Hepatitis C Virus E1E2 co-evolving networks unveil their functional dialogs and highlight original therapeutic strategies

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Abstract

Hepatitis C Virus (HCV) is a major health concern. HCV infects more than 170 million people worldwide and no vaccine is available yet. Initial HCV infection is most often followed by a chronic liver disease that may sometimes progress toward liver cirrhosis and hepatocellular carcinoma. The recent development of direct acting agents (DAAs) that target HCV proteases has led to serious advances in patient treatment but their use still presents limitations. Thus, there is today an urgent need to develop multitherapy strategies targeting different stages of HCV life cycle. HCV entry is the first step of the virus life cycle and may represent a promising target for therapies, but no virus-specific inhibitors directed against entry have been approved so far for HCV treatment. HCV entry is mediated by several cellular and viral actors, such as the two HCV E1 and E2 envelope glycoproteins assembled as heterodimer onto the virus surface. During entry process, E1E2 structurally rearrange to induce viral particle attachment to hepatocytes and virus membrane fusion with host cell membranes, allowing the release of the viral RNA into the cytosol. However, how E1 and E2 dialog, structurally rearrange and act together during these steps remain poorly defined.

In this work, we aimed to clarify the role and interrelation of E1E2 during virus entry through the identification of conserved dialogs or structural rearrangements critical for this process, thus opening ways to potential new therapeutic strategies. Our general working hypothesis was that different domains involved in a protein function co-evolve to maintain their most optimal direct or indirect interaction (or "dialogs).

We first examined the functional plasticity of E1E2. For this purpose, we investigated whether a strong genetic divergence between E1E2 heterodimers may highlight distinct functions and conformations. HCV is known to infect and replicate within B lymphocytes (B-cells) and HCV variants derived from B-cells are genetically divergent from serum and liver derived-variants in chronically infected patients. We assessed the ability of E1E2 to be a major determinant of the HCV lymphotropism, through the selection of particular genetic features and functions. We isolated full E1E2 sequences from B-cells and serum and observed *in vitro* that B-cell derived E1E2 were specialized for B-cell infection but not for hepatocyte infection. Our results suggested that new functions can emerge from the E1E2 conformational plasticity through the mediation of critical residues.

In a second time, we assessed E1E2 co-evolution in the context of closely related strains in order to precisely identify dialogs or interactions that are critical for HCV entry. Indeed, we identified experimentally a conserved dialog between E1 and the domain III of E2 (E2 DIII) that was critical for virus binding and fusion. Moreover, we also reconstructed through a computational model the entire co-evolving networks between E1 and E2. The model predictions highlighted a strong co-evolution between E1 and E2 and potential structural rearrangements. Moreover, results were consistent with our previous findings as well as with others reports from the literature. Altogether, this second approach suggested that HCV E2 is likely a fusion protein able to fold over via its domain III through the mediation of E1.

Altogether, these different works highlight that E1 and E2 are involved in complex and tight dialogs that regulate the heterodimer folding and functions. Our results suggest that E1E2 heterodimer is more likely a single functional and structural protein entity than an association of two proteins with specific functions.

Résumé

Le Virus de l'Hépatite C (VHC) est aujourd'hui un problème de santé majeure. En effet, ce virus infecte 170 millions de personnes à la surface de la planète mais aucun vaccin n'est encore disponible pour résorber l'épidémie. Ce virus provoque chez 80% des patients qu'il infecte une sévère pathologie chronique hépatique, qui peut parfois progresser vers une cirrhose du foie et un hépato-carcinome. Récemment, le développement d'inhibiteurs spécifiques (DAAs) ciblant certaines protéases du VHC ont permis d'important progrès dans le traitement de l'infection par le VHC mais leurs utilisations présentent encore des limitations. Ainsi, il y a aujourd'hui un besoin important de développer des multitherapies efficaces contre le VHC, capable de cibler simultanément différentes étapes du cycle viral. Le processus d'entrée du VHC dans les hépatocytes représente la première étape du cycle viral au cours de l'infection et pourrait donc constituer une cible prometteuse pour le développement de stratégie thérapeutique. Cependant, il n'existe encore actuellement aucuns inhibiteurs viraux spécifiques ciblant l'étape d'entrée du VHC. Le processus d'entrée du VHC est finement régulé par un nombre important d'acteurs cellulaires et viraux, comme les deux glycoprotéines d'envelope du VHC, E1 et E2, assemblé sous la forme d'un hétérodimère incorporé à la surface des particules virales. Au cours du processus d'entrée, E1 et E2 modifient continuellement leurs conformations pour permettre l'attachement de la particule virale aux hépatocytes et la fusion entre la membrane virale et les membranes de la cellule hôte. Cependant, comment E1 et E2 dialoguent, modifient leurs conformations et se coordonnent mutuellement au cours de l'entrée reste encore à être définit.

Dans ce travail, nous avons souhaité clarifier le rôle et l'interrelation entre E1 and E2 au cours de l'entrée à travers l'identification de dialogues conservés, interactions et conformations qui sont critiques pour ce processus, cela pouvant alors ouvrir la voie à de potentiels stratégies thérapeutiques. Notre hypothèse de travail a été basée sur l'idée que la co-évolution de domaines variables au sein d'une protéine permet de conserver des fonctions assurées par différents segments protéiques via leurs interactions directes ou indirectes (ou dialogues).

Nous avons tout d'abord examiné la plasticité fonctionnelle d'E1E2 et recherché si une importante divergence génétique entre des hétérodimères E1E2 pouvait être lié à l'existence de fonctions ou conformations particulières. Le VHC est connue pour être également capable d'infecter et de se répliquer dans les lymphocytes B (LB). De plus, il a été observé que les variants du VHC isolés à partir de LB sont génétiquement divergents des variants isolés du foie ou du sérum. Nous avons émis l'hypothèse que E1E2 pouvait porter des déterminants majeurs du lymphotropisme, à travers la sélection de fonctions ou de propriétés génétiques particulières. Nous avons isolé des séquences E1E2 complètes à partir de LB et de sérum et observé une forte spécialisation des E1E2 isolé des LB pour l'infection des LB, mais pas des hépatocytes. Nos résultats ont montré que de nouvelles fonctions peuvent émerger de la plasticité conformationel de E1E2, et sont dépendantes de l'existence et la régulation de nombreux dialogues entre ces deux protéines.

Dans un second temps, nous avons examiné la co-évolution de E1 et E2 en étudiant des souches du VHC génétiquement proche afin d'identifier précisément des dialogues ou

interactions importantes pour le processus d'entrée. En effet, nous sommes parvenus à identifier un dialogue conservé entre E1 et le domaine III de E2 (E2 DIII), critique pour les processus d'attachement et de fusion du VHC. Par ailleurs, dans une seconde approche, nous avons aussi reconstitué grâce à une approche bio-informatique les réseaux de co-évolution au sein de E1E2. Cette approche a mis en évidence une co-évolution très importante entre E1 et E2, mais a également suggérer de potentiel changement de conformations au sein de l'hétérodimère. Ces suggestions corrélaient également de manière importante avec nos précédents résultats expérimentaux, mais également avec des précédents résultats proposés dans la literature. Ainsi, il est apparu de ces résultats que E2 est sans doute une protéine de fusion, capable de se replier sur elle-même via le repliement de son domaine III et l'aide critique de E1.

