cell fusion was measured using a quantitative HTLV envelope cell fusion interference assay (CFIA), as previously described [9].

HTLV-1 Env-induced cell fusion was significantly diminished upon expression of the H1<sub>215</sub>SU subdomain in target cells, 12% ± 2% of control fusion (*P* < 0.001),



### Interference to HTLV Env-mediated cell fusion



**Figure 5**  
**HTLV-1 and -2 SU subdomains interfere with HTLV Env-mediated cell fusion.** Cell-to-cell fusion assays were performed by cocultivating fusogenic HTLV-1 Env-expressing cells with target cells expressing the Env derivatives indicated and schematically represented below each histogram. HTLV-1 Env-mediated cell fusion in the presence of target cells transfected with empty vector (Mock) yielded 200 to 1000 blue foci in 4 independent experiments and these levels were defined as 100% cell fusion. Cell fusion levels in the presence of HTLV SU mutants or the A<sub>397</sub>SU control Ampho-MLV SU subdomain is shown as percent of control. Mean fusion percentages were determined from three to four independent experiments. Error bars represent the standard error of the mean.

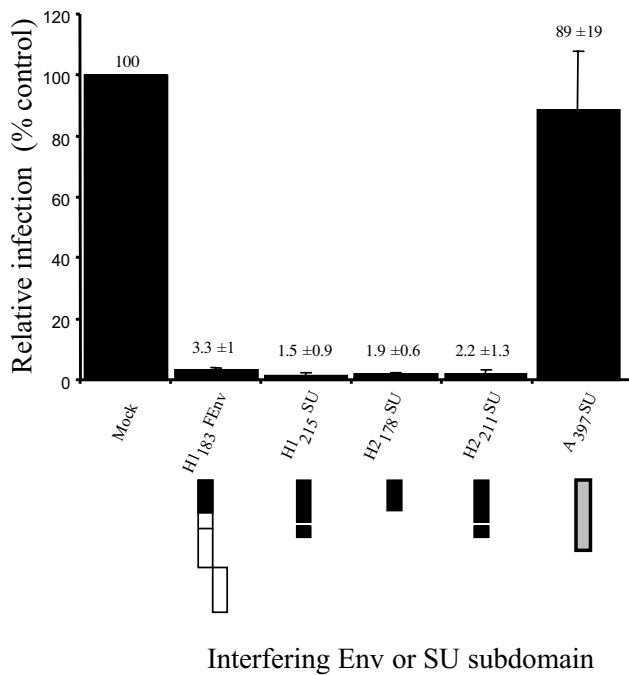
consistent with previous observations using the H1<sub>215</sub>FEnv chimera [9]. Significant interference to cell fusion was also observed with the H1<sub>183</sub>FEnv chimera, which lacked a PRRH, down to 26% ± 4% of control fusion ( $P < 0.001$ ) (Figure 5). The corresponding HTLV-2 SU subdomains produced a nearly identical cell fusion interference profile: interference by the H2<sub>211</sub>SU isolated domain, in which the PRRH was maintained, resulted in 15% ± 3% of control cell fusion levels, while the H2<sub>178</sub>SU subdomain, lacking the HTLV PRRH, inhibited HTLV-1 Env-induced cell fusion to 24% ± 6% of control levels ( $P < 0.001$ ) (Figure 5). It is noteworthy that similar data were obtained when comparing cell fusion interference by H1<sub>215</sub>FEnv and H1<sub>183</sub>FEnv. These effects were specific to

HTLV SU amino terminal domains as A<sub>397</sub>SU did not interfere with HTLV-1 Env-mediated cell fusion (83% ± 11% of control fusion) (Figure 5). Furthermore, no interference was observed when these truncated HTLV SU fragments and chimeric Env were tested against heterologous, fusogenic control Env such as AΔR Env, FΔR, XenoΔR and VSVG (data not shown). Altogether, these results confirmed our findings that receptor-binding determinants are present within the first 183 and 178 amino acids of the HTLV-1 and -2 Env, respectively. They also indicated that the PRRH (H1<sub>215</sub>SU and H2<sub>211</sub>SU), although unnecessary for receptor binding, modulates the efficiency of interference to HTLV Env-induced cell-to-cell fusion ( $P < 0.03$ ).

### Interference to HTLV Env-mediated infection by HTLV SU amino terminal subdomains

Interference, as described above, was based on the inhibition of cell-to-cell fusion induced by fusogenic Env expressed in the absence of other viral proteins. We further evaluated the abilities of the Env chimeras and soluble subdomains to specifically interfere with HTLV Env-mediated infection. HTLV Env-pseudotyped MLV virions, MLV(HTLV), were produced to infect 293T target cells. Because these recombinant cell-free virions are not competent for replication, this viral pseudotype infection assay tests a single round of infection, and does not measure replication and subsequent exponential viral dissemination. Therefore, relative infection values are expressed in linear rather than logarithmic scales.

Infection of mock-transfected target cells, devoid of interfering Env domains, resulted in a mean infection value of 9905 ± 1117 infectious units per ml (iu/ml), and this was taken as 100% control infection (Figure 6). Similar values, 8803 ± 1871 iu/ml or 89% ± 19% of control infection, were obtained upon infection of target cells expressing a heterologous SU subdomain, A<sub>397</sub>SU (Figure 6). Expression of the H1<sub>183</sub>FEnv and H1<sub>215</sub>FEnv chimeric Env in target cells significantly reduced MLV(HTLV) infection to 324 ± 98 iu/ml, 3.3% ± 1% of control infection, and to 307 ± 129 iu/ml, 3.1% ± 1.3% of control infection, respectively (Figure 6 and data not shown). Similarly, the H2<sub>178</sub>SU and H2<sub>211</sub>SU subdomains diminished MLV(HTLV) infection to 191 ± 56 iu/ml and 215 ± 122 iu/ml, 1.9% ± 0.6% and 2.2% ± 1.3% of control infection, respectively (Figure 6). The specificity of interference to infection by HTLV Env constructs was assessed by their lack of interference abilities toward Ampho-MLV Env-pseudotyped virions, MLV(Ampho) (data not shown). Thus, for both HTLV-1 and -2, the amino terminal domain upstream of the PRRH was sufficient for specific interference to HTLV Env-mediated infection. Furthermore, in contrast to the cell fusion interference assays described above, the PRRH did not detectably influence MLV(HTLV) infection.



**Figure 6**  
**HTLV-1 and -2 SU subdomains interfere with infection by HTLV envelope-pseudotyped virions.** 293T cells ( $5 \times 10^5$ ) expressing the indicated interfering Env derivatives were infected with cell-free HTLV-2 Env-pseudotyped virions MLV(HTLV) carrying a LacZ reporter gene. Infected cells were detected 2 days later by X-gal staining. Infection values are represented as percent of control infection, i.e., relative to infection of mock (pCDNA3.1) transfected target cells, calculated as infectious units per ml of virus containing supernatant (i.u./ml). Data are representative of at least three independent experiments performed in duplicate. Error bars represent the standard error of the mean.

Because HTLV dissemination appears to occur mostly via cell-to-cell contact, we also tested envelope interference to infection by HTLV-1 SU amino terminal domains using a cell-to-cell transmission interference assay. In this assay, cells harboring interfering chimeric Env and soluble subdomains were cocultured with cells producing MLV(HTLV) virions. Transfection of either chimeric Env or soluble subdomains into HeLa target cells decreased MLV(HTLV) infection to levels similar to those observed in the cell fusion interference assay presented in figure 5 (data not shown).

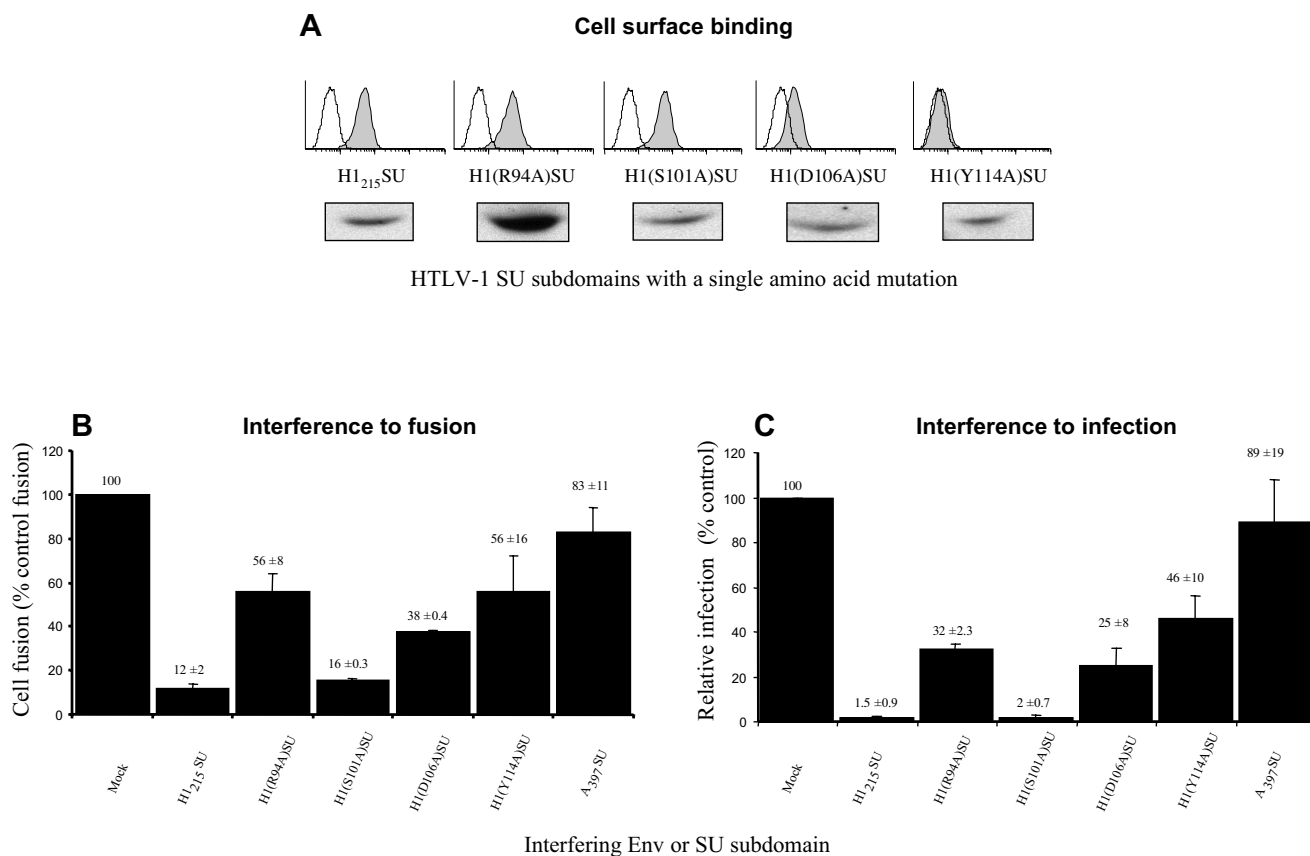
**Identification of residues within the HTLV SU amino terminal domain that modulate receptor binding and HTLV Env-mediated interference**

Two key residues contained in the HTLV SU RBD and conserved between HTLV-1 and -2, arginine 94 (Arg<sub>94</sub>) and

serine 101 (Ser<sub>101</sub>) for HTLV-1 Env which correspond to Arg<sub>90</sub> and Ser<sub>97</sub> in HTLV-2 Env, have been shown to alter cell-to-cell fusion and infection when mutated [36,37]. To determine whether mutations of these residues had an effect on receptor binding, we generated H1<sub>215</sub>SU subdomains with either Arg<sub>94</sub> or Ser<sub>101</sub> mutated to Ala, yielding the mutant H1(R94A)SU and H1(S101A)SU subdomains, respectively. We also evaluated mutations of Asp<sub>106</sub>, mutant H1(D106A)SU, and Tyr<sub>114</sub>, mutant H1(Y114A)SU, both residues found to be highly conserved between all human and simian T cell leukemia viruses (unpublished observations). Surprisingly, cell surface binding profiles of H1(R94A)SU and H1(S101A)SU mutants were not significantly altered when compared to binding by the parental H1<sub>215</sub>SU, whereas the H1(D106A)SU mutant presented reduced binding to HTLV receptor-bearing cells and the H1(Y114A)SU mutant showed a nearly complete abrogation of cell surface binding (Figure 7A). Loss of binding observed with the two latter mutants was not due to decreased soluble SU fragment production, as assessed by immunoblotting of transfected-cell culture media (Figure 7A). Moreover, equivalent binding profiles were obtained when the same mutations were introduced into the HTLV-2 soluble RBD H2<sub>178</sub>SU (data not shown). Altogether, these experiments demonstrated that Tyr<sub>114</sub> and to a lesser extent Asp<sub>106</sub> are key residues involved in HTLV Env receptor binding.

We next tested the abilities of these mutants to interfere with HTLV Env-mediated cell fusion and infection, using the assays described above. As mentioned above, all wild-type and mutant HTLV SU subdomains were produced and secreted with a similar efficiency (Figure 7A). Expression of the H1(D106A)SU and H1(Y114A)SU mutants, with decreased capacities to bind the HTLV receptor, correlated with decreased interference to HTLV Env-mediated cell fusion and infection. Indeed, H1(Y114A)SU, which had nearly undetectable level of binding, showed the lowest levels of interference and thus allowed the highest levels of HTLV Env-mediated cell fusion and infection ( $56\% \pm 16\%$  and  $46\% \pm 10\%$ , respectively) (Figure 7). Nevertheless, levels of fusion and infection were lower than that observed when the heterologous A<sub>397</sub>SU was used as a negative control of interference ( $83\% \pm 11\%$  and  $89\% \pm 19\%$  for cell fusion and infection, respectively). Thus, overexpression of mutant HTLV SU fragments with highly decreased receptor binding abilities can still exert, albeit to a significantly lesser extent, interference to HTLV Env-mediated cell fusion and infection.

We found that similar levels of interference to HTLV Env-mediated cell fusion and infection were observed when either the parental H1<sub>215</sub>SU or the mutant H1(S101A)SU were expressed in target cells (Figure 7B and 7C). This is consistent with the capacity of this mutant to bind target



### Figure 7

**HTLV-1 SU amino terminal domain mutants.** (A) H1<sub>215</sub>SU constructs were generated with the following SU amino terminal point mutations; R94A, S101A, D106A and Y114A. The abilities of these soluble H1<sub>215</sub>SU constructs to bind 293T cells were assessed by flow cytometry (gray histograms). The levels of expression of the various soluble SU subdomains are shown under each histogram. The abilities of the H1<sub>215</sub>SU mutants to interfere with (B) HTLV Env-induced cell fusion and (C) MLV(HTLV) pseudotype infection was assayed as described in Figs. 5 and 6. Data are representative of at least three independent experiments performed in duplicate. Error bars represent the standard error of the mean.

cells at levels similar to that of wild type H1<sub>215</sub>SU. However, interference to HTLV Env-mediated cell fusion and infection did not always correlate with cell surface binding profiles. While the H1(R94A)SU mutant inhibited cell fusion and infection, its effects were significantly lower than those of the wild-type H1<sub>215</sub>SU (56% ± 8% and 32% ± 2.3%, respectively) (Figure 7B,C). Thus, although neither Arg<sub>94</sub> nor Ser<sub>101</sub> of the HTLV-1 SU appears to play a direct role in binding, Arg<sub>94</sub> modulates HTLV Env-mediated fusion and infection (Figure 7), likely via post-binding effects rather than binding *per se*. In conclusion, Tyr114 appeared as the main determinant identified so far for HTLV Env binding, whereas the effects previously described with Arg<sub>94</sub> and Ser<sub>101</sub> are most likely associated with post-binding events.

### Discussion

Here, we report the generation of MLV Env with chimeric HTLV/MLV SU and truncated HTLV-1 and -2 amino terminal SU subdomains that can be expressed in and secreted from eukaryotic cell lines in functional, soluble form. Using these constructs, we demonstrated that the amino terminal 163 and 158 residues (i.e., expunged of their Env signal peptide) of the mature HTLV-1 and -2 Env SU, respectively, were sufficient to exert both HTLV receptor binding and efficient interference to diverse HTLV Env-mediated functions, including binding, cell-to-cell fusion and cell-free as well as cell-to-cell infection. Although the PRRH sequence comprising amino acid residues 180 to 215 of the HTLV-1 Env and 176 to 211 of the HTLV-2 Env was previously thought to be a receptor binding site, our

data preclude a major role for this region in the binding properties described above. Indeed, whereas a synthetic peptide composed of amino acids 197 to 216 and located within the HTLV-1 PRRH, has been reported to interfere with HTLV Env-induced syncytia formation [22], this peptide was later shown to compete neither with receptor binding of the entire HTLV-1 Env SU [38], nor with infection [26]. It is therefore likely that the effects reported for PRRH-derived peptides, as measured by syncytia formation, are solely due to post-receptor binding events. However, we identified Tyr<sub>114</sub> of the HTLV-1 Env, which corresponds to Tyr<sub>110</sub> of the HTLV-2 Env, as a key residue in HTLV Env binding and for all the aforementioned HTLV Env-mediated functional assays. We could not detect binding of H1(Y114A)SU by flow cytometry, while this mutant exerted residual, albeit significantly decreased, interference to HTLV Env-mediated cell fusion and infection. Altered folding outside of the binding domain *per se*, rather than direct alteration of the receptor-binding site, could also account for the lack of binding of this mutant. However, we favor the latter hypothesis, since the H1(Y114A)SU mutant was properly folded and transported to the plasma membrane and secreted in the medium as efficiently as wild type RBD, thus arguing against gross misfolding of this mutant. Accordingly, Tyr<sub>114</sub> appears to be conserved in all known human and simian T cell leukemia viruses strains, which share the same receptor.

The receptor-binding site in MLV RBD is composed of a combination of several cysteine loops located upstream of the PRR [11,39] which is linked to a conserved anti-parallel  $\beta$  core [13]. The isolation of an F-MLV SU amino terminal subdomain allowed crystallization of MLV RBD and the modeling of the RBD cysteine loop arrangement [13]. The precise organization of cysteine loops, likely to harbor the receptor binding determinants, within the HTLV SU amino terminus remains to be established. Nevertheless, the identification of Tyr<sub>114</sub> as a key HTLV-1 RBD residue points at this determinant as a very likely receptor-binding core. This, together with previous works relying on syncytia formation and cell-to-cell transmission [36,37], will help to distinguish between *bona fide* receptor binding determinants and determinants involved at a post-binding level.

Another recently identified determinant, the Pro-His-Gln SU motif conserved among gammaretroviruses such as MLV and feline leukemia viruses (FeLV), has been determined to play a major role in viral entry during post-binding events [40]. The mechanism of this effect involves a direct interaction of MLV SU soluble forms with Env attached SU carboxy terminus [41-46]. This interaction between the SU amino and carboxy termini leads to the T cell-restricted tropism of a natural isolate of FeLV, FeLV T,

in which the SU Pro-His-Gln motif is mutated. Indeed, FeLV T is restricted in cat to T cells because they naturally express an endogenous soluble FeLV RBD-related factor called FeLIX that trans-complements the lack of the SU Pro-His-Gln motif in the FeLV T Env and restores its post-binding defect [47]. Despite the HTLV-1 and F-MLV SU homologous modular organization and the assignment of several common motifs between the two latter SU, no obvious Pro-His-Gln motif homologue is present in the HTLV SU amino terminus. Whether a FeLIX-like molecule that interacts with HTLV Env exists in human T cells remains to be addressed. Furthermore, the fact that the Pro-His-Gln has been shown to play a major role in trans-activation of viral infection in several gammaretroviruses which are efficiently infectious as cell-free virions [42,44,48], raises the question whether the apparent lack of such a motif in the HTLV simple oncovirus-like SU is linked to the relative inefficiency of HTLV Env-mediated infection by cell-free virions. The HTLV SU subdomains described here should prove to be valuable in addressing such questions.