Ainsi, ces différents travaux soulignent l'implication de E1 et E2 au sein de dialogues à la fois fins et complexes, qui régulent à la fois les conformations et les fonctions de l'hétérodimère. Ainsi, cela suggère que l'hétérodimère E1E2 représente plutôt une unité fonctionnelle et structurale unique, plutôt que l'association de deux protéines aux fonctions distinctes.

Table of Content

Abbreviations

A
AA   Amino Acid
Ago   Argonaute
AID   Activation-induced cytidine deaminase
Alb   Abumin
ALT   Alanine aminotransferase
Apo   Apolipoprotein
ASPG-R   Asialoglycoprotein receptor

B
BDV   Border disease virus
BRG   Balb/c Rag2$^{-/-}$ γ-c$^{-/-}$
BVDV   Bovine viral diarrhea virus

C
CDC   Center for disease control and prevention
CE   Cholesterol ester
CEE   Central european encephalitis
CLDN1   Claudin-1
cLDs   Cytosolic lipid droplets
CLR   Classic C-type lectin receptors
CRD   C-terminal carbohydrate recognition domain
CSFV   Classical swine fever virus

D
DAAs   Direct acting agents
DC   Dendritic cells
DC-SIGN   Dendritic cell-specific intracellular adhesion molecule-3grabbing non-integrin
DGAT-1   Diacylglycerol O-acyltransferase 1
DHF   Dengue hemorrhagic fever
DI   Domain I
DII   Domain II
DIII   Domain III
dsRNA   Double stranded RNA
DV   Dengue virus

E
EC2   Second extracellular loop
EGF   Epithelial growth factor
EGFR   Epidermal growth factor receptor
eIF   Eukaryotic initiation factor
EL1   First extracellular loop

EMCV	Encephalomyocarditis virus
EphA2	Ephrin A2 receptor
ER	Endoplasmic reticulum
ERKs	Extracellular signal-regulated kinases
EWI-2wint	EWI-2 without its N-terminus

F

Fah	fumarylacetoacetate hydrolase
FRG	Fah$^{-/-}$ Rag2$^{-/-}$ γ-c$^{-/-}$

G

GAG	Glycosaminoglycans
GFP	Green fluorescent protein
gt	Genotype

H

HA	Influenza hemagglutinin
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVcc	Cell-culture grown HCV
HCVpc	Primary culture derived HCV
HCVpp	HCV pseudoparticles
HCVsp	Patient serum-derived HCV particles
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
HSPG	Heparan sulfate proteoglycan
Huh7	Human hepatoma cells 7
HVR1	Hypervariable region 1

I

IDL	Interlediate density lipoprotein
IFN	Interferon
IFNR	IFN receptor
IG	Immune globulin
IGIV	i.v. immune globulin
Igs	Immune globulins
IgVR	Intergenotypic variable region
IL	Interleukin
IL2	Interleukine 2
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
ISG	IFN-stimulated genes
IU	Infectious units

J

JAK	Janus kinase
JAM	Junction adhesion molecule
JEV	Japanese encephalitis virus
JFH-1	Japanese fulminant hepatitis
JNKs	c-Jun amino-terminal kinases

K

kDa	Kilodalton
KUN	Kunjin virus

L

L-SIGN	Liver-lymph node specific ICAM-3 grabbing non-integrin
LD	Lipid droplets
LDL	Low-density lipoprotein
LDL-r	Low-density lipoprotein receptor
LEL	Large extracellular loop
LPL	Lipoprotein lipase
LSEC	Liver sinusoidal endothelial cells
luLD	Lumina lipid droplets
LVP	Lipo-viro-particle

M

MAPK	Mitogen-activated protein kinase
MC	Mixed cryoglobulinaemia
miR-122	MicroRNA-122
MLV	Murine leukemia virus
MOI	Multiplicity of infection
MTase	Methyltransferase
MTP	Microsomal triglyceride transfer proteins
MTP	Microsomal triglyceride-transfer protein

N

NCR	Non-coding region
NHL	Non-Hodgkin's lymphoma
NK	Natural killer cells
NPC1L1	Niemann-Pick C1-like 1 cholesterol absorption receptor
NS	Non-structural protein
Nt	Nucleotides
Nt Abs	Neutralizing antibodies
NTBC	2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione
NTPase	Nucleoside triphosphatase

O

OCLN	Occludin
ORF	Open reading frame

P

PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PHH	Primary human hepatocytes
PI3K	Phosphoinositide 3-kinase
PRRs	Pattern recognition receptors

R

RdRp	RNA-dependent RNA-polymerases
rER	Rough ER
RF	Replicative form
RIG-I	Retinoic acid-inducible gene I
RISC	RNA-induced silencing complex
RSSE	Russian spring-summer encephalitis
RTK	Receptor tyrosine kinase
RTPase	RNA triphosphatase

S

sE2	Soluble E2
SEC	Sinusoidal endothelial cells
sER	Smooth ER
siRNA	Small interfering RNA
SR-BI	Scavenger-receptor BI
STAT1	Signal transducer and activator of transcription 1
SVR	Sustainable viral responses

T

TBEV	Tick-borne encephalitis microdomain
TEAs	Tetraspanin-enriched areas
Tf	Transferrin
TfR	Transferrin receptor
TG	triacylglycerols
TLR	Toll-like receptor
TMB	Transmembrane

U

uPA/SCID	Urokinase plasminogen activator/severe combined immunodeficiency
UTR	Untranslated region

V

VLDL	Very low-density lipoprotein

VLPs	Virus like particles
VR	Variable domain
VSV	Vesicular stomatitis virus
VSV-G	VSV envelope glycoprotein G

W

WHO	World health organisation
WNV	West nile virus

Y

YFV	Yellow fever virus

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Preamble

Make a long story short

The hepatitis C Virus (HCV) was recognized as a distinct viral hepatitis-causing agent in 1975. After being qualified as a non-A non-B hepatitis virus, the identification of the infectious agent encountered several difficulties for more than a decade until the virus was fully identified in 1989. This identification was allowed by the isolation of a cDNA clone obtained from the serum of a non-A non-B infected chimpanzee, opening the way for fundamental and clinical research. During the twenty four years that followed, impressive advances have been continuously made to increase our understanding of this virus, from the characterization of its life cycle to the resolution of some of its proteins structure. Importantly, these progresses have led to the development of more and more efficient therapeutic strategies in order to restrain the pandemic and improve patients care.

However, Hepatitis C Virus is still today one of the major causes of chronic liver disease worldwide and more particularly, the leading cause of liver transplantation. The recent development of direct acting-agents (DAAs) against nonstructural viral proteins has led to serious progress in patient treatments. However, these compounds still raise some issues. Their use as combination agents with non-specific treatments induces serious side effects and patient contradictions. In contrast, their use as monotherapy agent seriously increases the risk of emergence of resistant variants in the next few years. These facts highlight the need to continue to increase our fundamental knowledge about the HCV life cycle. Especially, viral entry and viral assembly, two critical steps of the virus life cycle, constitute promising targets for the establishment of multi therapies against HCV.