The recent identification of Glut1, the ubiquitous glucose transporter of vertebrates [49], as a receptor for HTLV Env [8] adds an additional similarity between the Env of HTLV, a deltaretrovirus, and that of gammaretroviruses. All these virus Env recognize multimembrane-spanning metabolite transporters [50,51]. This and the common modular organization of the HTLV and MLV SU raise questions regarding the origin of the HTLV Env. It has previously been reported that envelopes of invertebrate retroviruses may have been "captured" from other viruses [52-54]. As HTLV and MLV have strongly divergent overall genomic organizations, "envelope capture" from related ancestor genes might account for the close relationship between the Env of these phylogenetically distant viruses [10].

## Conclusions

We have generated truncated domains of the HTLV Env amino terminus, upstream of residues 183 and 178 of the HTLV-1 and -2 Env, respectively, that were sufficient to bind target cells of different species through interaction with the HTLV Env receptor. We also identified a tyrosine at position 114 and 110 in HTLV-1 and -2 Env, respectively, as a key determinant for this binding. In addition to their use for further exploration of the mechanisms involved in HTLV entry, the tagged HTLV-1 and -2 RBD subdomains described here are novel tools for the detection of Glut1 cell surface expression and intracellular trafficking. Indeed, we tracked intracellular expression of EGFP-tagged HTLV SU subdomains by time-lapse microscopy, and found that they are preferentially routed toward cell-cell contact areas (unpublished observations), where Glut1 is particularly abundant [55] and our unpublished

observations). Furthermore, those HTLV SU derivatives could be of particular importance in view of the key roles played by Glut1 in various biological processes, including T cell survival and activation [31,56], tumor genesis [57,58], and neuronal activity [59]. Interestingly, soluble HTLV SU subdomains inhibit Glut1-mediated glucose transport, and accordingly, expression of mutants with diminished receptor binding ability resulted in less pronounced inhibition [8] and data not shown). Thus, these HTLV SU derivatives could also be used as glucose transport inhibitors. These data demonstrate the potential for the novel and broad utility of these reagents in the study of HTLV infection as well as biological processes involving glucose transport and metabolism.

## Materials and methods

### Construction of chimeric Env and HTLV-1 and -2 SU subdomains

To exchange the PRR and PRRH regions, we introduced an allelic *MfeI* restriction site in the HTLV-1 and F-MLV Env. Introduction of this site in F-MLV resulted in the substitution of a glutamine and leucine (QL) dipeptide for the parental arginine and valine (RV) residues of the GPRV-PIGP motif, at the start of the MLV Env PRR. Introduction of the *MfeI* site in the PSQL motif of the HTLV-1 SU maintained the parental QL residues, at the start of the HTLV Env PRRH. By exchanging domains at the *MfeI* sites, we derived the H1<sub>183</sub>FEnv chimera containing the amino terminal 183 residues of the HTLV Env followed by the F-MLV PRR. In this chimera, the PSQL/PIGP hybrid sequence is generated at the exchange border, and the PRRH of HTLV is replaced by the F-MLV PRR (Figure 2A). In contrast, the entire PRRH of HTLV-1 is present in the H1<sub>215</sub>FEnv chimera – this Env chimera has been previously described and designated HHproFc [9]. The H1<sub>183</sub>FEnv and H1<sub>215</sub>FEnv chimeras, as well as the parental HTLV-1 and F-MLV Env, were inserted in an allelic fashion into the previously described pCEL retroviral Env expression vector [60]. The HTLV-2 Env expression vector, pCSIX/H2, was constructed by inserting the HindIII – EcoRI fragment from pHTE-2 (a gift from M-C Dokheler) encompassing the HTLV-2 *env* gene, the pX region and the 3' LTR into pCSI (CMV promoter, SV-40 intron) [61] at the HindIII and EcoRI restriction sites.

The H1<sub>215</sub>SU, H2<sub>211</sub>SU, H1<sub>179</sub>SU, and H2<sub>178</sub>SU subdomains, corresponding to the HTLV-1 and -2 SU amino terminus with and without their respective PRRH, were generated by PCR and subcloned into the pCSI expression vector as fusion proteins harboring a carboxy terminal rFc or HA tag (Figure 2B). The H1(R94A)SU, H1(S101A)SU, H1(D106A)SU, and H1(Y114A)SU substitution mutants were generated by oligonucleotide-directed PCR mutagenesis on the H1<sub>215</sub>SU vector and subcloned into the pCSI expression vector. All PCR-generated DNA fragments

were sequenced using an ABI Prism 310 sequencer. Cloning details are available upon request.

### Protein expression and immunoblots

Approximately  $5 \times 10^5$  293T cells per 35 mm well were transfected with 5  $\mu$ g of vectors using a calcium-phosphate-Hepes buffered saline (HBS) transfection protocol. Transfection medium was replaced with 3 ml of fresh culture medium twenty hours post-transfection. Forty-eight hours post-transfection cell culture medium (supernatant) was recovered and filtered through a 0.45  $\mu$ m pore-size membrane to remove cell debris. Twenty  $\mu$ l were directly analyzed by SDS-PAGE (15% polyacrylamide gel), and the rest was aliquoted and stored at -20°C for later use in binding assays (see below). Cell extracts were collected 48 h post-transfection in 1 ml of cell lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, 0.5% deoxycholate, and a cocktail of mammalian protease inhibitors [Sigma]) and clarified by two successive centrifugations at 13,000 rpm for 10 min at 4°C in a microcentrifuge. Approximately 20  $\mu$ l of each extract, adjusted after normalization for protein concentration using the Bradford assay (Sigma), were subjected to electrophoresis on SDS-15% acrylamide gels, followed by transfer onto nitrocellulose (Protran; Schleicher & Schuell). Membranes were blocked in phosphate-buffered saline (PBS) containing 5% powdered milk and 0.5% Tween 20, probed with a 1:1000 dilution of a goat anti-RLV gp70 polyclonal antibody (Viomed) followed by a horseradish peroxidase-conjugated anti-goat immunoglobulin (for detection of chimeric Env), or goat anti-rabbit-IgG-horseradish peroxidase-conjugated immunoglobulins (for detection of rFc-tagged SU subdomains). Immunoblots were subsequently washed three times with PBS-0.1% Tween 20 and revealed by chemiluminescence (ECL+, Amersham).

### Binding and binding interference assays

Binding assays were performed as previously described [31]. Briefly,  $5 \times 10^5$  target cells were detached with a PBS-EDTA solution, collected by centrifugation, incubated for 30' at 37°C with 300  $\mu$ l of rabbit Fc-tagged soluble HTLV-1, HTLV-2, or Ampho-MLV truncated SU, washed, labeled with an anti-rabbit-IgG FITC-conjugated antibody, and analyzed on a FACSCalibur (Becton Dickinson). Data analysis was performed using the CellQuest software (Becton Dickinson). For interference studies, 293T cells were transfected with 4  $\mu$ g of Env or Env SU subdomain expression vectors (carboxy terminal rFc-tagged forms) using the calcium-phosphate-HBS method. Under these conditions, transfection efficiencies ranged from approximately 80 to 90% of the target cells. Twenty-four and 48 hours post-transfection, cells were collected and transfected 293T cells expressing the different interfering HTLV or Ampho-MLV domains were incubated with a

challenging HA-tagged soluble HTLV-2 SU amino terminal subdomain (H2<sub>178</sub>SU-HA). Cells were stained using a primary 12CA5 anti HA antibody followed by an anti-mouse-IgG FITC-conjugated antibody before detection by flow cytometry.

#### **Envelope interference to cell fusion assay**

Briefly, the HTLV/MLV Env chimera, H1<sub>183</sub>FEnv, was used to interfere with challenging HTLV Env. The interfering non-fusogenic H1<sub>183</sub>FEnv and truncated HTLV SU subdomains were transiently transfected into HeLaCD4LTRLacZ, a cell line highly susceptible to HTLV Env-induced fusion that contains a stably integrated Tat-dependent LacZ expression vector [62]. These transfectants were cocultured with Tat-expressing NIH3T3(TK-) cells (NIH3T3(TK-)Tat) that were transiently transfected with the challenging HTLV Env. The NIH3T3(TK-)Tat cell line is resistant to HTLV-Env-induced syncytia formation, despite its ability to express the HTLV receptor and to bind HTLV Env, and thus can be used to precisely monitor fusion of the HeLaCD4LTRLacZ target cells [9,29]. H1<sub>183</sub>FEnv Env and truncated HTLV SU subdomains plasmid DNA (2 to 3 µg) was transfected into HeLaCD4LTRLacZ cells, while challenging, fusogenic HTLV-1 Env plasmid (1 µg) was transfected into NIH3T3(TK-)Tat. The interfering Env or SU subdomain-presenting cells were detached 24 hours post-transfection and 1–2 × 10<sup>5</sup> cells were cocultured for 24 hours with 1–2 × 10<sup>5</sup> challenging HTLV-1 Env-presenting NIH3T3(TK-)Tat cells. Subsequently, the cocultured cells were fixed and stained for β-galactosidase expression as described previously [60]. Transfection efficiencies of the HeLaCD4LTRLacZ target cells were approximately 50%. Mock transfections were performed with similar amounts of control plasmid DNAs. Env interference was measured by the decreased number of blue foci and was expressed as percent blue foci of control fusion (mock-transfected target cells). Data are represented as mean interference (± standard deviation), and statistical significance of interference levels was determined using a pairwise Student's *t* test.

#### **Envelope interference to infection assay**

MLV(Ampho) and MLV(HTLV) pseudotyped virions were produced after transfection of 10<sup>6</sup> 293T cells with 5 µg pCSI/Ampho or pCSIX/H2, respectively, 5 µg pCL/Gag-Pol [29] and 10 µg of pCLMFG-LacZ [63], using a calcium-phosphate-HBS transfection protocol. Supernatants were recovered 48 hours post transfection and filtered through 0.45 µm pore-size membrane to remove cell debris, and stored at -80°C. The pCLMFG-LacZ plasmid is a retroviral expression vector that provides a packageable RNA coding for the LacZ gene marker. pCSI/Ampho is an expression vector encoding the Ampho-MLV Env, and the HTLV-2 Env expression vector, pCSIX/H2, is described above.

Virion-containing supernatants were used to infect target 293T cells expressing the chimeric Env or HTLV RBD subdomains. Transfection efficiencies of target 293T cells were >80% in all experiments. Infections were performed 36–48 hours post-transfection on cultures grown in 12 well plates (Costar) at 37°C, medium was changed 24 hours later, and confluent cell monolayers were fixed, stained for β-galactosidase activity before counting blue foci. Interference to infection was determined by infecting transfected target cells with approximately 100 and 1000 iu. Infection was evaluated as described above, and the number of LacZ-positive blue colonies counted was normalized by multiplying by the appropriate dilution factor. The resulting infection values were analyzed as iu/ml of virus containing supernatant. Subsequently the relative infection levels in cells expressing the HTLV SU domains were compared to those of mock transfected cells and were expressed as percentages of control infection (% control).

#### **List of abbreviations used**

HTLV Human T-cell leukemia virus  
 SU envelope extracellular surface component  
 Env envelope glycoprotein  
 MLV murine leukemia virus  
 F-MLV Friend-MLV  
 RBD receptor-binding domain  
 PRR proline-rich region  
 PRRH proline rich region homologue  
 Ampho amphotropic  
 HA influenza hemagglutinin  
 rFc rabbit immunoglobulin constant fragment  
 A<sub>397</sub>SU Ampho-MLV Env fused to a carboxy terminal rFc tag  
 CFIA cell fusion interference assay  
 iu/ml infectious units per ml  
 Arg<sub>94</sub> arginine 94  
 Ser<sub>101</sub> serine 101  
 Tyr<sub>114</sub> tyrosine 114

FeLV feline leukemia viruses

HBS Hepes buffered saline

PBS phosphate-buffered saline

SDS sodium dodecyl sulfate

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

FJK designed and realized or supervised most of the experiments and co-wrote the manuscript. NM participated to some molecular constructions, set up, realized and analyzed most binding assays and FACS analyses and participated to the redaction of the manuscript. ENG set up and performed the cell-to-cell transmission assay and performed the corresponding experiments, CV constructed some of the RBD point mutants and tested them, MS initiated the project, co-participated in the design of the study, co-coordinated its realization and co-wrote the manuscript, and JLB realized some of the molecular constructs, performed some of the experiments, co-participated in the design of the study, co-coordinated its realization and co-wrote the manuscript. All authors read and approved the final manuscript.

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## Emergence of vertebrate retroviruses and envelope capture

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### Abstract

Retroviruses are members of the superfamily of retroelements, mobile genetic elements that transpose via an RNA intermediate. However, retroviruses are distinct from other retroelements in that their “transposition” is not confined to single cells but extends to neighboring cells and organisms. As such, the “transposition” of these elements is defined as infection. It appears that a key step in the conversion of a retrotransposon into a retrovirus is the modular acquisition or capture of an envelope glycoprotein (Env) which facilitates dissemination from its initial host cell. Here we present several examples of retroviruses for which envelope capture has been identified. Indeed, capture may explain the notable conservation of *env* sequences among otherwise phylogenetically distant retroviruses. In a recent example, sequence homologies reported between the *env* of the phylogenetically distant murine leukemia viruses (MLV) and human T cell leukemia viruses (HTLV) argue in favor of an *env* capture by the latter. Env acquisition can provide new adaptive properties to replication-competent viruses in addition to altering their host range. Also, the captured *env* can alter the spectrum of physiological affects of infection in new host cells and organisms. The elucidation of such envelope exchanges and properties thereof should contribute significantly to the clarification of retroviral phylogeny, insight into retroviral pathogenesis, and to the discovery of new retroviruses.

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**Keywords:** Vertebrate retroviruses; Human T cell leukemia viruses; Envelope capture

### Retrotransposons and retroviruses

#### *Genomic retroelements*

Nearly half of the human genome can be recognized as derived from transposable genetic elements, most of which are retrotransposable (Lander et al., 2001; Ostertag and Kazazian, 2001; Smit, 1999). Retrotransposition is characterized by a step in which the transposon-derived RNA is reverse-transcribed into DNA before integration into the cell genome (for review see Links 1 and 2). Among the retroelements, viral retrotransposons comprise long terminal repeat (LTR) sequences at the 5' and 3' extremities surrounding *gag-pol* coding sequences (Fig. 1). Endogenous retroviruses (ERV) are viral retrotransposons which are present in the vertebrate host germline as proviruses and, as such, are part of the host cell genomic patrimony (Link 2). A significant percentage of ERV is the vestige of ancient infections of the germline by exogenous infectious retroviruses (Benit et al.,

2001; Pavlicek et al., 2002). It has been hypothesized that replication-competent retroviruses have evolved from transposable genetic elements (Temin, 1980). The human genome contains numerous endogenous retroviruses, or HERV, distributed among at least 26 multigenic families, some comprising several hundred elements (Lower et al., 1996; Tristem, 2000) and occupying 5–8% of the genome (Lower et al., 1996; Ostertag and Kazazian, 2001; Smit, 1999). Although the majority of ERV sequences are found in the generally nontranscribed heterochromatic regions of the host genome, their direct influence in viral evolution (see below) and in gene expression has been well established (Robins and Samuelson, 1992; Samuelson et al., 1996).

#### *The envelope brings out the retrovirus*

Infectious retroviruses harbor a virus-encoded envelope glycoprotein, designated Env (Fig. 2, and see below), which is inserted into the viral envelope formed by the host cell plasma membrane. The viral Env confers properties that are essential for viral entry and the dissemination of retroviral particles (Hunter and Swanstrom, 1990). The initial steps of the retroviral infection cycle comprise assembly, budding, and release of infectious viral particles from the cell (see

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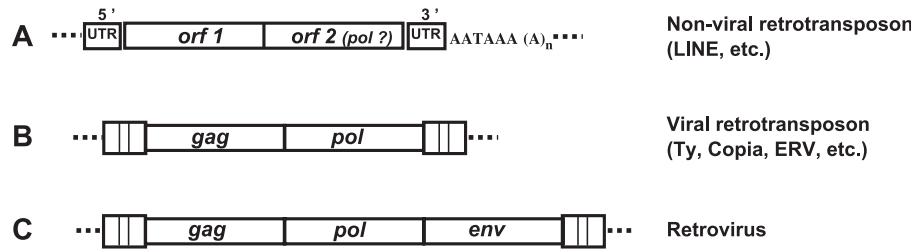


Fig. 1. Schematic representation of retrotransposons and retroviruses. (A) Nonviral (i.e., non-LTR) retrotransposons, such as LINES, containing a gag-like open reading frame (*orf*) and *pol* sequences comprising endonuclease, reverse transcriptase, and in some cases RNaseH encoding sequences; (B) viral retrotransposons (i.e., flanking, 5' and 3'-LTR) such as Ty (of *Saccharomyces cerevisiae*), Copia (of *D. melanogaster*), and certain endogenous retroviral sequences (see text); (C) retrovirus harboring the three reading frames *gag*, *pol*, and *env*.

Link 3). The Env does not appear to be essential for particle assembly, as virus-like particles (VLP) can form in the absence of Env. Furthermore, extracellular budding of *env*-less VLP is observed in the context of artificially overexpressed Gag, suggesting that the *env* is not necessary for viral egress (Garoff et al., 1998). However, in naturally expressing cells, VLP accumulation driven by Gag occurs in the cytosol or via budding into the endoplasmic reticulum or the nucleus (Fig. 3A, and see Assembly and Maturation chapter of Link 4). Adding *env* to this process can considerably alter the topology of particle expression, for example, by favoring assembly at the plasma membrane or by directing polarized, basolateral assembly of viral particles in epithelial cells (Garoff et al., 1998; Owens et al., 1991) (Fig. 3B). Moreover, we recently showed that the envelope glycoprotein of murine leukemia virus (MLV) redirects Gag and viral RNA trafficking from lysosomes to the plasma membrane and extracellular release via endosomal vesicles (Basyuk et al., 2003). This influence of *env* on vesicle routing may constitute one of the initial selective advantages leading to the formation of

*env*-containing retroviruses. Thus, the infectious properties of *env*-containing retroviruses may have developed through the cumulative acquisition of at least three *env*-dependent properties: (i) the ability to exit cells via endosomal membrane trafficking; (ii) a protective affect conferred by the cellular lipid bilayer to viral capsids that are released to extracellular environments; and (iii) the ability to disseminate to neighboring cells and organisms (Fig. 3). Splicing-dependent expression of *env* mRNA could have subsequently evolved from the large panel of alternative and cryptic splice sites that are present in the *gag-pol* sequences of simple retroviruses (Dejardin et al., 2000).

The colinear insertion of an *env* gene into the genome of retrotransposons thus appears to be a key step in the emergence of replication-competent infectious retroviruses (see Link 5). *env* capture by retrotransposons recapitulates the evolution of a sporadic trans-complementation system (Figs. 3A–B) into one of a constitutive cis-complementation (Fig. 3C). Nevertheless, viral Env's conserve their capacity for trans-complementation, as illustrated by the phenomenon

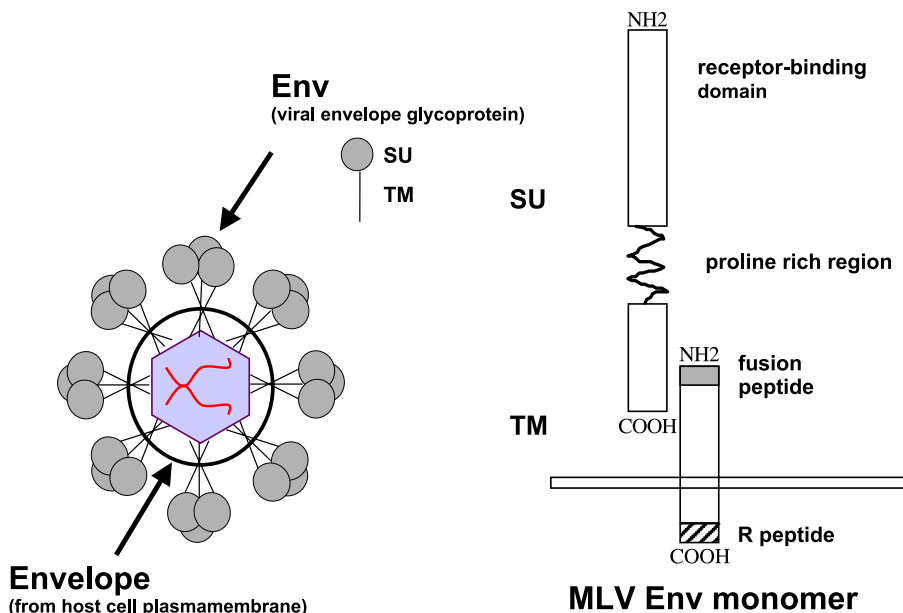


Fig. 2. Schematic representation of a virion and a retroviral Env. (Left) Env trimers anchored on the surface of a virion. (Right) Schematic representation of a murine leukemia virus (MLV) Env monomer composed of a receptor-binding-domain-containing SU and a transmembrane fusion protein TM.

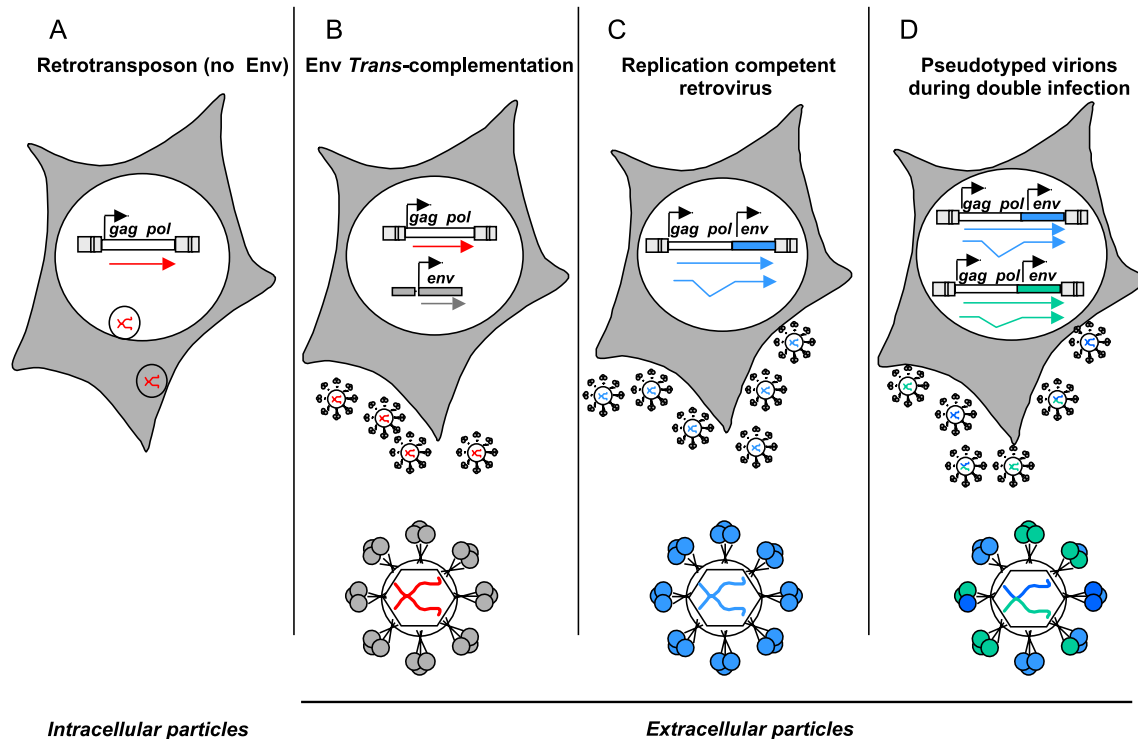


Fig. 3. Schematic diagrams of retrotransposon and retrovirus particle formation. (A) Retrotransposons without *env*. Particles form and bud within the cytoplasm or nucleus (see Link 4). (B) Virion pseudotyped by Env trans-complementation. Association of Env with forming particles allows extracellular budding of *env*-less retrotransposons or endogenous retroviruses lacking functional *env* expression. (C) Replication-competent provirus and cis-complementation by homologous Env expression. Shown are the full-length genomic RNA molecule and the singly spliced *env* mRNA. (D) Double infection resulting in Env heteromultimers and pseudotyped virion containing *env* and genomic RNA from infecting viruses. This type of phenotypic mixing broadens the host range of the resulting pseudotyped virions and can result in chimeric retroviruses.

of viral particle pseudotyping, in which the Env from one retrovirus is used by heterologous viruses during coinfection (Fig. 3D) (Sitbon et al., 1985). Pseudotyping following activation of endogenous retroviral envelope sequences may lead to extended spreading of replication-competent retroviruses or defective retroviral vectors (An et al., 2001; Bonham et al., 1997).

The evolution of retroviruses through *env* capture by retrotransposons does not preclude the coexisting reverse evolutive process, that is, the extinction of Env expression in infectious retroviruses. Such Env extinction has likely contributed to the stable insertion of exogenous retroviruses in the germ line of the infected organisms. While extinction by open reading frame mutations is commonly observed for HERV (Griffiths, 2001), the excision of an entire *env* gene remains more difficult to ascertain.

#### *Env, the viral envelope glycoprotein*

The Env of vertebrate retroviruses is encoded by a third reading frame of the retroviral genome, the *env* gene situated 3' of the viral *gag* and *pol* genes (Fig. 1). *env* mRNA is the product of the spliced viral genomic RNA (Fig. 3C) and its accumulation in the cytoplasm is dependent on cellular factors and virus-encoded proteins in the case of complex retroviruses (Cullen, 1991).

Despite their diversity, all retroviral Env-mediated viral entry comprises two principle steps: (i) binding to a cell surface receptor(s), preceding viral entry, and (ii) *env*-induced fusion of viral and cellular membranes (Weissenhorn et al., 1999). For vertebrate retroviruses, these two functions are performed by the entirely extracellular Env surface component (SU) and the membrane-anchored transmembrane component (TM), respectively. The *env* SU of vertebrate retroviruses contains receptor-binding determinants that affect viral tropism (Gallaher et al., 1995; Hunter and Swanstrom, 1990) and is one of the most variable regions of the viral genome, suggesting that it is the target of positive selective pressures (Pancino et al., 1994). The TM structure is highly conserved among the retroviruses (Fig. 2) (Benit et al., 2001; Kobe et al., 1999). Despite its characteristic variability, the SU harbors a modular organization as well as certain determinants that appear to be conserved among some highly divergent retroviruses (see below).

In addition to their role in viral infection, several Env domains influence other aspects of host physiology such as cellular membrane structure (Kozak et al., 2002), immune response (Benit et al., 2001; Wyatt and Sodroski, 1998), cell signaling (Rai et al., 2001), cell metabolism (unpublished observations), and proliferation (Kinet et al., 2002).

## env capture

### env capture by invertebrate retrotransposons

The emergence of infectious invertebrate retroviruses evidences the de novo acquisition or “capture” of *env* genes by retrotransposons. One of the best-documented examples of such capture is the *Gypsy* retrotransposon of *Drosophila melanogaster* (Pelisson et al., 2002; Terzian et al., 2001). *Gypsy* is in fact an infectious insect retrovirus (genus *errantivirus*) partly due to the capture of an *env*-like gene from baculovirus (double-stranded DNA insect virus) (Malik et al., 2000; Pearson and Rohrmann, 2002; Song et al., 1994). Further evidence of *env* capture by invertebrate retroviruses has been suggested. For example, the *env* of the nematode (*Caenorhabditis elegans*) retrovirus Cer shares homologies with a phleboviral fusion protein; and Tas, a retrovirus of *Ascaris lumbricoides*, appears to have captured the gB glycoprotein of an ancestral herpesvirus (Malik et al., 2000).

The capture of *env*-like genes as described above suggests that gene acquisition may occur via intragenomic recombination events after integration of retrotransposons into the genome of these large dsDNA viruses (Pearson and Rohrmann, 2002). Evidence in support of this capture mechanism is provided by the TED retrotransposon of the lepidopteran *Trichoplusia ni*, whose *env* gene is homologous to the baculovirus *F* gene, combined with the observation that integrated forms of TED have been found in the genome of the baculovirus ACNV (Pearson and Rohrmann, 2002). A similar mechanism may also explain the capture of herpesvirus gB sequences by an ancestral retroelement of Tas (Malik et al., 2000). Capture may not require the integration of retroelements into the genome of infecting viruses. However, according to the above examples, infection by large dsDNA viruses such as baculovirus or herpesvirus appears to provide conditions favorable to the sporadic capture of *env* by host retroelements—and the emergence of new infectious retroviruses.

### env capture by vertebrate retroviruses

The first and most thoroughly described example of the de novo capture of a genetic element by an infectious retrovirus is the acquisition of the oncogene *src* by Rous sarcoma virus (Stehelin et al., 1976). However, de novo capture of an *env* gene by an initially *env*-less vertebrate viral retrotransposon remains to be demonstrated. In contrast to the scenario of *env* capture by invertebrate retrotransposons described above, vertebrate *env* capture has been described only in terms of the acquisition of heterologous *env* sequences among different endogenous or exogenous retroviruses. This is evidenced by the presence of chimeric retroviral sequences in a broad range of animals, including chickens, mice, cats, sheep, goats, monkeys, and humans. A recent example of this phenomenon is the emergence of a highly infectious subgroup J avian retrovirus which is at

least partly due to the acquisition of a functional *env*, closely related to an endogenous sequence found in chickens (Denesvre et al., 2003).

### Cat and mouse retroviruses

Infection of both mice and cats may lead to the emergence of new retroviruses through recombination between the incoming exogenous viruses and endogenous viral sequences. The mouse mink cell focus-forming (MCF) viruses, also designated polytropic MLV, were the first retroviruses identified as resulting from such de novo recombinations following MLV infection (Fischinger et al., 1975; Hartley and Rowe, 1976). Subsequent to infection by an ecotropic MLV, acquired *mcf env* sequences broaden the cellular tropism of the parental ecotropic MLV through the acquisition of heterologous receptor-binding determinants in the *env* SU (Fan, 1997). Generation of new *env*-recombinant viruses also occurs upon infection of cats with feline leukemia viruses (FeLV). Thus, infection with the FeLV-A subtype can result in the generation of FeLV-B subtypes with markedly modified tropism (Stewart et al., 1986).

The acquisition of heterologous viral sequences, however, is not limited to *env*, and it is important to note that recombination among or with *mcf* sequences involves other regions of the MLV genome as well (Evans and Cloyd, 1985). Irrespective of the region of the viral genome, it is generally accepted that recombination between endogenous *mcf* and exogenous MLV sequences occurs after coencapsulation of heterologous genomic RNA into virus particles (Fig. 3D) (Katz and Skalka, 1990). The production and characteristics of the resulting recombinant viruses depend upon the subspecies or strain of mouse and the type of exogenous infectious virus inoculated (Chesebro et al., 1983; Lavignon et al., 1997). It is also noteworthy that the initial dissemination of these recombinant viruses depends largely on pseudotyping with *env* from the infecting, exogenous MLV (Sitbon et al., 1985) (Fig. 3D).

### Retroviral chimerism and predatory chains

Interspecies retroviral *env* capture is exemplified by the remarkable series of acquisitions that led to the formation of the endogenous feline retrovirus RD114 (Fig. 4). Moreover, emergence of this chimeric virus evokes a scenario of infection during predatory exchanges among feline and primate species. As presented in Fig. 4, at least four distinct retroviruses are implicated in the emergence of RD114: baboon endogenous retrovirus (BaEV), *Papio cynocephalus* endogenous virus (PcEV), simian endogenous retrovirus (SERV), and *Felis catus* endogenous virus (FcEV). BaEV is a replication-competent endogenous retrovirus (Benveniste et al., 1974) found in several simian species including mandrills, baboons, mangabeys, and African green monkeys (van der Kuyl et al., 1995). BaEV is a chimeric retrovirus composed of type C *gag* and *pol* sequences

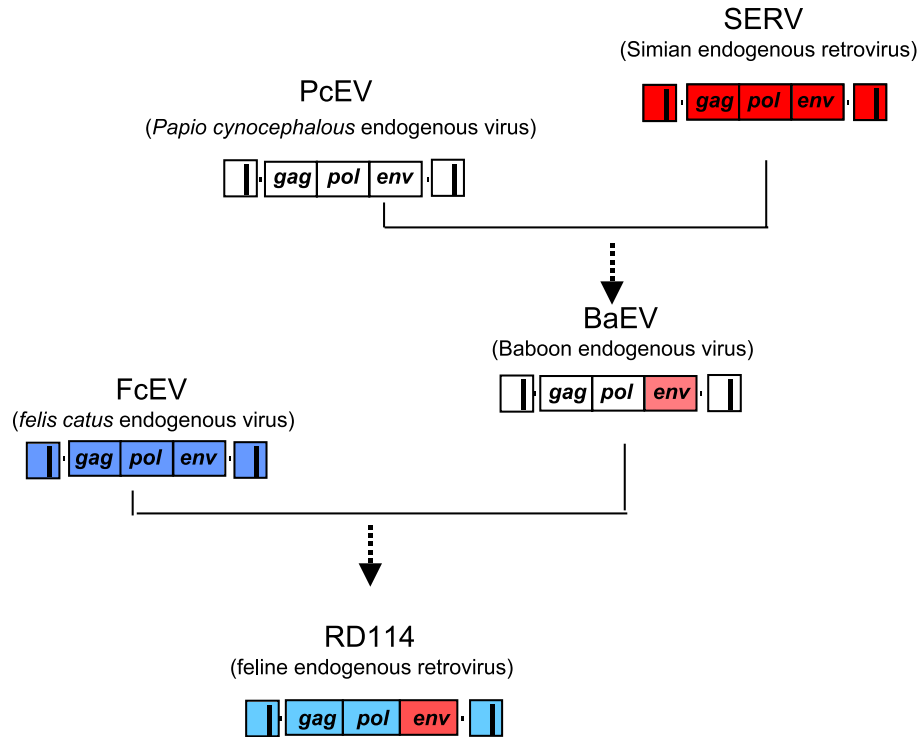


Fig. 4. Schematic representation of *env* captures leading to RD114. RD114 is a chimeric retrovirus composed of *gag-pol* genes derived from *Felis catus* endogenous retrovirus (FcEV) and an *env* gene derived from baboon endogenous retrovirus (BaEV). The emergence of RD114 is the result of a chain of recombination events involving at least two distinct *env* captures, as outlined from top to bottom of the diagram. The Env of RD114 enables this feline retrovirus to infect primate cells, including human cells (van der Kuyl et al., 1999). Different shades are used to illustrate closely related but not identical sequences.

originating from another endogenous baboon retrovirus, PcEV, and the *env* of a type D simian endogenous retrovirus, SERV (van der Kuyl et al., 1999). The emergence of RD114 is thus explained by at least two *env* acquisition events: the capture of the SERV *env* by PcEV, to produce BaEV; and the capture of the BaEV *env* by FcEV, that resulted in the emergence of RD114 (van der Kuyl et al., 1999) (Fig. 4).

The type C gibbon ape leukemia virus (GaLV) may reveal another cascade of *env* captures resulting from murine–feline–primate predatory relationships. According to its genomic organization and sequence, GaLV is highly related to MLV, and it has been suggested that the origin of GaLV may be traced to an ancient infection of monkeys by a xenotropic murine retrovirus (Wolgamot et al., 1998 and references therein). However, the Env SU of GaLV binds the same cell surface receptor as the FeLV-B feline leukemia virus (Takeuchi et al., 1992). Based on these data, one possible scenario for the emergence of GaLV could involve a successive transmission of viral sequences. A murine retrovirus exhibiting an interspecies host range emerged (possibly an MLV-like virus generated through MCF sequence acquisition as described above) and infected feline predators, resulting in the emergence of infectious feline retroviruses such as FeLV-A (see above). Subsequently, *env* recombinant retroviruses, such as FeLV-B, may have then been generated and disseminated from their feline host to

predatory primates (or through the inverse predatory relationship) to form GaLV.

In addition to scenarios implicating infection after blood exchange during predation, examples of chimeric retroviruses involving animals with no apparent predatory relationship have also been described. For example, the ovine or caprine retrovirus Jaagsiekte retrovirus (JSRV), the etiologic agent of ovine pulmonary adenocarcinoma, is a type D virus with an *env* derived from a type B virus (York et al., 1992). Related type D or B chimeric viral sequences have been described not only in sheep and goats, but also in other ungulates, including the vast majority of the genus *ovis* and genus *capra*, as well as in domestic cattle, suggesting an ancient *env* capture and interspecies dissemination of chimeric viruses (Hecht et al., 1996).

*Highly divergent human and murine retroviruses share homologous env*

Recent comparative analysis of the *env* of the human T-cell leukemia or lymphoma virus (HTLV) and the murine leukemia virus (MLV) demonstrated that these phylogenetically highly divergent viruses (Tristem, 2000) share remarkable homologies of modular organization and motifs within the *env* SU that are usually particularly variable even among viruses of the same species. Thus, we showed that the amino acid sequence LLTLVQ in the *env* SU of HTLV-1



and LLNLVQ of Friend-MLV delimits homologous and interchangeable functional domains (Fig. 5 and Kim et al., 2000, and submitted for publication).

Such SU close homology and motif conservations within the genomes of otherwise highly divergent retroviruses suggest a common ancestral origin of the HTLV and MLV *env* and are compatible with a scenario of *env* capture by HTLV (Kim et al., 2000, 2003, and submitted for publication). The routes of interspecies transmission and the acquisition of new sequences by ancestors to these viruses may be deduced by precise phylogenetic analyses of *env* sequences of HTLV, its simian homologues STLV, and other simian, feline, and murine C-type retroviruses. Such analyses and the identification of yet to be identified HTLV-related endogenous sequences, *env* or other, in human genomes may help elucidate an apparently ubiquitous phenomenon of recombinatory sequence acquisition by retroviruses.

#### The origin(s) of *env*?

The acquisition and recombinatorial exchange of heterologous *env* is well documented. However, the origin of the *env* itself remains enigmatic. The general structure of the SU receptor binding domain associates it with the immunoglobulin superfamily (Fass et al., 1997), and the membrane fusion capacity of *env* TM naturally implies a common origin with cellular fusion proteins and membrane proteins involved in membrane trafficking (Poumbourios et al., 1999; Weissenhorn et al., 1999). As such, one possible scenario suggests

that *env* genes could have formed de novo by stochastic recombination of distinct protein coding sequences (Lerat and Capy, 1999; Lerat et al., 1999), possibly via mechanisms such as exon shuffling (Boeke and Pickeral, 1999; Pavlicek et al., 2002). The remarkable conservation of TM among highly divergent viruses, in contrast to the SU, suggests the possibility of modular acquisition of SU and TM as distinct domains (Benit et al., 2001; Kobe et al., 1999). Thus ancestral TM-harboring highly conserved functional domains such as the fusion peptide, immunodominant and immunosuppressive peptides (Gallagher et al., 1995; Pancino et al., 1994), and the highly conserved coiled-coiled extracellular domain (Kobe et al., 1999) may have acquired SU of various origins. This scenario is supported by experiments demonstrating that functional, truncated receptor-binding domains of the SU can be expressed independent of other *env* domains (Battini et al., 1995; Kim et al., submitted for publication; Manel et al., 2002), and that *env* function can be reconstituted by complementing defective *env* with these independently expressed, soluble SU domains (Barnett and Cunningham, 2001; Lavillette et al., 2000).