With the objective to decipher the molecular mechanisms of viral entry, the present work aims to bring new material for this purpose. The first part of this manuscript will be dedicated to put this work in context. We will first review the large viral family from which HCV is classified. Then, we will resume more particularly the current knowledge about molecular and structural HCV biology, virus life cycle and

pathogenesis, with a particular emphasis on the virus entry process. The second part of the manuscript will describe the experimental and theoretical results we obtained on virus entry. Studies on HCV envelope glycoproteins dialogs and co-evolution networks, extra-hepatotropism entry determinants and on potential new therapeutic approaches will be presented. Finally, the third part of this manuscript will be dedicated to the conclusions and perspectives highlighted by our works.

PART

I
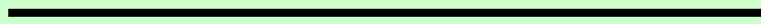
INTRODUCTION

CHAPTER I

The *Flaviviridae* Family

When the Hepatitis C Virus (HCV) was identified in 1989, important analogies were observed between its genome organization (i.e. viral proteins sequences) and that of *Flaviviridae*, a large positive RNA virus family. However, despite these similarities, HCV presents unique molecular and genetic features. These particular properties led HCV to be classified as a unique member of a new *Flaviviridae* genus, the hepacivirus. Prior to focus on HCV, this chapter will be dedicated to the presentation of the three other genera of the *Flaviviridae* family. Indeed, a better understanding of the HCV biological mechanisms and pathogenesis necessarily requires to place our analysis in a larger context. Understanding the origins and relations of some HCV features with other related viruses is critical to know whether these features are unique adaptations or not, as well as to take one more step towards the elucidation of several protein functions or mechanisms that still remain poorly characterized for HCV. First, flaviviruses and pestiviruses will be introduced and compared together. These two genera both harbor well characterized viruses that present a large tropism such as Dengue virus (DV) or the Bovine viral diarrhea virus (BVDV). Then, pegiviruses and non-HCV hepaciviruses will be reviewed. Although these viruses are still poorly understood, they harbor a restricted tropism and are more related to HCV than any other *Flaviviridae*.

I.   General Presentation

The *Flaviviridae* family is classified into four genera: the flaviviruses, the pestiviruses, the hepaciviruses and the pegiviruses (Figure 1) (Calisher et al., 2003; Lindenbach et al., 2007; Kolekar et al., 2012). These enveloped viruses are structured by a lipid membrane bilayer harboring one or different species of glycoproteins. The lipid bilayer surrounds a nucleocapsid, where a positive single stranded RNA genome is complexed with capsid proteins (Lindenbach et al, 2007). These viruses can infect a very large panel of cell types including immune cells and epithelial cells, and tissues such as hepatic tissues, neurons and the gastrointestinal tract. Commonly, the viral RNA encodes for a large polyprotein cleaved by several cellular or viral proteases into a set of structural and non-structural proteins involved respectively in virus structure or in viral replication and assembly.
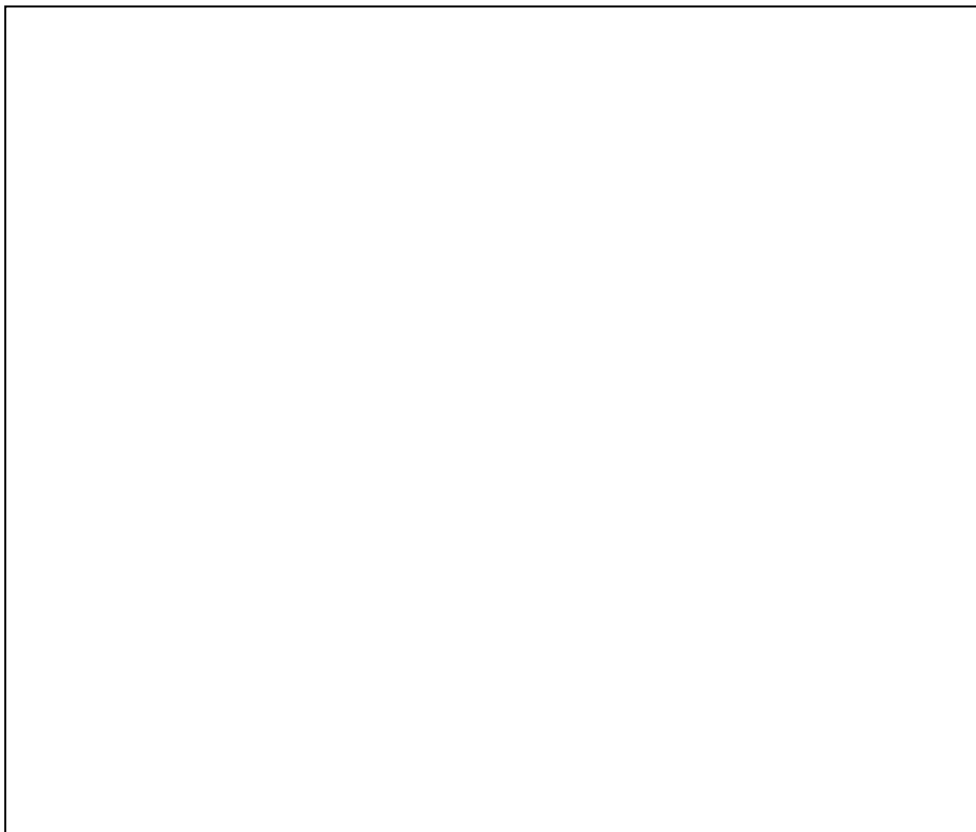
Figure 1. Phylogenetic analysis of the RNA dependent RNA polymerase (RdRp) of the representative members of the hepacivirus, pegivirus, pestivirus, and flavivirus genera using RD-based alignment-free method (Adapted from Kapoor et al., 2011).

Flaviviridae usually bind to specific cellular receptors through their envelope glycoproteins and penetrate into the cell by a receptor-mediated endocytosis process (Figure 2). The acidification of the endosome then lowers down the pH and induces the fusion between the viral membrane and the endosomal membrane, allowing the release of the viral RNA into the cytosol. Viral replication and assembly occur into the cytosol, in rearranged membranous complexes close to the nucleus (Figure 2). Viral RNA is translated as a large polyprotein processed by several cellular or viral proteases to release structural and non-structural proteins. The RNA-dependent RNA polymerase (RdRp) uses the viral RNA as a template to produce minus-stranded RNA intermediate to allow production of new positive sense RNA genomes (Figure 2). After viral genome replication and encapsidation, viral particles assemble by budding into intracellular membranes (most likely the endoplasmic reticulum membrane) and are released from the cell after transit via the secretory pathway (Figure 2).
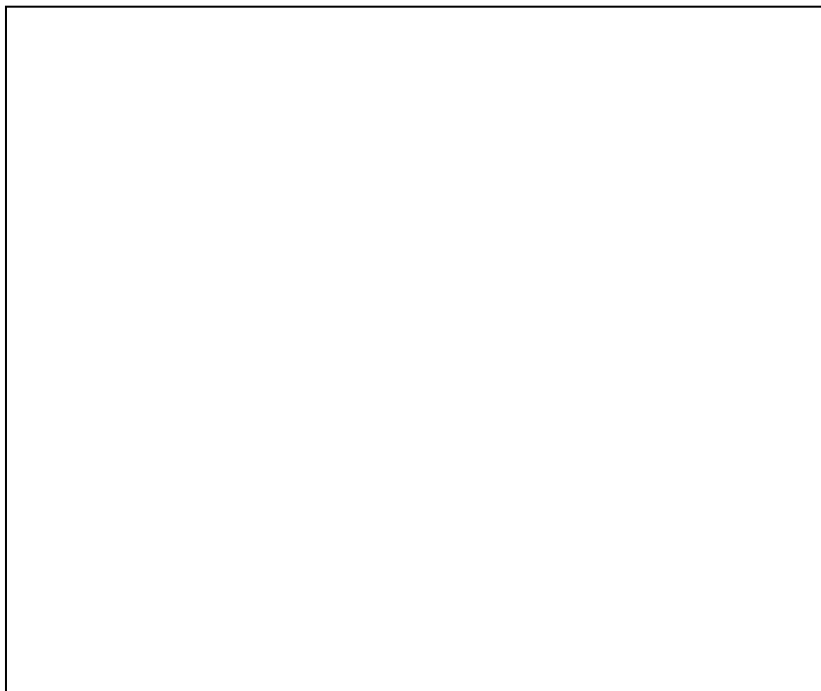
Figure 2. **Flaviviridae Life Cycle**. After virus entry through endocytosis, viral RNA is translated into the cytosol as a large polyprotein co- and post-transcriptionally cleaved into several structural and non-structural proteins. Viral RNA is used as a template to produce minus-stranded RNA intermediates use to subsequently synthesize new positive-stranded RNA. Viral particles are assembled through budding into intracellular membranes and released into the extracellular environment after transit via the secretory pathway. (Lindenbach et al., 2007).

II.   Flaviviruses and Pestiviruses

1. **Epidemiology and prevalence**

The Flaviviruses represent the larger *Flaviviridae* genus with around 70 viruses dispersed all over the globe. These viruses are mainly arthropods-borne human viruses whereas few others infect only insects. Among these viruses, several are responsible of major health concern worldwide including Dengue virus (DV), the Japanese encephalitis virus (JEV), the Yellow Fever Virus (YFV) or the West Nile Virus (WNV) (Mackensie et al., 2004). These pathogens induce a large variety of diseases in humans such as fever, encephalitis or even hemorrhagic fever as dengue hemorrhagic fever (DHF) (Martina et al., 2009; Pfeffer et al., 2010). As mentioned previously, a major characteristic of these viruses that explains their rapid spread around the world is their ability to be arthropod-borne (Pfeffet et al., 2010; Cleton et al., 2012). Indeed, these viruses are mainly vectored by particular genera of mosquitoes termed *Aedes* (i.e *Aedes aegypti* or *albopictus*) or *Culex* (i.e. *Culex pipiens*, *tarsalis* or *quinquefasciatus*) that are present in a large variety of countries and areas around the world (Cleton et al., 2012).

In strong contrast to Flaviviruses, the number of Pestiviruses that have been identified and characterized is far less important. Pestiviruses are worldwide pathogens that infect mainly ruminants or swine (Thiel et al., 1996; Lindenbach et al., 2007). The most defined Pestiviruses are represented by the type member Bovine Viral Diarrhea Virus (BVDV) divided into two main species BVDV-1 and BVDV-2, the Classical Swine Fever Virus (CSFV) and the Border Disease Virus (BDV) (Liu et al., 2009). However, the recent identification of new Pestiviruses isolated from other species (such as Girafe) may highlight that we only have a restricted view of these viruses and of their potential host species (Thiel et al., 1996).

Among Flaviviruses, Dengue virus is one of the major concerns for health authorities in the world. Indeed, 2.5 billion people live in risk areas, represented by around 100 countries where *Aedes* mosquitoes are endemic (Figure 3). So far, Dengue virus

infects 50 to 100 million people worldwide and causes the death of 20000 patients each year (World Health Organization – Dengue and Hemorrhagic fever). Today, the risk areas tend to dramatically extend as global warming is increasing and climate-temperate countries are now suitable for *Aedes* mosquitoes. Continuous growth of industrialized cities in risk areas, increase of global aerial transportation and decrease in mosquitoes control efforts also dramatically contributed to the expansion of Dengue virus during the last decades (World Health Organization). West Nile virus also represents an important health issue worldwide by being endemic in Europe, North America, Central and West Asia, Africa and Oceania (Debel et al., 2001). In North America, the virus initiated a brutal outbreak in New York in 1999 that killed 18 people before spreading in the rest of the United States. Between 1999 and 2009, the West Nile Virus infected more than 37000 people in the United States and killed up to 1500 of the infected patients (Center for disease control and prevention - USA). Although the large majority of Flaviviruses are transmitted by mosquitoes, tick-borne viruses represent also a significant clade within the Flavivirus genus. For example, the tick-borne encephalitis virus (TBEV) is the major cause of meningitis or meningoencephalitis in humans (Kaiser et al., 2008).
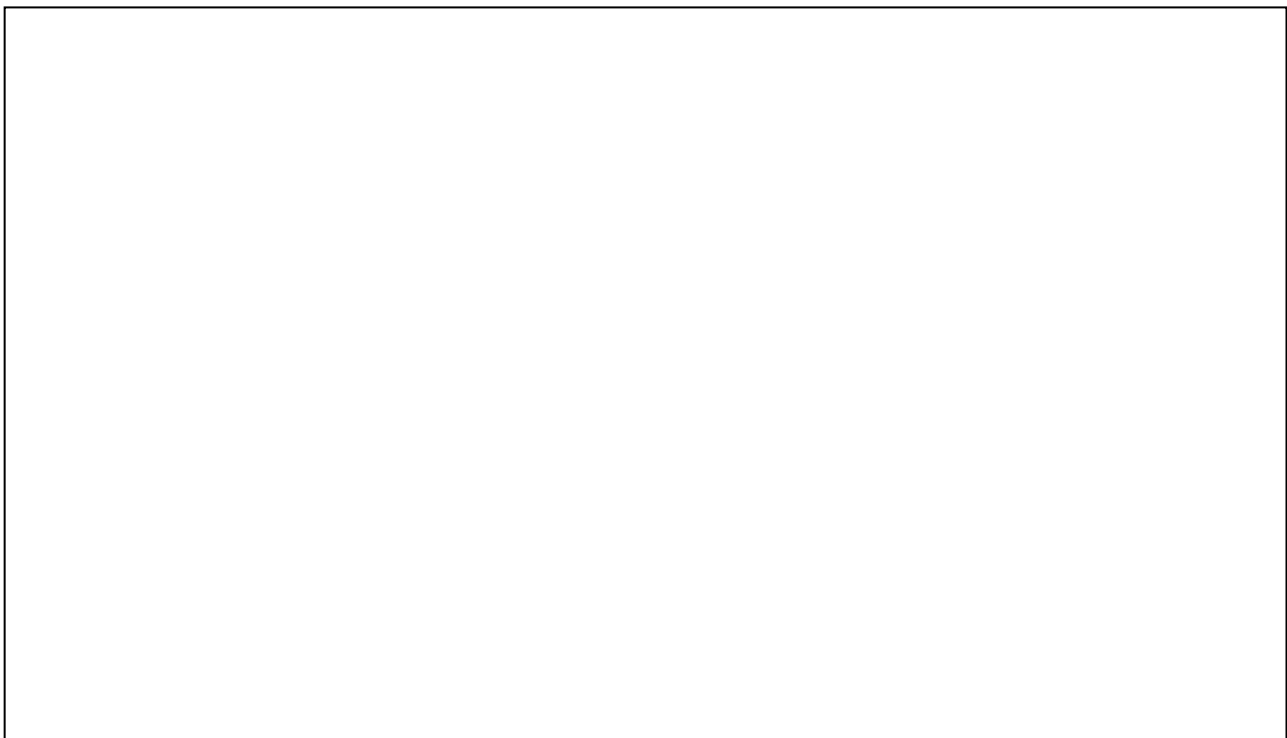
Figure 3. World distribution of Dengue Virus (WHO 2007, CDC 2008, ProMed-Mail 2008).