In addition to the role of Env in driving viral infection, endogenous *env* sequences can also exert cellular functions (Blond et al., 2000; Britt et al., 1984; Frendo et al., 2003; Kattstrom et al., 1989; Mi et al., 2000) or confer either resistance or increased susceptibility to infection and pathogenesis (Anderson et al., 2000; Chesebro et al., 1983; Ikeda and Sugimura, 1989). A major focus of current research is to determine causal relationships between endogenous retroviral sequences and idiopathic pathologies including certain

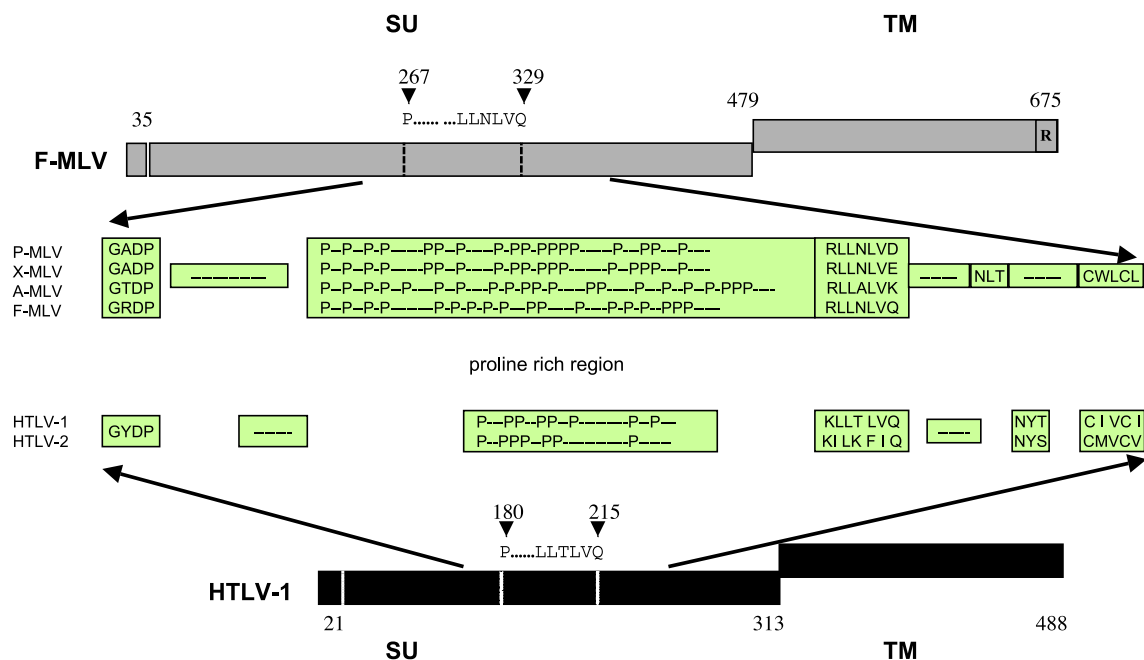


Fig. 5. Organization and sequence homologies of the SU of MLV and HTLV *env* (Kim et al., 2000, and submitted for publication). Key homologous regions are indicated for prototype polytropic (P-MLV), xenotropic (X-MLV), amphotropic (A-MLV), Friend ecotropic (F-MLV) murine leukemia virus, and HTLV-1 and -2. Variable amino acid residues among the indicated blocks of homology are represented by dashed lines. The proline residues (P) of the “proline rich region” are shown and the other amino acid residues within this domain are represented by dashed lines.

leukemias, and neurodegenerative and autoimmune diseases (Lower, 1999; Perron and Seigneurin, 1999; Portis, 2002; Power, 2001).

## Conclusion

Retroviruses and retroelements in general constitute a continuously evolving catalog of sequences driving the production of new viruses. Retroviral envelope “capture” appears to be a recurrent phenomenon in the generation of new infectious retroviral species. At least three general mechanisms can be at the origin of such a new insertion in a retroviral genome: (i) recombination after coencapsidation of heterologous retroviral genomes (Katz and Skalka, 1990), or cellular mRNA as it is generally believed for oncogene “capture”; (ii) intragenomic exon shuffling of retroelements (Boeke and Pickeral, 1999; Pavlicek et al., 2002); (iii) recombination after integration of retrotransposons or retroviruses into the genome of large dsDNA viruses such as herpes or baculovirus (Isfort et al., 1992; Malik et al., 2000; Pearson and Rohrmann, 2002).

These recombination events illustrate the permanent co-evolution of retrotransposons and retroviruses with their host organisms. As a key element in the dissemination of retroelements to new hosts, *env* capture and the consequent dissemination of new retroviruses may be considered a deleterious event as generally perceived; however, it may also be considered a fundamental component in the evolution of genomes.

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Table and nomenclature of retroelements (<http://www.ncbi.nlm.nih.gov:80/books/bv.fcgi?call=bv.View.ShowSection&rid=rv.table.3431>).

Properties of retroelements (<http://www.ncbi.nlm.nih.gov:80/books/bv.fcgi?call=bv.View.ShowSection&rid=rv.section.3525>).

Budding of retroviral particles (<http://www.ncbi.nlm.nih.gov:80/books/bv.fcgi?call=bv.View.ShowSection&rid=rv.section.2497>).

Assembly and maturation of retroelements (<http://www.ncbi.nlm.nih.gov:80/books/bv.fcgi?call=bv.View.ShowSection&rid=rv.chapter.2494>).

Modular acquisition of retroviral genes (<http://www.ncbi.nlm.nih.gov:80/books/bv.fcgi?call=bv.View.ShowSection&rid=rv.section.3542>).

> Les rétro-éléments constituent un moteur de la diversité du monde vivant du fait de leurs propriétés de transposition. Parmi eux se distinguent les rétrovirus, dont la «transposition» se propage en une véritable infection de la cellule initiale aux cellules et organismes voisins. Une étape clé de la conversion d'un rétrotransposon en agent infectieux est l'acquisition d'une glycoprotéine d'enveloppe, désignée Env. Nous présentons ici différents exemples où a pu être retracée cette «capture d'enveloppe», qui a conduit à la constitution de rétrovirus infectieux de mammifères, et montrons qu'un tel événement de capture s'est probablement produit dans le cas de la protéine Env du rétrovirus humain HTLV. Ces captures expliqueraient l'étroite parenté de la protéine Env de différents rétrovirus pourtant phylogéniquement distants. L'élucidation des diverses « étymologies » rétrovirales devrait aider à mieux comprendre la phylogénie des rétrovirus et la physiopathologie des infections rétrovirales, voire mener à la découverte de nouveaux rétro-éléments.

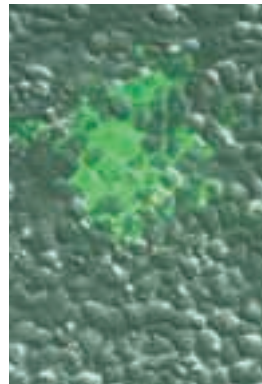
## Rétrotransposons et rétrovirus

### Rétro-éléments et génomes

La transposition des rétro-éléments [1] nécessite la transcription inverse d'un ARN en ADN, qui s'intègre alors dans le génome cellulaire. Ces rétrotransposons sont abondants et participent directement au phénotype de l'hôte [2] : ils ont ainsi accompagné la spéciation de l'homme, chez lequel les séquences rétrovirales endogènes (HERV) représentent 5% à 8% du génome [3, 4]. Comme les rétrovirus, les rétro-éléments de type viral portent des séquences *gag-pol* encadrées de longues séquences répétées (LTR, *long terminal repeat*) (Figure 1). En revanche, ces rétro-éléments ne codent généralement pas pour des glycoprotéines virales d'enveloppe (Env) et ne sont habituellement pas capables de «dissémination» extracellulaire.

# La capture d'enveloppe par les rétrovirus

Felix . Kim, Nicolas Manel, Jean-Luc Battini, Marc Sitbon



### Les rétrovirus, des rétrotransposons bien enveloppés ?

L'enveloppe rétrovirale arrachée aux membranes cellulaires lors du bourgeonnement de la particule porte la gly-

coprotéine virale Env. L'adjonction d'Env permet à la particule de disséminer à l'extérieur de la cellule en apportant deux fonctions principales : la liaison à un récepteur cellulaire par sa composante de surface (SU) et la fusion aux membranes cellulaires par sa composante transmembranaire TM (Figure 2). Si des particules rétrovirales extracellulaires peuvent être produites abondamment en l'absence de la protéine Env, par surexpression des protéines Gag et Pol, les particules endogènes naturelles dépourvues d'Env ne sont généralement pas relarguées à l'extérieur en l'absence de cette surexpression artificielle, mais s'accumulent à l'intérieur de la cellule (Figure 3A) (voir aussi [5], chapitre *Assembly and maturation*). En revanche, l'ajout d'Env favorise l'assemblage de virions à la membrane plasmique et polarise la production virale à l'extérieur de cellules épithéliales [6, 7] (Figure 3B). Il a d'ailleurs été montré que la protéine Env déroutait l'ARN et les virions de la voie lysosomiale vers la voie endosomiale d'export extracellulaire [8]. Ces propriétés suggèrent que l'émergence de rétrovirus à partir de rétrotransposons aurait bénéficié du routage extracellulaire des particules par Env (Figure 3A) et de l'effet protecteur d'une enveloppe dérivée de la membrane cellulaire.

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Sur la base de ces avantages sélectifs, la «capture d'enveloppe» par un rétrotransposon récapitulerait l'évolution d'un effet sporadique de *trans*-complémentation (Figure 3B) en une *cis*-complémentation constitutive (Figure 3C) donnant au rétrovirus ainsi constitué de nouvelles capacités infectieuses, tropiques et, parfois, pathogènes. L'Env capturée par un rétrovirus reste susceptible d'être utilisée *in trans* par d'autres rétrovirus ou par des rétro-éléments défectueux (pseudotypage<sup>1</sup>) (Figure 3D) [9].

## Rétrotransposons et capture de gènes *env*

### Rétrotransposons d'invertébrés aux *env* venues d'ailleurs

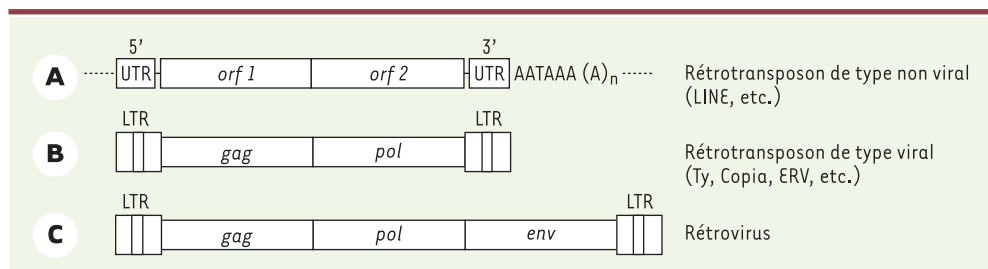
De récents travaux ont fourni des indices précis sur la «capture d'enveloppe» par les rétro-éléments de type viral d'invertébrés. Ainsi, les rétrovirus du groupe Gypsy de *Drosophila melanogaster*, ainsi que d'autres rétro-

<sup>1</sup> Le pseudotypage est l'incorporation d'une enveloppe hétérologue par une particule virale, conférant à ce virus le tropisme de l'enveloppe hétérologue.

transposons apparentés (*errantivirus*), sont infectieux [10] du fait de la présence du gène *Id130* codant pour une protéine Env analogue à la protéine F des baculovirus (virus d'insectes à grand génome ADN double brin) [11, 12]; de même, l'Env des rétrovirus Cer du nématode *Caenorhabditis elegans* est apparentée à une protéine des phlébovirus (virus à ARN simple brin); enfin, le rétrovirus Tas du ver *Ascaris lumbricoides* semblerait avoir capturé une Env apparentée à la glycoprotéine gB d'un herpes virus (virus à ADN double brin) [11].

Des indications supplémentaires sur un mécanisme éventuel de capture sont fournies par le rétrotransposon TED du lépidoptère *Trichoplusia ni*, dont le gène *env* est également apparenté au gène *Id130* du baculovirus. Des formes intégrées de TED ont été retrouvées dans le

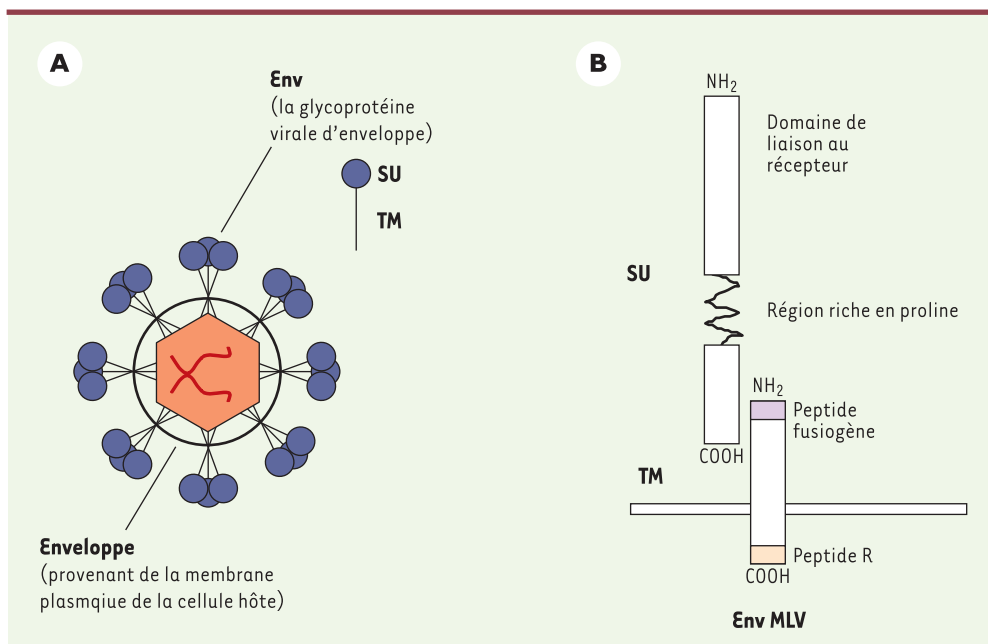
**Figure 1. Principaux rétrotransposons.** **A.** Rétrotransposon de type non viral, tel que les LINE (*long interspersed nuclear element*), dépourvu de LTR (*long terminal repeat*) et contenant deux cadres de lecture

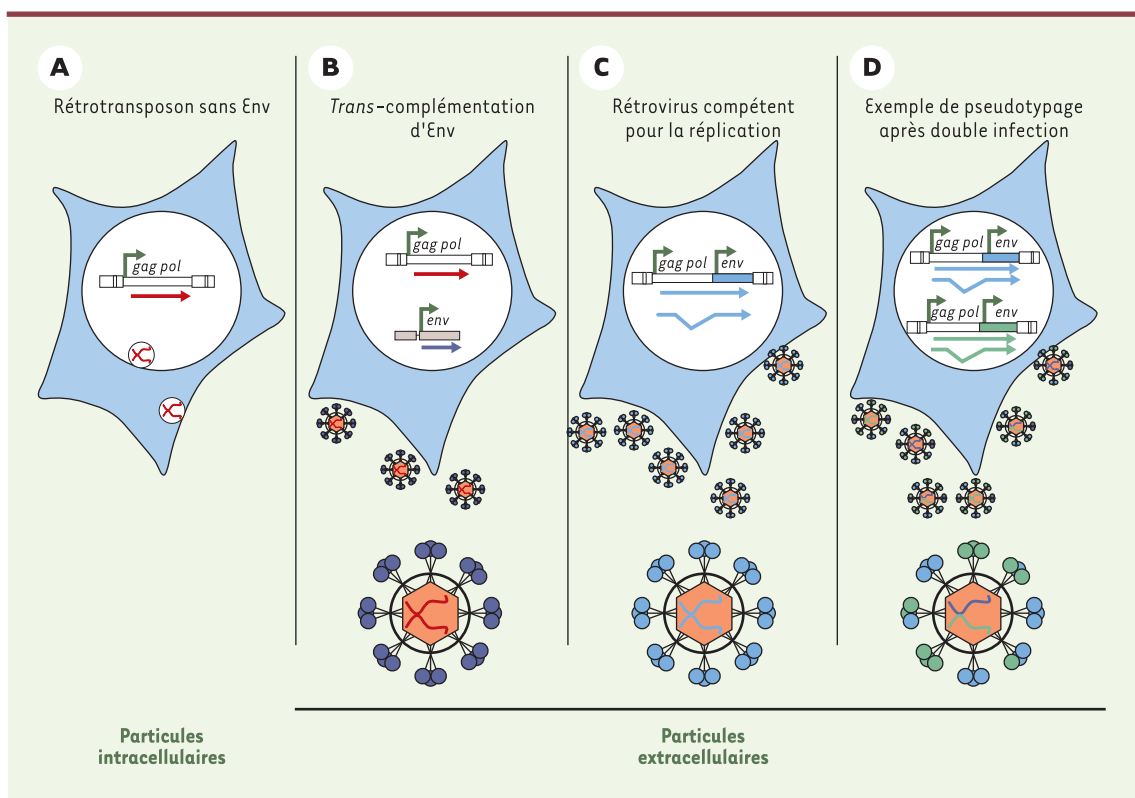


ouverts (ORF1 et ORF2, *open reading frame 1 and 2*). L'ORF 1 code pour des protéines de type Gag, tandis que l'ORF 2 porte les séquences codant pour les protéase, transcriptase inverse, RNase H et intégrase caractéristiques des rétrovirus. **B.** Rétrotransposon de type viral délimité par des LTR, mais dépourvu du gène *env*, tel que les rétrotransposons Ty de levure, Copia de drosophile et certaines séquences endogènes rétrovirales (ERV). **C.** Rétrovirus infectieux, de type exogène ou endogène, arborant les trois cadres de lecture *gag*, *pol* et *env* [5].

### Figure 2. rganisation de l'enveloppe rétrovirale.

**A.** Trimères de molécules d'Env à la surface d'un virion et monomère isolé, avec ses deux composantes de surface SU et transmembranaire TM. **B.** Organisation en modules de la protéine Env prototype des MLV de souris et de celle, apparentée, du rétrovirus humain HTLV. Une molécule d'Env comprend deux sous-unités issues du clivage d'une seule protéine précurseur: la sous-unité de surface (SU), entièrement extracellulaire, et la sous-unité d'ancrage transmembranaire (TM); sont représentés le domaine de liaison au récepteur et la région riche en prolines de la sous-unité SU, ainsi que le peptide fusogène aminoterminal et le peptide R carboxyterminal de la sous-unité TM impliqués dans la fusion membranaire [14, 20, 21].