This pathogen is present in Europe and Asia mainly in the form of two related viruses, the central European encephalitis (CEE) and the Russian spring-summer encephalitis (RSSE) (Ecker et al., 1999; Mansfield et al., 2009; Hubalek et al., 2012). These viruses may cause permanent neurologic damage in 10 to 20% of infected patients whereas infection is fatal in only 2 to 3% of the cases.

Pestiviruses can cause a large panel of disease and may represent severe economic issues for agronomic industry (Baker et al., 1995; Thiel et al., 1996). BVDV viruses are extensively present all around the world. BVDV-1 dominates Europe where important numbers of cattle have been identified to present a strong proportion of seropositive animals (Lindberg et al., 2006). For example, it is estimated that 95% of the ruminants in United Kingdom present anti-BVDV-1 antibodies (Paton et al., 1998). A strong link between animal density in farm and seropositivity has been established (Vega et al., 1997). BVDV-1 infection often leads to minor clinical syndrome in adult animals, explaining why traces of this virus are so important in European cattle. In contrast, BVDV-2 infection causes severe diseases such as acute and critical hemorrhagic syndrome in cattle (Pellerin et al., 1994). As BVDV-2, CSFV also causes severe hemorrhagic syndrome that may lead to significant mortality (Dahle et al., 1992; Leifer et al., 2013). Pestivirus infection is mainly transmitted by persistently infected animals through saliva, nasal secretions, urine or feces. Infection can also be transmitted through placenta to future new-born animals (Fray et al., 2000). Infection can be immediately lethal or cause persistent infection (Peterhans et al., 2010) in the new born that may then result in a lethal mucosal disease. Development of a severe mucosal disease in animals is commonly induced by the passage of the virus from a non-cytopathogenic to a cytopathogenic state (Peterhans et al., 2010).

The development of treatments against Flaviviruses remains challenging. Today, no efficient and specific anti-viral compounds or vaccines exist to treat the flaviviruses of greatest concern, such as Dengue or West Nile virus. The control of mosquito's population through eradication campaigns or prevention still remains the best tool to reduce the spread of the virus (World Health Organization). In contrast to Flaviviruses, vaccination with live attenuated Pestiviruses strains or inactivated

Pestiviruses is available (Blome et al., 2006). However, vaccination of infected animals often leads to severe side effects that may be lethal.

2. Viral Particle Structure

Flaviviruses particles are small (~50nm) particles surrounded by a lipid bilayer where two glycoproteins, E and M, are incorporated (Lindenbach et al., 2007; Smit et al., 2011; Zhang et al., 2013). Pestivirus particles are similar in size, from 40 to 60 nm in diameter (Ohmann et al., 1990). However, their buoyant density is quite lower than flaviviruses particles, ranging between 1.12-1.15 g/ml versus 1.20 to 1.23 for flaviviruses (Lindenbach et al., 2013). Mature Dengue particle have a relatively smooth surface, with 90 E dimers adopting a head-to-tail antiparallel conformation and lying parallel to the lipid bilayer (Figure 4A) (Kuhn et al., 2002; Zhang W. et al., 2003). During virus maturation, viral particles are immature and larger than mature particles (~60nm). Immature particles harbor 60 PrM-E complexes associated as trimeric heterodimers that protrude onto the particle surface (Figure 4B) (Zhang Y. et al., 2003). When viral particles are secreted outside the cell, trimers dissociate and heterodimers dramatically rearrange as 90 antiparallel dimeric complexes to form mature viral particle (Figure 4B) (Zhang et al., 2004). During virus fusion, E dimers rotate and rearrange as trimeric structures that protrude onto the viral surface and fold over to allow the merging of the viral membrane with the endosomal membrane (Modis et al., 2004; Kielian et al., 2006; Modis, 2013).
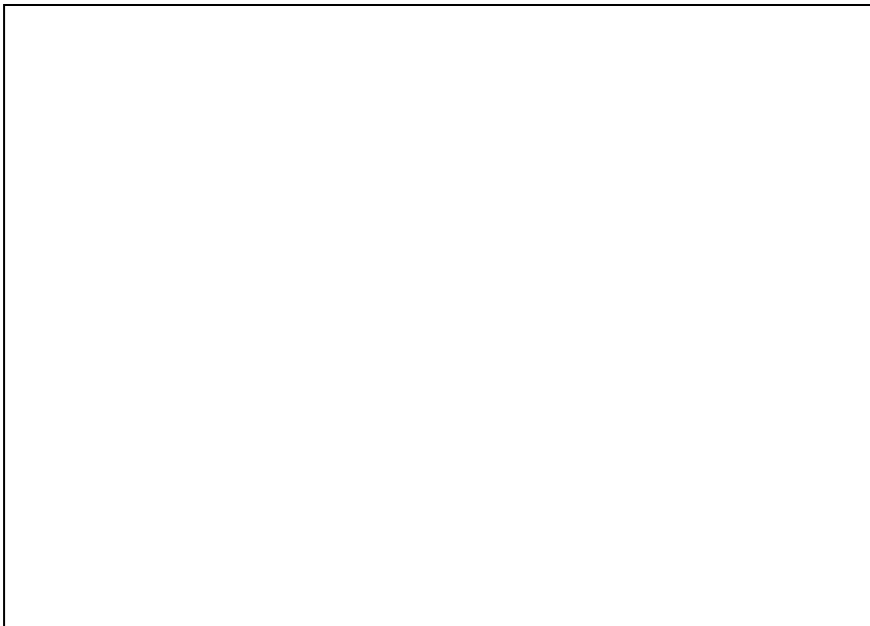
Figure 4. A. Structure of mature Dengue viral particle at 28°C. Particle harbor an icosahedral symmetry and is composed of 90 dimers of the surface glycoprotein, protein E (Adapted from Rey et al., 2013). **B.** Dengue envelope proteins structural organization in immature and mature viral particles (Adapted from Lindenbach et al., 2007).

Consistently with their lower buoyant density, pestivirus particles are associated with serum lipid components in patient although the precise chemical composition of the viral particles is not clear yet (Laude et al., 1987). Interestingly, pestivirus particles can survive to a large range of pH without being inactivated, in contrast to flavivirus particles (Liess et al., 1981). Pestiviruses harbor onto their surface two envelope glycoproteins, E1 and E2 that mediate the binding and the fusion of the viral particles with host cells.

3. Genome organization and translation

Despite some disparities, flaviviruses and pestiviruses have a relatively similar genome organization. Flaviviruses and pestiviruses both harbor a single stranded RNA positive genome of respectively 11 and 12.3 kb encoding for a single open reading frame (ORF) flanked by a 5′ and a 3′ non-coding region (NCR) (Figure 5). 5′ NCR of pestiviruses remains larger than the one of flaviviruses (~380 nucleotides versus ~100 nucleotides) whereas 3′NCR of flaviviruses is the largest one (400 to 700 nucleotides versus ~200 nucleotides) (Lindenbach et al., 2007). Moreover, although both genus RNA lack a 3′ poly-(A) tail, only flaviviruses RNA have a 5′cap and thus a viral translation that is cap dependent (Figure 5) (Thurner et al., 2004). Pestiviruses RNA do not present any 5′capping and use an internal ribosome entry site (IRES) to direct cap-independent translation (Figure 5) (Brock et al., 1992). Flaviviruses NCRs are poorly conserved (Markoff et al., 2003). However, despite variability in primary sequences, these regions present conserved structural similarities and patterns that are critical for translation and RNA replication (Brinton et al., 1988; Thurner et al., 2004). Similarly, pestiviruses NCRs contain several conserved terminal sequences and stem loops essential for translation and replication (Figure 5). More precisely, 5′ NCR of both genera are essential for initiation of RNA translation and to mediate positive-strand RNA synthesis during RNA replication (Frolov et al., 1998; Markoff et al., 2003; Deas et al., 2005). On the other hand, 3′ NCR are involved in the enhancement of viral translation and direct minus-stranded RNA synthesis (Khromykh et al., 2001; Markoff et al., 2003; Isken et al., 2004; Holden et al., 2006).

Viral translation of flaviviruses and pestiviruses produces a large polyprotein of respectively 3400 or 4000 amino acids co- and post-translationnaly cleaved into 11 or 12 distinct proteins respectively (Figure 6). Regarding the flaviviruses ORF, the 3 first proteins translated represent the structural protein (Capsid, PrM and E) whereas the 7 that follow represent the flaviviruses non-structural proteins involved in translation, replication and assembly (NS1-NS2A-NS2B-NS3-NS4A-2K-NS4B-NS5) (Lindenbach et al., 2007).
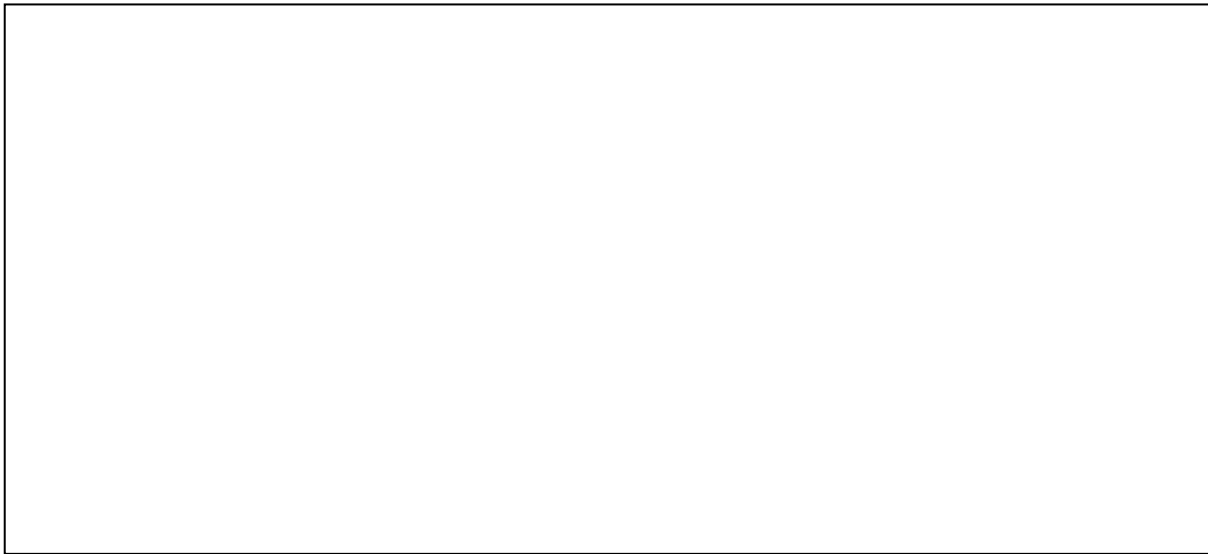
Figure 5. Genomic organization of flavivirus and pestivirus viral RNA. ORF lengths are indicated for each virus genus. Below the ORF, Non Coding regions (NCR) are highlighted and structural NCR features, hairpins loops and domains are indicated. ST, Stem loop; CS or RCS, Conserved sequences (Adapted from Lindenbach et al., 2007) .

Unlike any other *Flaviviridae,* the first protein translated and produced from the pestiviruses viral RNA is a non-structural protein, N^{Pro}, an auto protease that auto-cleaves its junction with the Capsid protein that is the next translated protein within the ORF (Wiskerchen et al., 1991). Capsid protein translation is followed by the translation of the three other structural proteins, E^{rns}, E1 and E2 and of seven non-structural proteins (p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B) (Figure 6).

During translation, the host signal peptidase is involved in the cleavage of the flaviviruses and pestiviruses. This peptidase dissociates flaviviruses Capsid, PrM and E from each other as well as the NS1-2K and the 2K-NS5 polypeptides whereas it targets pestiviruses C/E^{rns}, E1/E2, E2/p7 and p7/NS2 junctions (Figure 6) (Heinz et

al., 1993; Stark et al., 1993; Rodenhuis-Zybert et al., 2010). The enzyme responsible for the pestiviruses E^{rns}/E1 cleavage is not yet identified.

The release of the non-structural proteins of the two genera presents however more disparities. The flaviviruses NS2B-NS3 protease complex ensures the cleavage of the non-structural protein junctions from NS2A to NS5 (Figure 6) whereas the enzyme that cleaved the NS1/NS2A junction, likely ER resident enzyme, has not been identified yet (Figure 6). In contrast, the pestiviruses non-structural proteins release requires first the auto-cleavage of the NS2/NS3 junction by NS2 (Figure 6). This initial cleavage then allows the processing of the NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions by the NS3-NS4A serine protease complex (Heinz et al., 1993; Stark et al., 1993; Rodenhuis-Zybert et al., 2010).
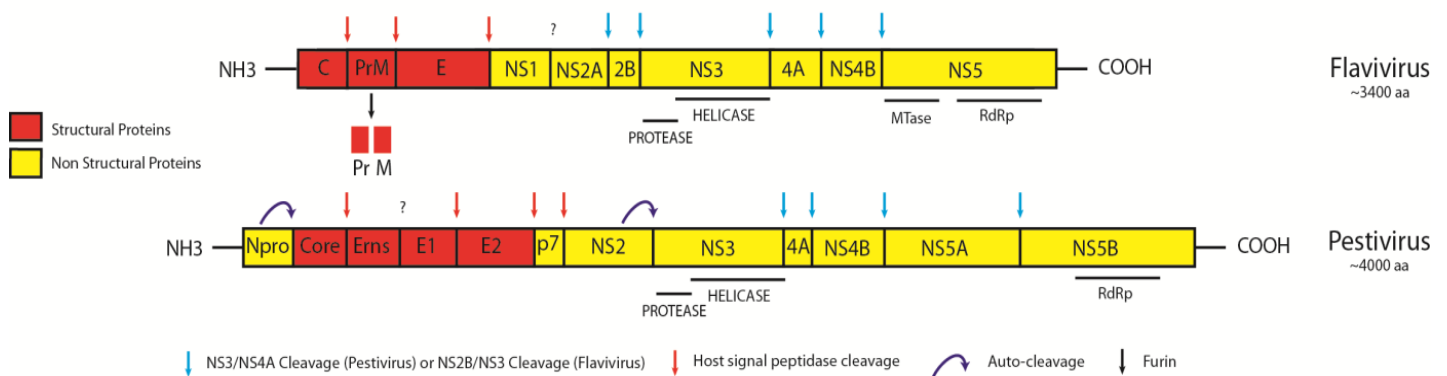
Figure 6. Flavivirus and pestivirus polyprotein organization and processing. Polyprotein lengths are indicated below the genus name. Red and yellow blocks represent respectively the structural and non-structural proteins. Polyprotein processing is indicated: Red and blue arrows indicate junctions processed respectively by the host signal peptidase and by the NS3/NS4A or NS2B/NS3 serine protease. Black arrow represents the processing of PrM by the furin. Curved arrow indicates the auto-processing of the NS2/NS3 junction by NS2. Particular protein functions are indicated. RdRp, RNA-dependent RNA-polymerase.