**Figure 3. Voies de production de particules rétrotransposables.** **A.** Rétrotransposons dénués du gène *Env*, et donc de la glycoprotéine d'enveloppe *Env*: les particules bourgeonnent à l'intérieur de la cellule [5]. **B.** Pseudotypage d'*Env* par complémentation en *trans*: cette complémentation par une *Env* hétérologue permet aux particules en formation d'acquérir une protéine *Env* et, ainsi, de bourgeonner à l'extérieur de la cellule; sont représentés les gènes *gag* et *pol* du rétrotransposon et un gène *env* hétérologue. **C.** Complémentation d'*Env* en *cis* dans le cas d'un rétrovirus entier: sont représentés le provirus entier et les ARN dérivés génomique entier et épissé *env*. **D.** Coproduction d'*Env* hétérologues: cas de deux rétrovirus produisant des pseudotypes d'*Env* et un génome rétroviral hétérodimère. Une variante de ce schéma est qu'une des deux *Env* provient d'un rétrovirus endogène défectueux; ce pseudotypage étend le spectre d'infection de la particule.

génomique du baculovirus AcNV (*Autographa californica nucleopolyhedrosis virus*). La capture d'un gène *env* par le rétro-élément ancêtre de Gypsy et de TED a donc pu se produire dans une cellule par recombinaison d'un rétrotransposon (moins de 10 kb) intégré dans l'ADN d'un baculovirus (plus de 100 kb) [12].

L'infection par un virus exogène ADN à grand génome, baculovirus ou herpès virus, pourrait donc, sporadiquement, fournir les conditions favorables à la capture d'*env* par un rétro-élément et à l'émergence de nouveaux rétrovirus chez les invertébrés.

### Captures d'*env* chez les rétrotransposons de vertébrés

Chez les vertébrés, l'acquisition de l'oncogène *src* par le virus aviaire du sarcome de Rous a été la première description de capture d'un gène hétérologue par un rétrovirus. L'origine des *Env* des rétrovirus de vertébrés reste cependant inconnue et l'acquisition *de novo* d'un gène *env* n'est pas documentée. Seule la «capture» de séquences *env* par recombinaison avec d'autres séquences *env* rétrovirales hétérologues a été décrite. Cependant, des rétrovirus aviaires ont été retrouvés intégrés

dans un herpès virus [13]: par analogie avec ce qui a été décrit pour les rétrovirus d'invertébrés, cette capacité d'un rétrovirus à s'intégrer dans le génome d'un virus à grand génome ADN pourrait constituer une étape propice à la capture d'*env*.

### Des chats, des souris et des rétrovirus

Le modèle murin fournit de nombreux exemples du rôle des rétro-éléments dans l'évolution des génomes et dans la production de nouveaux rétrovirus. Ainsi, les virus leucémogènes murins MLV-MCF (*murine leukemia virus-mink cell focus-forming virus*), également appelés MLV polytropes, et MLV 10A1 apparaissent à la suite de recombinaisons entre un MLV infectieux et des séquences rétrovirales endogènes de type *env*. Ces séquences élargissent le tropisme du MLV par l'acquisition de capacités de liaison à de nouveaux récepteurs (pour revue, voir [14]). Toutefois, la dissémination *in vivo* de ces virus recombinants dépend paradoxalement aussi de leur pseudotypage par l'*Env* du MLV infectieux [9] (Figure 3D). Un processus similaire de recombinaison en cours d'infection a été identifié chez les rétrovirus leu-

cémogènes de chat (FeLV), où la capture d'*env* par un FeLV de type A produit un nouveau rétrovirus, FeLV B, de tropisme différent [15].

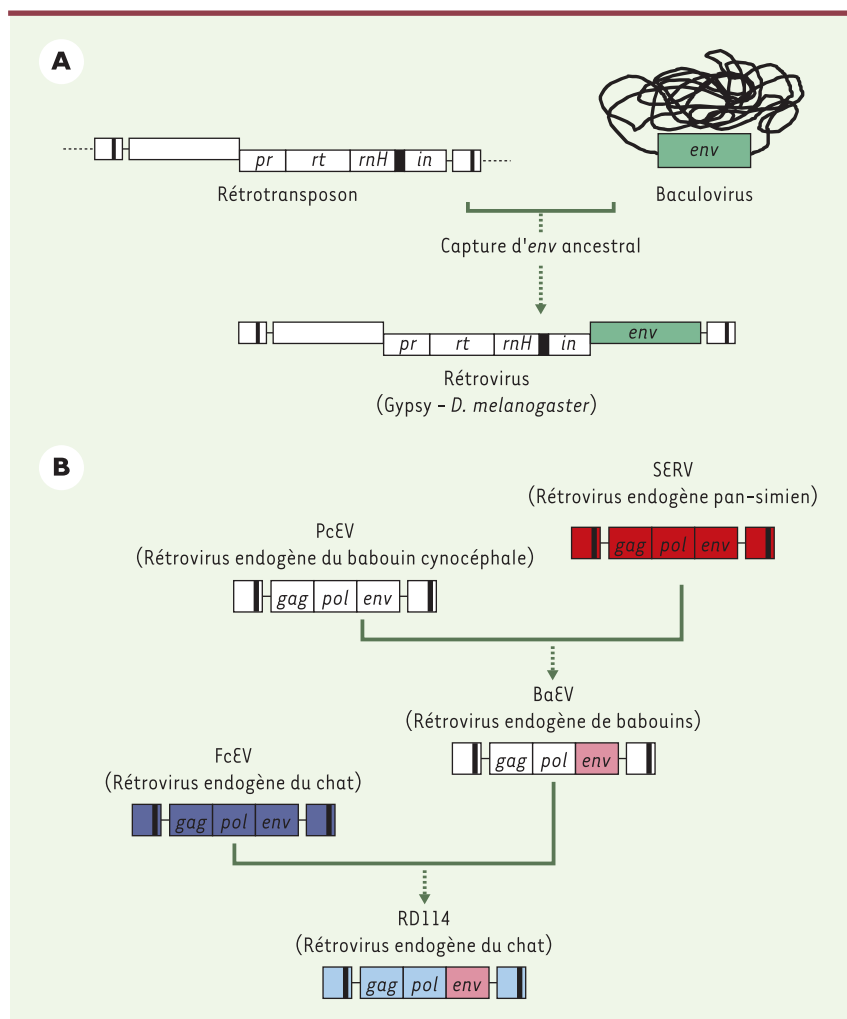
### Gènes *env*, chaînes de prédation et infections inter-espèces

Un des exemples retraçant le mieux la capture d'*Env* par un rétrovirus de vertébrés est l'échange d'*env* entre des rétrovirus de babouin et de chat (Figure 4). BaEV (*baboon endogenous virus*) est un virus endogène infectieux retrouvé dans plusieurs familles de singes (mandrills, babouins, mangabeyes et singes verts africains). BaEV est composé des gènes *gag* et *pol* d'un rétrovirus endogène de type C du babouin *Papio cynocephalus* (PcEV) et d'un gène *env* du virus endogène simien (SERV) de type D [16]. Ce même BaEV est impliqué dans l'émergence du virus infectieux endogène félin RD114. Ce dernier est un rétrovirus chimère

composé du gène *env* du BaEV type D et des gènes *gag-pol* de type C provenant du virus endogène de chat FcEV (*felis catus endogenous virus*) [16]. À l'origine du RD114 félin existent donc au moins deux événements de capture d'*env* par recombinaison: la capture du gène *env* de SERV par PcEV, qui aurait permis l'émergence du BaEV, et la capture du gène *env* de BaEV par FcEV, donnant naissance au rétrovirus RD114. On peut raisonnablement penser que ces infections inter-espèces prennent place lors de contaminations sanguines occasionnées par des rapports de prédation.

Une cascade de captures d'enveloppe est peut-être aussi à l'origine de l'émergence du GaLV (*gibbon ape leukemia virus*), rétrovirus de type C du singe gibbon asiatique. En effet, l'*Env* du GaLV est apparentée aux *Env* murine (du MLV) et féline (du FeLV). Sur la base de cette parenté étroite, il est tentant d'établir le schéma d'une transmission successive de ces séquences rétrovirales suivant la voie de prédation ascendante la plus probable, souris-chat-primat. Cette transmission horizontale mettrait en jeu des rétrovirus exogènes de souris (MLV écotropes) capturant des *env* rétrovirales endogènes de souris, de type « xénotropes », capables ainsi d'élargir leur tropisme à d'autres espèces [14, 17]. Cela leur permettrait d'infecter leurs prédateurs félins, entraînant ainsi l'émergence de nouveaux rétrovirus exogènes félins tels que FeLV-A. De nouveaux rétrovirus félins recombinants d'enveloppe, tels que le FeLV-B, pourraient passer au prédateur primat et y favoriser l'émergence de virus tels que GaLV.

On peut donc attendre une parenté étroite entre gènes rétroviraux provenant d'espèces phylogénétiquement éloignées, mais reliées par une « chaîne alimentaire ». De tels échanges de rétrovirus ne dépendraient pas exclusivement de relations de prédation, mais utiliseraient aussi d'autres voies d'échanges entre espèces partageant le même habitat. Ainsi, des recombinaisons au niveau des gènes *env* ont été décrites entre des rétrovirus de différentes espèces de mammifères pourtant strictement herbivores. Le rétrovirus ovin/caprin JSRV (*Jaagsiekte retrovirus*), responsable de l'adénocarci-



**Figure 4. Captures d'*env*.** A. Capture *de novo* d'un gène *env* par un rétrotransposon d'invertébré, conduisant à la formation du rétrovirus répliatif ancestral Gypsy de drosophile [11]. B. Acquisition séquentielle d'un gène *env* de rétrovirus de primates par le rétrovirus endogène félin RD114. Cette capture d'une *Env* de virus de type BaEV par le rétrovirus endogène de chat FcEV survient après des recombinaisons entre les virus endogènes simiens SERV et PcEV [16]. L'enveloppe du RD114 dérivée de cette capture permet l'infection efficace de cellules de primates, y compris humain [45] (d'après [46]).

nome pulmonaire ovin, porte ainsi des séquences *gag* de type D et le gène *env* d'un virus de type B [18]. Des séquences chimériques de type B et D, apparentées à celle de JSRV, sont présentes non seulement dans le génome du mouton, mais également chez d'autres ongulés, dont la quasi-totalité du genre *capra* et les vaches domestiquées [19], suggérant une ancienne capture d'*env* et une dissémination inter-espèce.

### Le V commun de virus leucémogènes murins et humain

Lors de nos travaux sur l'enveloppe du rétrovirus de la leucémie humaine HTLV (*human T-cell leukemia/lymphoma virus*), nous rapportions que, bien que les MLV et HTLV soient phylogénétiquement très éloignés sur la base du gène *pol*, leurs composants de surface SU présentaient des blocs d'homologies remarquables. Un bloc, en particulier, est relativement conservé entre HTLV et MLV, délimité en aval chez les MLV de Friend et HTLV-1 par les résidus VQ avec, respectivement, les séquences RLLNLVQ et KLLTLVQ; les domaines de SU délimités par ces séquences sont fonctionnellement interchangeables entre HTLV-1 et MLV de Friend [20, 21]. Homologies et conservations de motifs d'Env au sein des génomes MLV et HTLV, par ailleurs très divergents, évoquent la capture d'un gène *env* ancestral commun. L'analyse phylogénétique de ces séquences devrait fournir de nouveaux indices sur la parenté entre les HTLV, leurs équivalents simiens STLV et les rétrovirus de type C simiens, félins et murins. Des voies de transmission inter-espèces et d'acquisition de nouvelles séquences par les ancêtres de ces virus, par ailleurs très distants, pourraient également être élucidées. Une autre question est celle de la présence de séquences endogènes, *env* ou autres, apparentées aux HTLV, au sein des génomes de primates, et de leur rôle éventuel dans la physiologie et la physiopathologie humaine.

### Des *env* et des génomes

Si l'origine de l'enveloppe de certains rétrovirus d'invertébrés est bien établie et repousse la question aux origines mêmes de ces séquences hétérologues, l'origine des Env rétrovirales de vertébrés reste plus énigmatique. Du fait des propriétés fusogènes des Env, une recherche de parenté entre les glycoprotéines Env et les partenaires cellulaires de la fusion ou de l'endocytose membranaire reste privilégiée. Toutefois, les propriétés de la composante de surface SU permettent également d'envisager une parenté avec des gènes codant pour des ligands de récepteurs membranaires (immunoglobulines, cytokines...).

Ces deux origines pourraient être conciliées dans le cadre de la formation d'Env par recombinaison stochastique de domaines distincts des protéines [22, 23], tel que cela est décrit, par exemple, dans le mécanisme de brassage des exons [24-26]. La plus forte conservation, au sein des enveloppes rétrovirales, des séquences TM par rapport aux séquences SU, suggère une acquisition modulaire en deux temps, au moins [27]. À une TM ancestrale bardée de domaines conservés (tels que peptides fusogène, immunodominant et immunosuppresseur [28, 29]) se

serait arrimée une SU d'origine plus variable. Cela est étayé par la possibilité de produire des domaines isolés de SU reconnaissant le récepteur [20, 30, 31]: ces domaines tronqués de la SU nous ont d'ailleurs permis d'identifier le récepteur de la protéine Env de l'HTLV [32, 33]. Il a également été montré que des fonctions de la protéine Env impliquées dans l'entrée virale pouvaient être reconstituées par un domaine de reconnaissance du récepteur soluble, physiquement dissocié du reste de l'Env [34]. En ce qui concerne le maintien d'un grand nombre de séquences endogènes *env* dans les génomes de vertébrés, des fonctions physiologiques essentielles ont été suggérées pour certaines enveloppes endogènes [35-37], ainsi que des fonctions vaccinales potentielles (pour revue, voir [14]). À l'inverse, une Env endogène peut aussi faciliter une infection virale. Ainsi, une SU tronquée d'Env endogène de FeLV sert de corécepteur cellulaire lors de l'infection par un isolat thymotrope de FeLV [38]. L'abondance de rétrovirus chimères naturels et l'altération du spectre d'hôte qui découle de la capture d'*env* présagent de l'influence permanente des interactions entre rétro-éléments endogènes et exogènes. Cette influence se manifeste par la production de nouveaux rétrovirus et leur impact dans certaines maladies, mais aussi par leur impact sur l'évolution des génomes, du fait de leur capacité à passer dans la lignée germinale de leurs hôtes. Ainsi, l'émergence récente chez les rétrovirus aviaires du sous-groupe J fortement infectieux est au moins en partie due à l'acquisition d'une séquence *env* fonctionnellement apparentée à une séquence endogène du poulet [39].

### Conclusions

Les événements de « capture » d'un gène *env* sont susceptibles de se produire par au moins trois mécanismes de recombinaison: après co-encapsidation de génomes rétroviraux hétérologues [40], par brassage intragénomique d'exons par les rétro-éléments [24, 26] et, enfin, après intégration de rétrotransposons ou de rétrovirus dans le génome de virus à ADN tels que les baculovirus ou les virus de type herpes [11, 12].

Ces recombinaisons témoignent de la co-évolution étroite, permanente, des rétrotransposons et des rétrovirus avec les organismes hôtes. L'infection rétrovirale peut aussi être considérée comme l'« introduction d'[une] espèce dans un écosystème: en fonction des avantages sélectifs, la nouvelle espèce peut s'installer de manière stable et permettre un nouvel état de l'ensemble. L'espèce peut aussi s'éteindre ou au contraire proliférer et/ou provoquer un déséquilibre mortel » [41]. Un des axes actuel de recherche est d'établir des liens entre Env rétrovirales et différentes maladies idiopathiques, dont certaines leucémies, maladies dégénératives et syndromes auto-immuns [42-44]. Cependant, bien que la capture d'*env*, en tant qu'élément clé de la dissémination à de nouveaux organismes, puisse effectivement constituer un événement dont l'impact est délétère, elle reste aussi un acteur essentiel de l'évolution des génomes. ♦



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## SUMMAR

### Envelope capture by retroviruses

Retroelement transposition is a major source of diversity in genome evolution. Among the retrotransposable elements, the retroviruses are distinct in that their « transposition » extends from their initial host cells to neighboring cells and organisms. A determining step in the conversion of a retrotransposable element into an infectious retrovirus is the acquisition of an envelope glycoprotein, designated Env. Here, we review some examples of envelope « capture » by mammal retroviruses and provide evidence for such a mechanism by HTLV. This phenomenon may explain the notable conservation of env genes observed between phylogenetically distant retroviruses. Elucidation of these recombination processes should help to clarify retroviral phylogeny, better understand retroviral pathogenesis, and may lead to the identification of new retroelements. ♦

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TIRÉS PART  
M. Sitbon



calcium dans le segment externe qui ont le pouvoir d'induire l'apoptose.

### Le rôle pathogène de l'opsine non liée

Ainsi, les photorécepteurs des souris *Rpe65*<sup>-/-</sup> sont soumis à un phénomène ambigu: du fait de la très faible quantité de rhodopsine, ils sont protégés contre les fortes expositions de lumière, mais l'activité de l'opsine, correspondant à une exposition faible en permanence, entraîne une dégénérescence irréversible. Le phénomène est d'autant plus intéressant que les souris *Rpe65*<sup>-/-</sup> sont aussi un modèle pour la carence en vitamine A dans laquelle les bâtonnets, pri-

vés de rétinale mais contenant de l'opsine, subissent aussi une dégénérescence.

C'est la première fois qu'un tel mécanisme physiopathologique est démontré en pathologie humaine: en l'absence de mutation, un trouble fonctionnel d'un récepteur couplé à une protéine G est capable de produire des lésions. Il sera intéressant de rechercher si d'autres récepteurs couplés à des protéines G peuvent également être à l'origine de maladies humaines. ♦

**Spontaneous activity of opsin, one of the causes of Leber congenital amaurosis**

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### NOUVELLE

## GLUT-1 est le récepteur des rétrovirus humains HTLV

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fusion de la TM et son ancrage dans la membrane plasmique de la cellule cible. Si l'organisation modulaire de nombreuses Env rétrovirales et l'identité de certains récepteurs ont été élucidées, les régions

> HTLV (pour *human T-cell leukemia virus*) est le premier rétrovirus identifié chez l'homme [1]. Depuis, deux types, HTLV-1 et 2 ont été identifiés. On estime à plus de 20 millions le nombre de personnes infectées par les HTLV dans le monde, avec des foyers endémiques de forte prévalence sur tous les continents en dehors des populations caucasoïdes chez lesquelles les infections restent sporadiques. HTLV se transmet par voie sanguine et sexuelle et, semble-t-il plus communément, de la mère à l'enfant au cours de l'allaitement. Deux maladies sont associées à HTLV-1: le lymphome à cellules T de l'adulte (ATL) et la paraparésie spastique tropicale appelée aussi myélopathie associée au HTLV (TSP/HAM) [2]. Moins de 5% des personnes séropositives pour HTLV-1 développent une ATL, généralement plusieurs décennies après l'infection, et moins de 1% dévelop-

pent une TSP/HAM. D'autres syndromes sont également associés aux HTLV. Les mécanismes sous-jacents de ces effets pathogènes ne sont pas encore élucidés. Comme tous les rétrovirus, HTLV s'adsorbe à la surface de la cellule cible puis le contenu nucléoprotéique viral est introduit dans le cytoplasme après la fusion des membranes virale et cellulaire (Figure 1A). Cette fusion se produit après interaction de la glycoprotéine virale d'enveloppe (Env) avec des composants membranaires cellulaires faisant office de récepteurs. Chaque molécule d'Env comprend deux sous-unités issues du clivage d'une seule protéine précurseur: la sous-unité de surface (SU) entièrement extracellulaire et la sous-unité d'ancrage transmembranaire TM (Figure 1B). L'interaction Env/récepteur est accompagnée de changements conformationnels qui entraînent le démasquage du peptide de

de l'Env du HTLV qui interagissent avec la cellule cible et la nature des récepteurs cellulaires restaient mystérieuses. Cette ignorance est en partie due à l'expression ubiquitaire du récepteur HTLV sur toutes les cellules de mammifères en culture; à la faible efficacité des infections HTLV réalisées à partir de préparations virales acellulaires, la transmission semblant nécessiter un contact direct entre cellule infectée et cellule cible; enfin, la production d'une Env entière est cytotoxique empêchant toute étude cytologique appropriée.

En comparant les séquences primaires des Env des virus leucémogènes murins (MLV), de type simple, avec celles des HTLV, de type complexe (voir Encadré), nous avons mis en évidence des homologies inattendues au niveau de leurs SU [3, 4]. Sur la base de ces homologies, et sachant que le domaine de liaison au récepteur (DLR) de l'Env MLV avait été localisé précisément



dans la partie amino-terminale de la SU [5], nous avons réussi à dériver des Env chimères HTLV/MLV fonctionnelles, capables de reconnaître le récepteur HTLV [3]. Celles-ci nous ont permis de délimiter et de produire un module de 159 acides aminés amino-terminaux de la SU de HTLV (H<sub>DLR</sub>) et de montrer que ce module est nécessaire et suffisant pour la liaison au récepteur [6]. Produit sous forme soluble, lié à une étiquette fluorescente (Figure 1B), ce domaine de liaison au récepteur de l'Env HTLV n'est pas cytotoxique, nous offrant ainsi un outil de choix pour évaluer l'expression du récepteur du HTLV par cytométrie de flux ou par microscopie (Figure 1C).

### Récepteur HTLV, activation lymphocytaire et dissémination virale *in vivo*

L'action pathogène principale de HTLV-1 étant une ATL, nous avons évalué l'expression du récepteur HTLV à la surface des lymphocytes T. De façon inattendue, nous avons observé que les cellules T fraîchement isolées de donneurs sains, qui sont en phase G<sub>0</sub> du cycle cellulaire, n'expriment pas le récepteur HTLV. Au contraire, les cellules T activées par un antigène ou *in vitro* par des anticorps dirigés contre le TCR (récepteur des lymphocytes T), expriment de façon transitoire le récepteur HTLV à leur surface, démontrant que l'expression de ce récepteur dépend directement de l'état d'activation des cellules T [7]. Après leur infection par HTLV, et leur retour en quiescence, les lymphocytes peuvent constituer un réservoir *in vivo* du virus, compte tenu de leur très longue durée de vie (>20 ans). Ces données nous permettent également de spéculer que la transmission du HTLV puisse avoir lieu essentiellement dans les endroits où résident les cellules activées, c'est-à-dire les sites inflammatoires et les organes lymphoïdes secondaires.

Les modalités de la transmission préférentielle mère-enfant du HTLV ne sont pas connues. Or les lymphocytes T présents dans le sang de cordon, récents émigrants du thymus, présentent des caractéristiques d'activation distinctes de celles des cellules T périphériques de l'adulte. Une fois

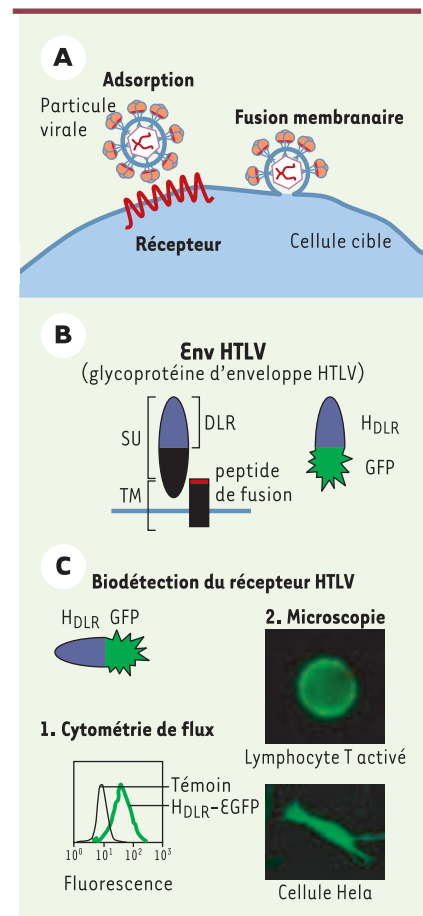
activées par l'IL-7, ces cellules, contrairement aux lymphocytes T de l'adulte [8, 9], expriment le récepteur HTLV, les rendant ainsi théoriquement susceptibles à l'infection par HTLV [7]. Cette sensibilité à l'IL-7 des cellules T naïves pourrait ainsi expliquer la transmission préférentielle du virus HTLV de la mère infectée à l'enfant, même en l'absence de stimulation antigénique.

### Identification du récepteur HTLV et impact sur le métabolisme cellulaire

La majorité des récepteurs rétroviraux furent identifiés au cours des vingt dernières années par une stratégie de clonage d'expression qui n'était pas envisageable dans le cas du récepteur HTLV car toutes les lignées cellulaires cultivées *in vitro* présentent ce récepteur à leur surface.

Tous les récepteurs des Env des différentes classes de MLV déjà identifiés ainsi que ceux des Env de rétrovirus apparentés de mammifères font partie de la superfamille des transporteurs de métabolites à plusieurs domaines transmembranaires [10]. Ainsi, ont été identifiés comme récepteurs deux transporteurs de phosphate inorganique, les transporteurs d'acides aminés cationiques et d'acides aminés neutres, ainsi que le transporteur du myo-inositol. L'homologie observée entre les SU des Env MLV et HTLV nous a conduits à émettre l'hypothèse selon laquelle le récepteur HTLV pourrait lui aussi être un membre de cette superfamille de transporteurs. Les cultures *in vitro* de cellules de vertébrés s'accompagnent généralement d'un changement de couleur du milieu de culture dû à une acidification révélée par l'indicateur colorimétrique rouge phénol; de couleur rouge, il vire au jaune pour un pH inférieur à 6,2 et au rouge pourpre pour un pH supérieur à 8. Au cours de nos travaux, nous avons remarqué que l'expression de Env bloquait ce changement de couleur. L'expression du H<sub>DLR</sub> suffisait à produire cet effet, qui était abrogé par l'expression de H<sub>DLR</sub> mutants incapables de lier le récepteur HTLV. L'acidification des milieux de culture est majoritairement due à la sécrétion par les cellules de protons et de lactate. Ce dernier, catabolite de la glycolyse anaérobie,

provient de la consommation de glucose à partir du milieu extracellulaire. Or l'absence d'acidification en présence de Env était corrélée à un blocage du transport de



**Figure 1. Infection cellulaire par HTLV.** A. Les premières étapes d'une infection rétrovirale. La glycoprotéine d'Env est responsable de l'interaction avec un récepteur à la surface cellulaire (étape d'adsorption), conduisant à la fusion des membranes et à l'infection virale. B. Organisation des glycoprotéines d'enveloppe du HTLV. Les glycoprotéines d'enveloppe (Env) sont constituées d'une sous-unité de surface totalement extracellulaire (SU) qui contient le domaine de liaison au récepteur (DLR) et d'une sous-unité TM d'ancrage transmembranaire qui porte le peptide inducteur de la fusion des membranes. C. Utilisation du H<sub>DLR</sub> comme marqueur du récepteur HTLV. Le DLR de HTLV, fusionné par exemple à la protéine fluorescente GFP (*green fluorescent protein*), sert d'outil comme marqueur du récepteur HTLV par cytométrie de flux ou par microscopie. DLR: domaine de liaison au récepteur.

glucose depuis le milieu extracellulaire vers l'intérieur de la cellule (Figure 2).

Le transporteur de glucose ubiquitaire des lignées cellulaires est GLUT-1 (*glucose transporter 1*). Parmi les 13 membres de la superfamille des GLUT, il est le seul à partager *in vivo* les caractéristiques d'expression du récepteur HTLV, en particulier il est indétectable sur les cellules T au repos et fortement exprimé au cours de l'activation. Par des mesures de cytométrie de flux et par co-immunoprécipitation nous avons montré que GLUT-1 interagit avec le H<sub>D</sub>LR. Afin d'évaluer le rôle de GLUT-1 dans l'infection par HTLV, nous avons développé trois systèmes expérimentaux: blocage de GLUT-1 par la cytochalasine B, un inhibiteur du transport du glucose; blocage du récepteur HTLV après introduction d'une Env, et levée de ce blocage par introduction de l'ADNc codant pour GLUT-1 [11]; inhibition spécifique de l'expression de GLUT-1 à l'aide d'ARN interférent (ARNi). L'ensemble de ces différents tests nous ont permis de conclure que GLUT-1 est capable de relayer une infection rétrovirale via l'Env du HTLV [12]. GLUT-1 est donc un nouveau membre de la famille des transporteurs de métabo-

lites qui sert de récepteur pour un rétrovirus. Par extension, il devient tentant de considérer les rétrovirus comme une réelle source de métabolites pour la cellule. Cependant, il n'existe encore pas d'explication formelle démontrant que les récepteurs rétroviraux ont été sélectionnés sur la base de leur fonction de transporteur. Notamment, il est possible qu'une propriété commune aux transporteurs de métabolites, mais indépendante de la fonction de transport *per se*, ait été sélectionnée: localisation, environnement lipidique, trafic vésiculaire [4, 6, 13]. Au-delà de l'interaction Env/récepteur, nos résultats suggèrent que l'environnement métabolique des cellules cibles conditionne fortement le cycle de réplication des rétrovirus. ♦

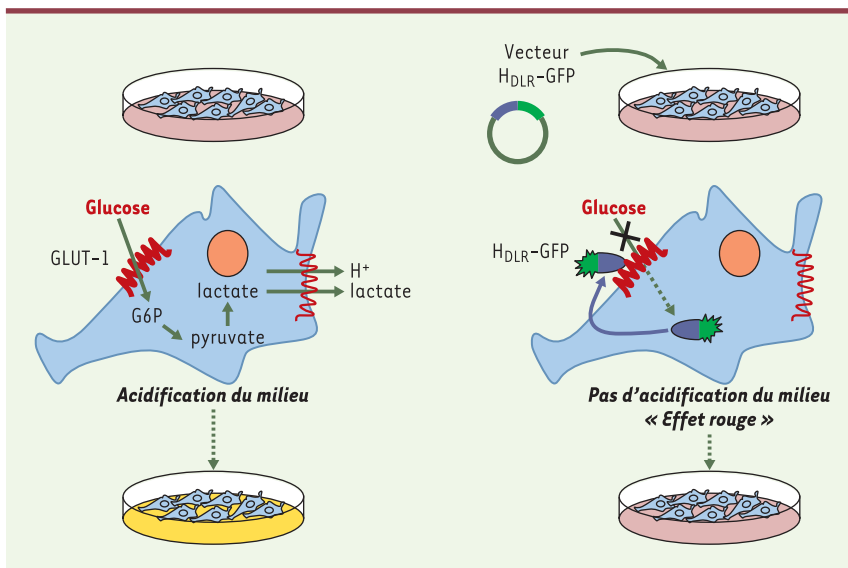
### GLUT-1 is the receptor of retrovirus HTLV

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Le génome des virus HTLV, comme celui de tous les rétrovirus, contient deux copies identiques d'une molécule d'ARN longue d'environ 9 000 nucléotides portant les gènes des protéines de capsid (*gag*), des enzymes virales (*pol*) et d'enveloppe (*env*). L'ARN du virus HTLV, comme celui du virus de l'immunodéficience humaine (VIH), code également pour des protéines dites régulatrices issues de diverses combinaisons d'épissages doubles de l'ARN viral qui sont caractéristiques des rétrovirus de type complexe. Au sein des virions, les deux molécules d'ARN sont associées à la transcriptase inverse dans un cœur protéique enveloppé par une bicouche lipidique dans laquelle sont ancrées les glycoprotéines virales d'enveloppe ou Env.

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**Figure 2. Blocage du transport du glucose via le transporteur ubiquitaire GLUT-1, par le DLR du HTLV.** Les cellules de vertébrés en culture fermentent le glucose extracellulaire en lactate, ce qui conduit à une acidification du milieu de culture (jaune). Lorsque le H<sub>D</sub>LR est exprimé dans ces cellules, l'import du glucose facilité par GLUT-1 est bloqué entraînant une chute de la fermentation, de la production du lactate et de l'acidification du milieu extracellulaire qui reste rougeâtre. DLR: domaine de liaison au récepteur.

# HTLV-1 tropism and envelope receptor

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**The identification of CD4 as the primary receptor for HIV followed shortly after the discovery of the virus, but the HTLV receptor remained long elusive, until its recent identification as the GLUT1 glucose transporter. In the present review, we describe the status of the literature that surrounded this discovery as well as the *in vitro* and *in vivo* observations that led to the identification of GLUT1. Also, we will explore a few tracks to conciliate the *in vitro* and *in vivo* data on HTLV-1 tropism within the context of the HTLV literature that has accumulated over the past 25 years. A close examination of these data leads us to conclude that the preferential detection of HTLV-1 in CD4+ T lymphocyte subsets *in vivo*, even in the absence of leukemia, is not likely to be directly related to envelope receptor interactions, but rather to an array of postentry selection bottlenecks *in vivo*. Furthermore, we propose that infection of other hematopoietic and nonhematopoietic cells is likely to take place during the lifetime of an individual, with a burst early during the infection.**

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## Envelope, receptor and tropism : a few definitions

### *Viral envelope versus Env glycoproteins*

The final step of virion release from retrovirus-infected cells consists of the coating of viral cores that contain the viral-encoded enzymes and the dimeric RNA viral genome surrounded by capsid proteins. This step occurs through budding and egress of viral particles at the cell surface and provides virions with an envelope that is constituted by a plasma membrane-derived lipid bilayer enriched in viral envelope glycoproteins (Env).

As is the case for all infectious retroviruses, HTLV Env derives from an envelope polyprotein precursor that is cleaved by cellular proteases yielding a mature Env that is inserted into the viral envelope (Figure 1). Env is composed of a surface amino terminal component (SU) and a transmembrane carboxy terminal component (TM). SU is entirely extracellular and in the case of

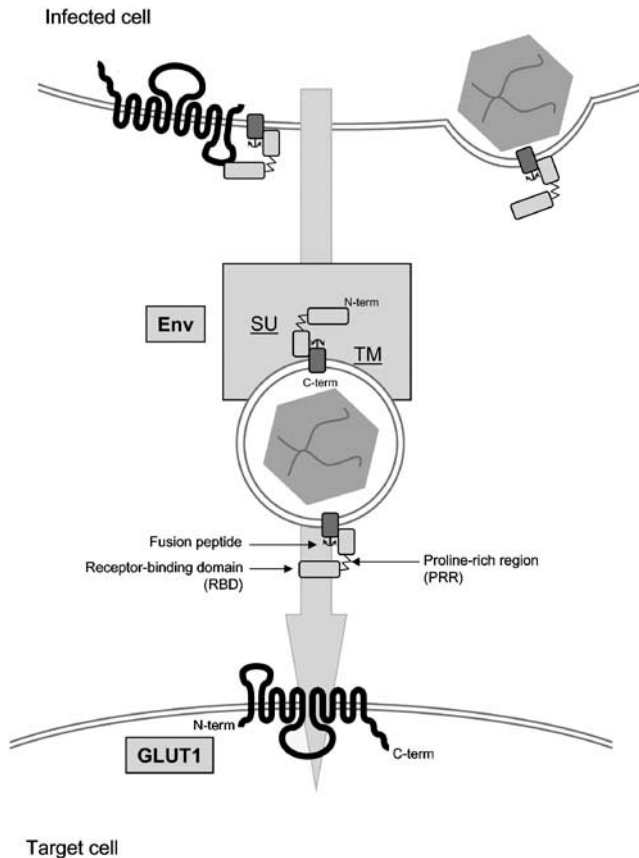
the prototypic MT-2 strain of HTLV-1, it is composed of 288 amino acids (not including the 20 amino-acid signal peptide that is cleaved during Env translation) with 4 N-glycosylation sites (Delamarre *et al.*, 1996) and an apparent mass of 46 kDa (gp46). SU is associated, most likely via labile disulfide bonds, to the membrane-anchored TM which is 180 amino acid long with one N-glycosylation site and an apparent mass of 21 kDa (gp21). Like all retroviral TM, HTLV-1 TMgp21 harbors an amino terminal fusion peptide (Figure 1).

Productive retrovirus infections, leading to the formation of viral progeny as opposed to nonproductive cell attachment and entry, appear to proceed only via interactions between virion-harbored Env molecules and specific cellular receptors present at the cell surface. It is generally accepted that those receptors, leading to either pH-independent direct fusion of the viral envelope at the plasma membrane or pH-dependent fusion within endocytic vesicles, constitute selective ports of entry for infectious particles. In the case of nonlentiviral mammalian retroviruses, virus entry has been thoroughly studied in different models, including mouse and feline leukemia viruses (MLV, FeLV). For these latter viruses, productive Env-mediated entry can be schematically described as the binding of an amino terminal receptor binding domain (RBD) in the SU to a cell surface receptor, leading to conformational changes that unmask the TM fusion peptide and initiate a membrane fusion process.

### *Receptors versus viral entry receptors*

Contacts between viruses and cells also occur outside of the *bona fide* Env-receptor interactions that lead to productive viral replication. In the absence of either viral Env or a receptor for viral entry, virions are likely to attach to cells and enter before degradation in phagosome-like structures (Marechal *et al.*, 1998, 2001). The crossing of the mucosal epithelial barrier by HIV has been proposed as another example of retrovirus entry into a cell wherein it will not replicate. Virions traverse from the cell apical lumen side to the basolateral side by transcytosis, before release to heterologous cells (Bomsel, 1997). Transcytosis occurs via an interaction between Env and glycosphingolipids that are harbored by the epithelial cells (Hocini and Bomsel, 1999). Another physiologically important situation that does not seem to depend on binding to the

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**Figure 1** Cell-to-cell transmission of HTLV. HTLV particles bud from GLUT1-positive infected cells in areas of the cell membrane enriched for the viral envelope glycoprotein (Env). HTLV particles bind to GLUT1 on target cells via the Env receptor-binding domain (RBD) located in the SU. Binding to GLUT1 is most likely followed by conformational changes that involve the proline-rich region (PRR), between the SU RBD and carboxy terminus (C-term), leading to the unmasking of the fusion peptide located in the Env transmembrane component (TM) and penetration of the viral core in the cytoplasm

receptor for virus entry involves dendritic cells (DC). DC can be used by several pathogens to escape immune surveillance but can also serve as carriers from the primary exposed mucosal site to target organs (Ludwig *et al.*, 2004). The transport of HIV virions by DC and their subsequent presentation to lymphoid targets seems to require Env-mediated virion binding to a DC-specific lectin, the intercellular adhesion molecule (ICAM)-3 grabbing non-integrin (DC-SIGN) (Geijtenbeek and van Kooyk, 2003; Steinman *et al.*, 2003). Nevertheless, transmission to lymphocytes several days after the initial exposure of DC to HIV requires Env-mediated virus entry and minimal viral replication (Nobile *et al.*, 2005). Similarly, both the integrin lymphocyte function-associated antigen 1 (LFA-1), via its interaction with ICAM-1 incorporated in HIV envelope (Fortin *et al.*, 1997), and the heparan sulfate proteoglycan syndecan, via a direct interaction with the HIV-1 SU (gp120) (Bobardt *et al.*, 2003), have been shown to favor adsorption of

HIV and SIV particles and enhance transmission to target cells.

Whether the nonproductive engulfing or transport of virions, described for HIV and other retroviral as well as nonretroviral pathogens, play a role in HTLV infection remains unknown. Nevertheless, it is important to note that the examples depicted above involve cell receptors that preferentially interact with virions, in an Env-dependent or independent manner. These receptors should be distinguished from cell receptors that mediate viral entry and production of a new viral progeny.