4. Viral proteins

a) Structural proteins

The flaviviruses and pestiviruses Capsid proteins are both highly basic proteins of respectively 11 and 14 kb (Ma et al., 2004; Lindenbach et al., 2007). Both capsid proteins C-terminus domains, which serve as signal peptide to allow the translocation of PrM and E^{rns} into the ER, are cleaved to generate mature capsid

proteins (by the flaviviruses NS3 or by the signal peptide peptidase in the context of pestiviruses infection) (Lobigs et al., 2004; Heinmann et al., 2006). Capsid proteins interact with a large number of viral or cellular components, including the viral RNAs. They form dimeric structures that assemble to form the viral nucleocapsid. It is hypothesized that interaction between flaviviruses Capsid dimer and viral RNA mediate assembly of nucleocapsid (Kiermayr et al., 2004).

The flaviviruses E and M proteins are the flavivirus envelope glycoproteins incorporated onto virion surfaces. E is the major flavivirus glycoprotein (53 kDa; ~500 aa) that mediates both the specific binding of the viral particles to the host cell and the fusion of the viral membrane with the endosomal membrane. This glycoprotein is N-glycosylated and the immunodominant one, as it is largely recognized and targeted by the host immune system (Lindenbach et al., 2007). In mature viral particle, E forms 90 dimers that lie onto the particle surface (Figure 7) and dramatically rearrange during virus fusion (See Introduction – Chapter V I.4.c). These rearrangement are mediated by a core of three functional domains, named DI, DII and DIII (D for domain) (Figure 7).The second glycoprotein, M (7-9 kDa; 75 aa), is a small protein fragment present onto the viral membrane and results from the proteolytic cleavage of a larger protein, PrM (26 kDa; 166 aa), during virus maturation. Although M has no reported role in virus entry, PrM has been shown to be critical for the correct folding of E during virus maturation. During virus maturation into the ER, PrM-E are associated as sixty trimeric heterodimer complexes that protrude onto the particle surface (Figure 4). During virus budding and passage in the trans-golgi network, heterodimers dramatically rearrange as dimeric complexes, the Pr-M cleavage site become accessible to furin and the Pr fragment is released from M (Kielian et al., 2006; Lindenbach et al., 2007). The Pr peptide remains complexed with E to protect the protruding fusion peptide from low pH until the virus exits the cell (Figure 4). The neutral environment of the extracellular media completes the maturation of the viral particles and of the heterodimer complexes by releasing the Pr fragment from the M-E heterodimer (Kunh et al., 2002; Zhang Y et al., 2004).
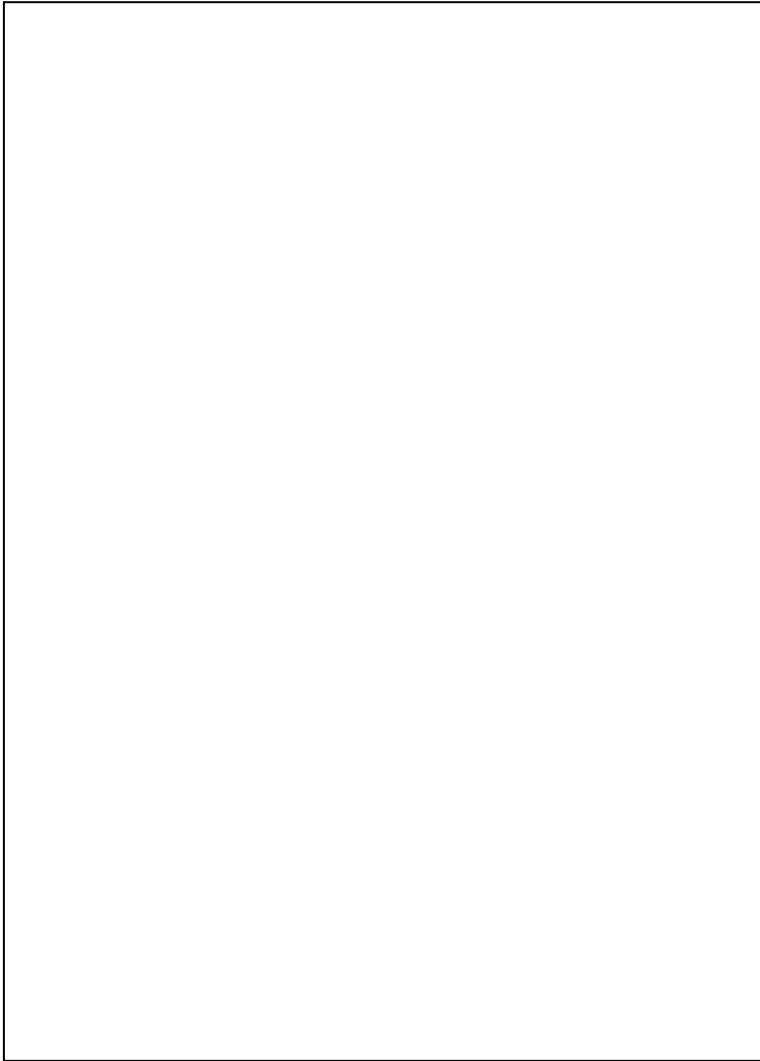
Figure 7. Dengue E envelope glycoprotein structure and organization. E dimer organization onto mature viral particle surface (A) and E dimer structural organization (B) (Modis et al., 2004). Dengue E domain I, II and III are colored respectively in red, yellow and blue. A schematic linear representation of E is showed in C.

Pestiviruses E1 and E2 glycoproteins are 2 N-glycosylated type I transmembrane proteins as Dengue E (Weiland et al., 1990). During polyprotein processing, an Enrs-E1-E2 precursor is produced and E2 is the first protein to be fully released and matured (Rumenapf et al., 1993). The Enrs-E1 precursor is then cleaved during later stage of polyprotein processing. During viral particle assembly and maturation, E2-E2 and E1-E2 heterodimeric complexes are formed and incorporated onto the particle surface via their transmembrane domains (Thiel et al., 1991). The E1-E2 heterodimer is recognized to be the fusion complex of the pestivirus, involved in particle binding and fusogenic conformational changes (Lindenbach et al, 2007).