#### Cell type-specific pathogenesis versus tropism

Viral tropism is regularly used to describe cell populations that either express viral products or are subjected to a cytopathic viral effect. In the absence of further precision, those uses may be misleading. From a strictly virological perspective, viral tropism refers to all cell types in which virus entry leads to the production of viral progeny, or cell types that maintain this production potential with 'dormant' viruses. As for all retroviruses, this is of particular importance in the context of HTLV, due to the stable integration of the viral genome in infected cells including T lymphocytes, whose *in vivo* lifespan may exceed a decade (Muul *et al.*, 2003). Pathogenic effects are thus additional properties, that obviously depend directly on cell tropism but that, strictly speaking, do not participate in its definition.

Env binding to a cell surface receptor is the first hurdle for the establishment of an infection, and as such Env/receptor binding constitutes a cornerstone of viral tropism. However, completion of the retroviral replication cycle also depends on numerous postbinding events and cell factors whose influence can drastically impact on infection outcome and hence tropism, independent of cell surface receptors. One classical example of a postbinding factor limiting cell tropism has been described for the T-cell tropic strain of the mammalian feline leukemia retrovirus (FeLV-T), whose Env receptor is the inorganic phosphate transporter PiT1 (see Table 1). Briefly, infection by FeLV-T is restricted to cells expressing the cat T-cell-encoded FeLIX glycoprotein, and as such the virus productively infects only T lymphocytes *in vivo* (Anderson *et al.*, 2000), although PiT1 is expressed in most tissues. Another block in the retroviral cycle has been elucidated due to the inability of HIV to replicate in mouse cells that are made fully permissive to viral entry. The block in this case is located after integration and caused by the inability of the mouse cyclin T1 to form a functional complex with HIV Tat (Bieniasz *et al.*, 1998). Several other preintegration and postbinding/post-entry blocks limiting retroviral tropism have been identified for several retroviruses (Nisole and Saib, 2004). Nevertheless, although envelope-independent restriction factors have been shown to play a role in the low infectivity of cell-free HTLV-1 virions (Derse *et al.*, 2001), the identities of such factors have yet to be elucidated.

The apparent *in vivo* tropism of a virus can be drastically skewed by the variables of a natural

**Table 1** Cellular receptors for mammalian gammaretroviruses are nutrient transporters

<i>Gammaretrovirus envelope</i>	<i>Original host</i>	<i>Receptor</i>	<i>Cell function</i>	<i>In vitro host range</i>	<i>References<sup>a</sup></i>
Ectotropic MLV	Mouse	CAT1	Cationic amino-acid transporter	Mouse	Albritton (1989)
Amphotropic MLV					Miller (1994)
10A1 MLV	Mouse	PiT2	Inorganic phosphate transporter	Mammals	Van Zeijl (1994) Battini (1999)
Xenotropic MLV Polytropic MLV	Mouse	XPR	Transporter structure, unknown function	Non-murine Mammals	Taylor (1999) Yang (1999)
M813 MLV	Mouse	SMIT1	Myo-inositol transporter	Mouse	Hein (2003)
10A1 MLV FeLV-B FeLV-T GaLV	Mouse Cat Cat Monkey	PiT1	Inorganic phosphate transporter	Mammals <sup>b</sup>	O'Hara (1990)
BaEV RD114	Monkey Cat	ASCT1;ATB <sup>o</sup>	Neutral amino-acid transporter	Mammals	Rasko (1999) Taylor (1999)
FeLV-C	Cat	FLVCR	Facilitative transporter	Mammals	Quigley (2000) Taylor (1999)
PERV-A	Pig	PAR1; PAR2	Transporter structure, unknown function	Mammals	Ericsson (2003)

<sup>a</sup>Refers to receptor identification. <sup>b</sup>FeLV-T entry requires the additional presence of the feline T-cell-encoded FeLIX factor (Anderson, 2000 #56)

infection. Thus, parameters that alter the availability and accessibility of potential target cells, due to the route and dose of the initial viral exposure, age at infection, status of the immune response, genetic background, etc., will play a major role in the tissue distribution of the virus. Additionally, combined viral and hosts properties that specifically reduce (via viral cytopathic effects, immune clearance, necrosis or apoptosis) or expand (via mitogenic and transforming effects) certain infected cell compartments will also considerably alter the apparent tropism. In the case of most retroviruses, which combine lytic, transforming and inflammatory pathogenic effects, the impact of these processes on the observed tropism are likely be magnified.

### HTLV-1 tropism: *in vitro* or *in vivo* veritas?

#### *In vitro* and *in vivo* tropism of HTLV-1

Studies documenting *in vitro* infection and dissemination of HTLV-1 to non-T cells, including nonlymphoid cells, were published soon after the identification of HTLV (Yamamoto *et al.*, 1982b; Clapham *et al.*, 1983; Hayami *et al.*, 1984). Productive transmission of natural HTLV-1 isolates to primary human endothelial cell cultures (Ho *et al.*, 1984; Hoxie *et al.*, 1984), monocyte and microglial cells (Hoffman *et al.*, 1992), as well as basal mammary epithelial cells (LeVasseur *et al.*, 1998) has been reported.

*In vivo*, HTLV-1 is readily detected in CD4+ T lymphocytes in both asymptomatic and symptomatic carriers ((Grant *et al.*, 2002) and references therein),

thus making it clear that the CD4+ cell compartment constitutes a privileged component of the *in vivo* HTLV-1 tropism (Richardson *et al.*, 1990). Nevertheless, this crucial observation and the quasi-exclusive association of *in vivo* HTLV-1-induced proliferation to the CD4+ T cell compartment has tended to diminish, if not obliterate, the *in vivo* tropism of HTLV-1 in many other cell types. Indeed, many earlier reports published in the mid 1980s described the potential infection of B lymphocytes by HTLV-1 *in vivo* (Yamamoto *et al.*, 1982a; Longo *et al.*, 1984; Franchini *et al.*, 1985). However, because of a step of *in vitro* culture, *in vitro* infection by contaminating HTLV+ T cells could not be formally excluded. In 1993, Koyanagi *et al.* (1993) thoroughly explored the *in vivo* infection by HTLV-1 in different hematopoietic cell compartments and in the absence of *in vitro* culture. Using PCR and RT-PCR to detect Tax DNA and RNA, they demonstrated that although the highest numbers of HTLV-1 DNA proviral copies and active transcription were generally found in CD4+ T lymphocytes, most asymptomatic HTLV-1-infected individuals, as well as HAM/TSP and ATL patients also exhibited infection of CD8+ lymphocytes, monocytes and B lymphocytes (Koyanagi *et al.*, 1993) (*in vivo* infection of nonhematopoietic tissues was not addressed: Y Koyanagi, personal communication). Interestingly, these authors also described an ATL patient in transient remission with no detectable HTLV-1 DNA in CD4+ cells, even though CD8+ cells, B cells and monocytes were infected. Others reports have largely confirmed the *in vivo* infection of HTLV-1 in CD8+ cells (Eiraku *et al.*, 1998; Hanon *et al.*, 2000; Nagai *et al.*, 2001; Grant *et al.*, 2002) and references therein. Accordingly, under certain condi-

tions, the *in vitro* transformation of unselected PHA-treated peripheral blood lymphocytes by coculture with an irradiated HTLV-1-infected cell line can lead to a vast majority of CD8+ transformants (N Taylor, unpublished results).

The search for HTLV sequences by *in situ* hybridization, in post-mortem brains of HAM/TSP patients has demonstrated that astrocytes can also be productively infected (Lehky *et al.*, 1995). Documented *in vivo* infection of noncultured adherent cells remains rare but has occasionally been reported in sweat gland epithelia (Setoyama *et al.*, 1998, 1999), salivary glands (Tangy *et al.*, 1999), vascular endothelial cells (Setoyama *et al.*, 1998) and mammary glands (Loureiro *et al.*, 2000).

In conclusion, although the CD4+ T-cell compartment appears to be the overwhelming reservoir, maintaining HTLV-1 infection during the lifespan of an infected individual, other hematopoietic cells (non-CD4+ T cell subsets, B lymphocytes, monocytes and macrophages, dendritic cells and megacariocytes) as well as glial cells (astrocytes and microglial cells) are also part of the HTLV-1 *in vivo* tropism (Macatonia *et al.*, 1992; Koyanagi *et al.*, 1993; Lehky *et al.*, 1995; Levin *et al.*, 1997; Eiraku *et al.*, 1998; Hanon *et al.*, 2000; Nagai *et al.*, 2001; Grant *et al.*, 2002).

#### Animal models: a broad *in vivo* tropism

Following the discovery of HTLV-1, several groups have developed rodent models of HTLV-1 infection (see Lairmore and Ratner, this issue). Thus, rabbit (Akagi *et al.*, 1985; Zhao *et al.*, 2002), rat (Suga *et al.*, 1991; Ishiguro *et al.*, 1992) and mouse (Fang *et al.*, 1998; Tanaka *et al.*, 2001) models of persistent HTLV-1 infection have been reported. In all these models, HTLV-1 was detected in a large spectrum of hematopoietic cell types and nonhematopoietic tissues, including brain, lung, kidney, heart, liver, thyroid, as well as thymic and endometrium epithelia, depending on the model and the timing of the inoculation.

HTLV-1 infection and spreading has also been closely examined after intravenous inoculation of adult squirrel

monkeys (*Saimiri sciureus*) (Kazanji *et al.*, 1997, 2000; Mortreux *et al.*, 2001). In this model, peripheral blood mononuclear cells were the main infected cell compartment, but other HTLV-1-infected tissues including salivary and thyroid glands, lung, liver, pancreas, intestine, muscle and spinal cord were sporadically detected in individual monkeys (Kazanji *et al.*, 2000).

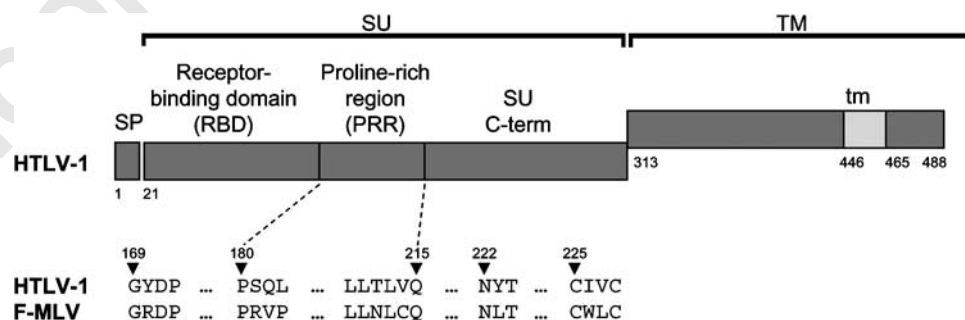
#### HTLV-1 envelope glycoprotein and receptor

##### *HTLV-1: a complex deltaretrovirus with a simple gammaretrovirus type Env*

The human HTLV and simian STLV retroviruses are deltaretroviruses that belong to the 'complex' retrovirus family, along with lentiviruses and spumaviruses, characterized by double spliced RNAs that code for regulatory proteins. Notably though, alignment of the HTLV-1 SU with that of the Friend-murine leukemia virus (F-MLV) reveals highly conserved microdomains within the SU and underscores a similar general organization of the SU (Kim *et al.*, 2000, 2004a) (Figure 2). Functional evaluation of different SU domains, as delineated in Figure 2, allowed us to demonstrate that, paradoxically to its 'complex' genetic organization, the HTLV SU has a modular organization that is identical to that of simple retroviruses (Battini *et al.*, 1995), with the RBD residing at the SU amino terminal (Kim *et al.*, 2000, 2004b). Furthermore, we found that this domain was sufficient for receptor binding, as measured by FACS analysis, fusion, infection and interference to infection (Kim *et al.*, 2000, 2004b), and did not require the central proline-rich region (PRR) of HTLV Env to exert these properties (Kim *et al.*, 2000, 2004b).

##### *The HTLV receptor enigma*

*Early studies on the HTLV-1 env receptor* Since the identification and isolation of HTLV-1 in the early 1980s, numerous efforts have been undertaken to identify its cellular receptor. It was rapidly shown by



**Figure 2** Schematic representation of the HTLV-1 envelope glycoprotein (Env). Mature Env is constituted of two subunits formed after cleavage of the amino terminal signal peptide (SP) and cleavage of the Env polyprotein precursor into the extracellular SU and the membrane-anchored TM. SU comprises three distinct subdomains: an amino terminal receptor-binding domain (RBD), a central proline-rich region (PRR) and a carboxy terminal domain (C-term). SU peptide landmarks in HTLV-1 SU homologous with those of the gammaretrovirus Friend murine leukemia virus (F-MLV) SU are indicated (Kim *et al.*, 2000, 2004a, b)

Env interference to superinfection that HTLV-1 and -2 (Clapham *et al.*, 1984) and related simian isolates (Sommerfelt and Weiss, 1990) share the same receptor. However, the search for a receptor was hampered by the combination of (i) a widely expressed receptor among vertebrate cell lines, (ii) an Env-mediated rampant syncytial effect and (iii) an inability to produce high titering cell-free virions.

In the quest for tools to help the identification of an HTLV-1 receptor, two human liver cell lines (Okuma *et al.*, 1999), the bovine MDBK cell line (Trejo and Ratner, 2000) and insect cell lines (Jassal *et al.*, 2001) were reported as lacking detectable expression of HTLV Env receptor or as being resistant to HTLV-1 Env-mediated pseudotype entry. However, it must be noted that binding of a tagged HTLV-1 SU to MDBK cells is significant, albeit at low levels (Jassal *et al.*, 2001).

**Receptor detection assays** Three main categories of read-outs have been used exclusively or in combination to evaluate the availability of functional HTLV receptors: (i) Infection with heterologous nonretroviral (Clapham *et al.*, 1984; Sommerfelt *et al.*, 1988; Sommerfelt and Weiss, 1990) or retroviral (Sutton and Littman, 1996; Trejo and Ratner, 2000; Manel *et al.*, 2003a) virions pseudotyped with HTLV-1 Env. An adaptation of this assay consists of the substitution of the HTLV-1 *env* gene for that of the VSV-G transmembrane protein into the VSV genome (Okuma *et al.*, 2003). (ii) Cell-to-cell fusion mediated by HTLV-1 Env. Fusion is generally measured by the formation of multinucleated giant syncytial cells (Hoshino *et al.*, 1983; Hildreth *et al.*, 1997; Tajima *et al.*, 1997), or by an adaptation of an HIV Tat-based assay (Rocancourt *et al.*, 1990) in which Env-receptor interactions are revealed by the transfer of HIV Tat molecules to indicator cells harboring a Tat-dependent marker gene (Denesvre *et al.*, 1995). The latter appears more suited to directly evaluate receptor function, since several cell lines that are fully susceptible to HTLV-1 Env-mediated viral entry are resistant to syncytia formation (Kim *et al.*, 2003). (iii) Binding of HTLV-1 Env, or RBD-containing Env derivatives, to receptor-harboring cells. The first version of these assays used whole tagged virions (Gavalchin *et al.*, 1993), while more refined versions rely on binding of either HTLV-1 SU fused to an immunoadhesin (Jassal *et al.*, 2001), or the isolated RBD as a diversely tagged soluble protein (Manel *et al.*, 2003a, b; Kim *et al.*, 2004b).

#### *HTLV receptor and coreceptor candidates*

Earlier suggestion that chromosome 17 encodes an HTLV-1 receptor, based on mouse cells resistance, has been abandoned. Thus, mouse cell lines are readily susceptible to HTLV Env-mediated binding (Jassal *et al.*, 2001), membrane fusion (Denesvre *et al.*, 1995) and retroviral infection (Trejo and Ratner, 2000).

Several groups have explored the identification of cell surface factors that influence HTLV Env-mediated receptor interactions, by measuring HTLV-1 Env-

induced syncytia formation. Tetraspanins, which are promiscuous proteins that interact with many membrane components, including integrins, have been reported to both positively and negatively affect syncytia formation (Imai *et al.*, 1992; Pique *et al.*, 2000).

The influence of ICAM and their integrin receptors in HTLV infection and pathophysiology has also emerged (Uchiyama *et al.*, 1996; Hildreth *et al.*, 1997; Al-Fahim *et al.*, 1999; Daenke *et al.*, 1999; Daenke and Booth, 2000). VCAM-1 has been reported to stimulate HTLV-1 Env-mediated syncytia, an event that is specifically blocked by an anti-HTLV SU antibody, independently of VLA-4, the integrin that binds VCAM-1 (Hildreth *et al.*, 1997). Others have reported an effect of integrins binding ICAM-1 and -3 in the production of syncytia mediated by HTLV-1 Env and an inhibition of this effect by antibodies directed against beta integrins (Daenke *et al.*, 1999; Daenke and Booth, 2000). Antibodies directed against GPI-anchored proteins (Niyogi and Hildreth, 2001) or class II histocompatibility molecules (Hildreth, 1998) have been found to specifically block HTLV-1-induced syncytia formation, likely by steric hindrance within lipid rafts (Niyogi and Hildreth, 2001). Using similar approaches, Sagara *et al.* (1998, 2001) have identified a membrane-associated HSC70 heat-shock cognate protein, as well as beta actin and a phosphatidyl glycerol, as parts of membrane structures wherein HTLV-induced syncytia formation proceeds.

Heparan sulfate proteoglycans (HSPG) are polyanionic-sulfated carbohydrate chains that branch a multitude of proteins at the cell membrane or in the extracellular matrix (Spillmann, 2001). They have been shown to bind and favor attachment and subsequent receptor-mediated entry of numerous enveloped and nonenveloped viruses, including human and animal herpes simplex viruses, flaviviruses, picornaviruses (Spillmann, 2001) and HIV (Bobardt *et al.*, 2003). The presence of HSPG at the cell surface also influences HTLV-1 Env-mediated binding and viral entry (Okuma *et al.*, 2003; Pinon *et al.*, 2003), whereas cholesterol seems to play a role at a postbinding level (Wielgosz *et al.*, 2005).

HTLV Env peptide mapping experiments were performed to identify functional determinants in the HTLV envelope (Sagara *et al.*, 1996). A peptide corresponding to SU amino acid 197 to 216 (P197) was found to inhibit HTLV-1 Env-induced cell-cell fusion. This region lies in the PRR central domain (Figure 2), which is recognized by neutralizing antibodies (Ralston *et al.*, 1989; Goetz *et al.*, 1997), suggesting that this region is important for fusion to proceed. Based on these data, P197 was considered to mirror the HTLV Env receptor domain. However, neutralizing antibodies that block viral entry do not necessarily map to the Env RBD and may interfere by steric hindrance with fusion and syncytia formation at a postbinding step. This is the case, for instance, of the 83A25 neutralizing monoclonal antibody which recognizes a murine leukemia virus Env epitope in the SU carboxyterminus, at the opposite side of the RBD,



downstream of the PRR (Evans *et al.*, 1990). Indeed, it has since been shown that peptide P197 does not compete with the cell binding properties of the HTLV-1 SU (Brighty and Jassal, 2001), indicating that this region is likely to be mostly important for postbinding events as previously shown for its MLV counterpart (Lavillette *et al.*, 1998). The mechanism by which P197 and anti-P197 antibodies inhibit fusion activity is unclear and possible nonexclusive mechanisms include blocking of the Env in a transitional conformation, alterations of interactions between Env molecules or interference with additional cellular factors that may play a role in postbinding viral entry steps.

From the studies mentioned above, it is therefore possible to draw the following sequence of events leading to HTLV-1 Env-mediated entry: (i) Attachment and increased mobilization of HTLV particles at the target cell surface via attachment to extracellular matrix structures such as HSPG, most likely in the context of cell-to-cell interactions (Igakura *et al.*, 2003; Jolly and Sattentau, 2004). (ii) Env interaction with a high-affinity receptor favored by attachment, followed by conformational changes that unmask the fusion peptide and direct entry in a structure compatible with productive infection; (iii) Formation or sequestration of virions within such structures of the plasma membrane, within or in the vicinity of lipid rafts, where fusion and viral entry can proceed.

*Mammalian gammaretroviruses use nutrient transporters as receptors* Gammaretrovirus receptors identified to date are either multimembrane-spanning nutrient transporters or, for those of unknown function, have a structure that is compatible with nutrient transport functions (Tailor *et al.*, 2003) (Table 1). The closely related structural organization of HTLV and gammaretrovirus Env (Figure 2) led us to predict that the HTLV receptor would also be a member of this family of molecules (Kim *et al.*, 2000).

Basyuk *et al.* (2003) have previously shown that MLV Env redirect endogenous retroviral RNA-Gag trafficking from intracellular lysosomes to exiting endosomes. Based on these observations and several phylogenetic considerations on the dichotomy between the evolutionary pattern of retroviral *env* genes and the remainder of the retroviral genome (Malik *et al.*, 2000; Benit *et al.*, 2001; Pearson and Rohrmann, 2002; Kim *et al.*, 2004a), we have postulated that infectious gammaretroviruses emerged upon *env* gene 'capture' by endogenous retrotransposons (Kim *et al.*, 2004a). Thus, it is possible that upon 'capture' of an *env* gene, intracellular interactions between Env and its cell receptor foster the routing of the virus out of the cell (Kim *et al.*, 2004a). Therefore, evaluating the contribution of Env-receptor interactions in viral egress might provide additional clues on the exclusive selection by gammaretroviruses and HTLV of nutrient transporters as Env receptors (Tailor *et al.*, 2003).

### GLUT1, the HTLV Env receptor

As described in Kim *et al.* (2004b), we were able to derive RBD-containing truncated domains of HTLV-1 and -2 SU as soluble amino terminal-tagged fusion proteins (HRBD). As expected for a *bona fide* receptor binding ligand, HRBD exerted full interfering abilities with exogenous HTLV Env. Thus, HTLV-1 and -2 Env binding, as well as Env-mediated cell-free and cell-to-cell infection were specifically blocked in HRBD-expressing cells, while remaining fully susceptible to MLV Env-mediated binding and infection (Kim *et al.*, 2004b).

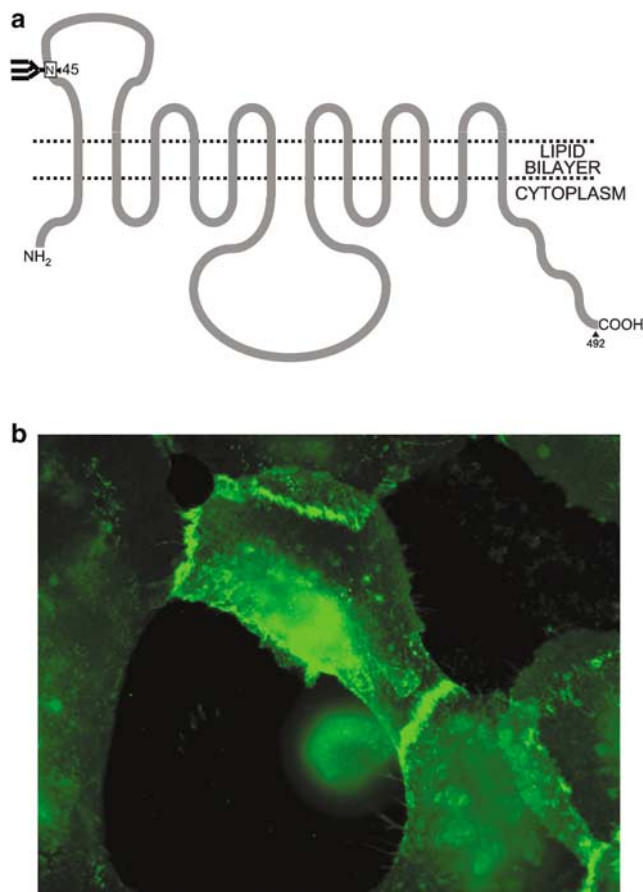
The elucidation of the modular structure of HTLV-1 Env, the subsequent production of HRBD-derived tagged constructs and two key observations that we made while monitoring receptor expression with those tools led us to the identification of the HTLV Env receptor.

The first observation was paradoxical with regards to the importance of T-cell infection in HTLV pathophysiology. Indeed, we (Manel *et al.*, 2003b), using HRBD constructs, and others using an entire SU (Nath *et al.*, 2003), observed no or little expression of the HTLV Env receptor in freshly isolated peripheral blood mononuclear cells, including CD4+ T cells. This *a priori* puzzling result was elucidated by the observation that T-cell receptor activation of both memory and naive T cells led to the rapid emergence, within 4–6 h, of cell surface HTLV receptor expression on CD4+ as well as CD8+ T lymphocytes (Manel *et al.*, 2003b; Nath *et al.*, 2003). Therefore, increased expression of the receptor at cell surface is an early marker of T lymphocyte activation, a feature that has most likely contributed to the receptor selection by HTLV. Moreover, *in vitro* activation of other hematopoietic populations was also associated with an HTLV receptor upregulation (Nath *et al.*, 2003).

The second observation was that introduction of HRBD by transfection into cultured cell lines block acidification of the cell culture milieu, as visualized by the persistence of a red tint in phenol red-containing culture supernatants (Manel *et al.*, 2003a). Proton enrichment from cultured vertebrate cell lines is largely due to the joint transport of protons with lactate as a glycolysis by-product. After experimentally excluding the potential role for widely distributed proton-linked lactate transporters (MCT) and the MCT-associated CD147 protein (Kirk *et al.*, 2000) as receptors for HTLV Env ((Manel *et al.*, 2003a) and unpublished observations), we focused on glucose transport, which fuels glycolysis. These studies quickly centered on GLUT1, the most largely distributed and conserved glucose transporter (Mueckler, 1994).

GLUT1 is a 12 membrane-spanning passive facilitator of glucose transport across the cell surface (Figure 3a). GLUT1 expression reflects intense metabolic activity, and its overexpression is used as a detection marker for numerous tumors (Smith, 1998; Younes *et al.*, 1996). Moreover, GLUT1, in accordance with the known properties of the HTLV Env receptor, is expressed on all evaluated mammalian cell lines. Indeed,





**Figure 3** Schematic representation of GLUT1 and its localization on the surface of primary human mammary cells. **(a)** Topological representation of human GLUT1, adapted from Hruz and Mueckler (2001). GLUT1 is a 12 transmembrane multispanning molecule delineating six extracellular five cytoplasmic loops with a single N-glycosylation site on the first extracellular loop (branching symbol at N45). **(b)** GLUT1 accumulates at cell contacts as revealed by binding of HTLV Env RBD-EGFP fusion protein to nonfixed primary human mammary cells

*in vivo* proliferation of tumors as well as *ex vivo* culture of cell lines are characterized by a switch from respiration to lactate fermentation, and as such have an increased requirement for glucose. Notably though, GLUT1 appears to be the mandatory glucose transporter used by mammalian cells cultured *ex vivo* via traditional methods.

We have shown that GLUT1 fulfills all characteristics of a retrovirus receptor (Manel *et al.*, 2003a), among which are the ability to directly interact with HTLV envelopes, mediate infection by viral surrogates harboring the HTLV envelope, and specifically alleviate HTLV Env viral interference to superinfection (Manel *et al.*, 2003a). Others have confirmed that GLUT1 increases HTLV infection in the relatively resistant MDBK cells (Coskun and Sutton, 2005). Furthermore, using GLUT1 and GLUT3 chimeric molecules, we identified the binding domain for HTLV envelopes on GLUT1, as well as separate domains required for subsequent viral

entry (N Manel, JL Battini and M Sitbon, unpublished results).

Following our identification of GLUT1 as the HTLV receptor, it was important to reassess data on HTLV receptor expression with results obtained by direct studies of GLUT1 (for a review, see Manel *et al.*, 2004). This was particularly of interest in the context of CD4+ T lymphocytes, the major *in vivo* HTLV reservoir. Indeed, as we and others reported for the as yet unknown HTLV receptor, GLUT1 is not detectable on resting lymphocytes and is a major marker of active lymphocyte metabolism (Chakrabarti *et al.*, 1994; Frauwirth *et al.*, 2002). Moreover, TCR engagement of naïve and memory T cells (Manel *et al.*, 2003b; Nath *et al.*, 2003) and TGF- $\beta$ -induced signaling of neonatal T lymphocytes (Jones *et al.*, 2005) rapidly induce GLUT1 expression at the cell surface, as measured with HTLV SU-derived ligands. Finally, it is notable that the homeostatic cytokine IL-7 induces surface GLUT1 expression on neonatal T cells to a significantly higher level than on adult T cells, as assessed by HRBD binding. This difference has biological significance as IL-7-induced neonatal T cells have a significantly increased glucose uptake as compared to their equivalently treated adult counterparts (S Kinet and NT, unpublished observations). The combined increase in surface GLUT1 and metabolic activity are likely to be crucial for propagating HTLV infection following mother-to-infant transmission of this virus.

### Conclusions and perspectives

Our recent mapping of distinct determinants on GLUT1 that play separate roles in binding and postbinding steps of HTLV Env-mediated infection (N Manel, JL Battini and M Sitbon, unpublished results) provides additional tools to elucidate a few of the enigmas surrounding HTLV-1 infection, dissemination and pathophysiology. It will thus be of interest to evaluate the sequence of events that involve GLUT1 and Env in the context of promiscuous factors promoting virus attachment, such as HSPG or ICAMs, as well as candidate coreceptors (D Ghez, Y Lepelletier, C Pique and O Hermine, personal communication).

It is also interesting that GLUT1 appears to concentrate in viral synapses (unpublished results of D Brighty cited in Jolly and Sattentau (2004)) such as those formed between HTLV-1-infected and noninfected lymphocytes (Igakura *et al.*, 2003). Accordingly, we have noted that GLUT1 molecules that are present at the cell surface tend to concentrate at regions of cell contact, even in the absence of HTLV Env (Figure 3b).

Despite the fact that tropism depends on many parameters that are independent of Env-receptor interactions, the discovery of GLUT1 as an HTLV receptor reinforces the notion of a broader *in vivo* tropism than usually perceived and provides additional clues to the pathophysiology associated with HTLV-1 infections (Manel *et al.*, 2004). Thus, other tissues where

GLUT1 is expressed include the endothelial blood–brain barrier, notably the luminal membrane (Cornford *et al.*, 1994), and various cell types of the eye, including the ganglion cell layer of the retina, the endothelium lining the Canal of Schlemm, the corneal endothelium and the basal cells of the corneal epithelium (Kumagai *et al.*, 1994). It will be important to explore whether this diverse expression of GLUT1 in the eye is related with the development of HTLV-associated uveitis (Mochizuki *et al.*, 1996).

Additionally, GLUT1 is expressed in glial cells of the central nervous system (Kumagai *et al.*, 1994; Virgintino *et al.*, 1997). In the peripheral blood, GLUT1 is detected primarily at the surface of red blood cells, where it accounts for up to 5% of the total content in membrane protein (Mueckler, 1994). Therefore, potential Env binding, with or without HTLV virion entry, in cell types as diverse as the cell types mentioned above provides an array of situations that may modulate local cell-to-cell interactions and HTLV transmission. One enlightening example centers on the importance of GLUT1 in the metabolic exchanges between intracerebral vessels and nerve terminals via glial cells in general and astrocytes in particular (Pellerin and Magistretti, 2003; Voutsinos-Porche *et al.*, 2003; Bonvento *et al.*, 2005). Indeed, the privileged anatomical relationship between astrocyte endfeet that project into the vasculature and individual endothelial cells of the blood–brain barrier (Kacem *et al.*, 1998) provide tracks to explore in HTLV-associated neuropathophysiological effects. *Ex vivo* studies of interactions between these different cell types placed in the presence of HTLV-1 or different types of HTLV-1-infected cells will help a

better understanding of HTLV spreading and pathology.

Altogether, the *in vitro* and *in vivo* observations and the different animal models of infection indicate that HTLV-1 spreads among many cell types *in vivo*, with a preponderance of CD4+ T cells (Grant *et al.*, 2002). The specific elimination of infected CD8+ cells (Hanon *et al.*, 2000), as well as the elimination, counterselection or nonproductive infection (Lehky *et al.*, 1995; Levin *et al.*, 1997; Grant *et al.*, 2002) of HTLV-1-infected non-CD4+ cells is likely to minimize the detectable spectrum of HTLV-1 tropism *in vivo*. More sensitive and noninvasive methods of detection (Yamano *et al.*, 2002; Heams and Kupiec, 2003; Spivack *et al.*, 2004) should further future studies of HTLV-1 expression *in situ*. Potential *in vivo* application of these methods to the early and systematic follow-up of nonhematopoietic epithelial and endothelial tissues, in different contexts of HTLV-1 infection, is likely to provide a more precise depiction of the *in vivo* HTLV-1 tropism.

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## **Annexe 2 – Brevets**

### **Brevet 1**

Felix J. Kim, **Nicolas Manel**, Marc Sitbon. Detection of retroviral sequences related to the envelope of Human and Simian T Cell Leukemia Viruses : identification of new HTLV variants. *International patent request PCT/FR03/01274 deposited on April 22, 2003* (formerly French patent request CNRS BF N° 02/05001 deposited on April 22, 2002).

### **Brevet 2**

**Nicolas Manel**, Felix J. Kim, Sandrina Kinet, Naomi Taylor, Marc Sitbon, Jean-Luc Battini. GLUT-1 as a receptor for HTLV envelopes and its uses. *International patent request PCT/EP2004/004624 deposited on April 30, 2004* (formerly European patent request 03 291 067.1 deposited on May 2, 2003).

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(54) Title: OLIGONUCLEOTIDES FROM SEQUENCES CODING FOR THE SURFACE COMPONENT OF PTLV ENVELOPE PROTEINS AND USES THEREOF

(54) Titre : OLIGONUCLEOTIDE ISSUS DES SEQUENCES CODANT POUR LA COMPOSANTE DE SURFACE DES PROTEINES D'ENVELOPPE DES PTLV ET LEURS UTILISATIONS

(57) Abstract: The invention relates to the use of oligonucleotides from the nucleotide sequences coding for the amino-terminal region of the surface component (SU) of envelope proteins of PTLV viruses in order to perform methods of detecting every PTLV strain or PTLV-related viruses, e.g. for the detection of novel PTLV variants or viruses comprising sequences related to PTLV SUs. The invention also relates to primer pairs which are used to perform said detection methods and the novel PTLV variants thus detected.

(57) Abrégé : L'invention a pour objet l'utilisation d'oligonucléotides issus des séquences nucléotiques codant pour la région aminoterminal de la composante de surface (SU) des protéines d'enveloppe des virus des PTLV, pour la mise en oeuvre de procédés de détection de toute souche de PTLV, ou de virus apparentés aux PTLV, notamment pour la détection de nouveaux variants des PTLV, ou de virus comportant des séquences apparentées aux SU des PTLV. L'invention a également pour objet des couples d'amorces pour la mise en oeuvre de ces procédés de détection, ainsi que les nouveaux variants de PTLV ainsi détectés.



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(54) Title: GLUT-1 AS A RECEPTOR FOR HTLV ENVELOPES AND ITS USES

(57) Abstract: The invention relates to the use of the ubiquitous vertebrate glucose transporter GLUT1, or of fragments or sequences derived thereof, for the in vitro diagnosis of cancers, when used as a tumor marker, or for the screening of compounds useful for the preparation of drugs for the prevention or the treatment of pathologies linked to an infection of an individual with a PTLV, or pathologies linked to an overexpression of GLUT1 on cell surfaces, or the *in vitro* detection of GLUT1 on cell surfaces. The invention also relates to pharmaceutical compositions containing GLUT1, or fragments or sequences derived thereof, and to their uses such as in the frame of the prevention or the treatment of pathologies linked to an infection of an individual with a PTLV.

**Auteur :** Nicolas Manel

**Titre :** Identification et caractérisation d'un récepteur d'enveloppe au virus de leucémie T humaine (HTLV), le transporteur de glucose GLUT1

**Titre en anglais :** Identification and characterization of a Human T-cell Leukemia Virus (HTLV) envelope receptor, the glucose transporter GLUT1

**Résumé :**

Le rétrovirus HTLV-1 (Human T-cell Leukemia Virus type 1) est l'agent responsable de la leucémie T de l'adulte (ATL) et de la paraparésie spastique tropicale (TSP/HAM). Le HTLV est présent de manière endémique au Japon, en Afrique centrale, en Océanie, en Amérique du sud et aux Caraïbes. Chez les patients, le HTLV est détecté principalement dans les lymphocytes T CD4+. Le récepteur du HTLV est présent chez tous les vertébrés, et toutes les lignées cellulaires de vertébrés semblent l'exprimer *in vitro*. L'organisation de la glycoprotéine d'enveloppe (Env) du HTLV est similaire à celle des MLV (Murine Leukemia Virus), avec un domaine de liaison au récepteur situé dans la partie N-terminale.

Au cours de cette thèse, nous avons dérivés des produits tronqués de l'Env, sur la base de cette organisation, nous permettant de mesurer l'expression de surface du récepteur HTLV. Nous avons ainsi pu mettre en évidence que le récepteur HTLV n'est pas détectable à la surface des lymphocytes T CD4+, mais que son expression est induite par l'activation lymphocytaire. Nous avons également montré qu'un traitement à l'IL-7 des lymphocytes T de sang de cordon, qui provoque la prolifération, induit l'expression du récepteur HTLV. Ces observations nous ont permis de conclure que le récepteur HTLV est un marqueur d'activation des lymphocytes associé à l'état prolifératif.

Par la suite, nous avons montré que l'expression de l'Env HTLV induit un blocage de la production de lactate. Nous avons pu établir que ce blocage est dû à l'inhibition par l'Env HTLV du transport de glucose dans la cellule, induisant un blocage de la glycolyse. Ces observations nous ont conduit à évaluer si le principal transporteur du glucose GLUT1 pouvait être un récepteur pour le virus HTLV. Effectivement, nous avons pu démontrer que GLUT1 permet à la fois la liaison à l'Env HTLV et l'entrée de particules virales.

Afin de comprendre cette interaction, nous avons construit des molécules chimériques entre GLUT1 et GLUT3. Nous avons ainsi observé que 7 acides aminés de la 6<sup>ème</sup> boucle extracellulaire de GLUT1 confèrent l'activité de liaison à l'Env. Cependant cette propriété n'est pas suffisante pour permettre l'entrée virale, et la présence concomitante de la 1<sup>ère</sup>, la 5<sup>ème</sup> et la 6<sup>ème</sup> boucle extracellulaire est requise pour permettre l'entrée virale, suggérant l'existence d'une nouvelle activité associée à la notion de récepteur rétroviral.

L'ensemble de ces données nous a enfin conduit à proposer de nouveaux modèles de pathogenèse. La prise en compte de l'inhibition de l'activité de transport de GLUT1 par l'Env autorise en effet de nouvelles hypothèses pour expliquer l'émergence de clones leucémiques et la démyélinisation associée à la TSP/HAM.

**Mots clés :** Rétrovirus, HTLV, enveloppe, SU, récepteur, GLUT1, GLUT3, lymphocytes, métabolisme, activation

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