Unlike flaviviruses, E1 (192 aa) and E2 (363 aa) are much smaller than E and present distinct features and functions. Indeed, both E1 and E2 pestiviruses envelope glycoproteins are considered as playing a role in viral entry and no cleavage during virus maturation has been reported for these proteins (Rumenapf et al., 1993; El

Omari et al., 2013). Pestiviruses E2 is the binding and immunodominant protein (Tscherne et al., 2008; Chang et al.; 2010) whereas it has been strongly suggested that E1 is the fusion protein as the recently described BVDV E2 structure does not present any fusion protein features (El Omari et al., 2013). Pestiviruses E2 structure is described in Introduction – Chapter V I.3.c. So far, the binding and fusion functions of pestiviruses would not be harbored by a single envelope glycoprotein but by two distinct proteins with complementary functions during entry.

The pestiviruses E^rns protein is a highly glycosylated protein assembled as homodimer at the viral particle surface through an unusual C terminal anchor (Langedijk et al., 2002). This protein has no reported role in viral entry but is secreted from the infected cells as a soluble protein (Rumenapf et al., 1993; Weiland et al., 1992). Surprisingly, this protein presents a ribonuclease activity that is important for virus infectivity as the use of antibodies targeting the ribonuclease activity neutralizes virus infection (Schneider et al., 1993). E^rns binds to cellular membrane and is also translocated into the cell cytoplasm, probably indicating that it targets particular intracellular proteins or functions (Langedijk et al., 2002). E^rns is also potentially involved in immune evasion by interfering with the IFN signaling (Luo et al., 2009).

b) Non-Structural proteins NS

The Flaviviruses NS1 protein is an N-glycosylated protein of 46kb largely retained within infected cells (Muller et al., 2013). This protein, which assembles as homodimer, is largely hydrophilic and surprisingly lacks a transmembrane domain even though it associates with ER-membrane. NS1 is essential for the early stages of RNA replication and is thus essential for virus production (Lindenbach et al., 1997; Muller et al., 2013). Infected cells secrete a slight proportion of soluble form of NS1, whose role is likely to enhance subsequent virus infection (Alcon-LePoder et al., 2005).

The flaviviruses NS2A and NS2B proteins are small ER-membrane associated hydrophobic proteins (Xie et al., 2013). Flaviviruses NS2A takes part in the RNA

replication complex by interacting with NS3, NS5 and the RNA genome (Rosi et al., 2007; Xie et al., 2013). NS2A is also involved in viral assembly as experiments using the Kunjin Virus (KUN), a non-pathogenic subtype of the West Nile Virus, have shown that this protein is involved in the biogenesis of virus-induced membranes (Leung et al., 2008). Moreover, NS2A is known to target host anti-viral responses by inhibiting interferon signaling during infection (Liu et al., 2006). NS2B is also critical for virus production. By forming complexes with NS3 as part of the NS2B-NS3 serine auto protease, this protein cleaves and releases the majority of the flaviviruses nonstructural proteins during polyprotein translation (Padmanabhan et al, 2006; Chappell et al., 2008). In contrast, *the pestiviruses NS2 protein* is a cysteine auto-protease involved only in the processing of the NS2/NS3 junction. However, the cleavage of this junction is essential for efficient polyprotein translation, virus assembly and production as well as for RNA replication (Agapov et al., 2004).

The flaviviruses NS3 protein is a large protein with an important number of functions. This protein is involved with NS2B in polyprotein processing through its N terminal third, allowing the release of most of the non-structural proteins (Lescar et al., 2008). NS3 generates the mature form of the capsid protein by cleaving the capsid C terminal anchor (Yamshchikov et al., 1994) and also processes its junction with NS2B (Lescar et al., 2008). The C terminal part of NS3 is involved in RNA replication and harbors a RNA-stimulated nucleoside triphosphatase (NTPase) function (Wengler et al., 1993; Lescar et al., 2008) and a RNA unwinding activity (Warrener et al., 1993; Lescar et al., 2008). These functions are encoded by regions that possess important similarities with RNA helicases. The flaviviruses NS3 protein has also been shown to possess a RNA triphosphatase activity (RTPase) involved in the 5′ RNA capping (Wengler et al., 1993) as well as an ability to induce cellular apoptotic effects through activation of particular caspases (Shafee et al., 2003) . *The pestiviruses NS3 protein* interestingly present similar functions. Its N terminal domain harbors a serine protease domain and its C terminal domain possesses RNA helicase and NTPase functions (Gorbalenya et al., 1989). However, although the flaviviruses NS3 protease activity requires the protein upstream within the ORF

(NS2B), the pestiviruses NS3 protease requires NS4B to express its protease activity as a co- factor and process the non-structural proteins (Xu et al., 1997). This protease activity is critical for RNA replication and virus particle production (Xu et al., 1997).

The flaviviruses NS4A and NS4B proteins are small hydrophobic proteins involved in RNA replication. NS4A interacts with NS1 and colocalizes in RNA replication complexes (Mackenzie et al., 1996; Lidenbach et al., 1999). NS4B colocalizes with NS3 and double stranded RNA in replication sites within the ER-derived membranes (Miller et al., 2006). These two proteins also inhibit type I interferon signaling (Munoz-Jordan et al., 2003). *The pestiviruses NS4A and NS4B proteins* are also hydrophobic proteins that present however more dissimilar functions than flaviviruses NS4 proteins. Pestiviruses NS4A is a cofactor with NS3 of the protease complex that processes the majority of the non-structural protein through a serine protease activity (Xu et al., 1997). On the other hand, NS4B has been shown as being important for virus cytopathogenicity (Fernandez-Sainz et al., 2010; Gladue et al., 2011), possibly through the mediation of ER-membrane rearrangements that are required for RNA replication. More particularly, this protein possesses an NTPase activity that is critical for virus life cycle and viral spread (Gladue et al., 2011).

The flaviviruses NS5 protein is a highly conserved protein that harbors a large panel of functions. It is involved in viral replication and RNA synthesis, modification of viral RNA structural features and in the inhibition of host defense (Davidson, 2009). The N terminal region of NS5 harbors a methyltransferase (MTase) activity that transfers methyl group to capped RNA structures (Egloff et al., 2002). On the other hand, the C terminal region of NS5 possesses a strongly conserved RNA-dependent-RNA polymerase (RdRp) activity that synthesizes the new strand of viral RNA (Rice et al., 1985; Davidson, 2009). NS5 also stimulates NS3 functions in replication sites and enhances virus spread and pathogenesis through recruitment of inflammatory cells to the site of infection (Yon et al., 2005). Finally, NS5 is able to bind to interferon-α/β/γ receptors and thus inhibits signal transducer and activator of transcription 1 (STAT1) (Best et al., 2005). *The pestiviruses NS5A* protein is known to regulate viral

RNA replication. It has been shown that NS5A localizes into the ER (He et al., 2012) and interacts with the 3'NCR and NS5B to regulate viral RNA synthesis (Chen et al., 2012). NS5A inhibits viral replication when its concentrations exceed the one of NS5B (Scheng et al., 2012). In parallel, as NS5, *the pestiviruses NS5B* protein harbors a strongly conserved RdRp activity involved in the synthesis of new RNA strands.

c) The particular pestiviruses non-structural proteins

Pestiviruses viral RNA encodes for two particular non-structural proteins that possess no equivalent in the flavivirus genome. The first one is *the N^pro protein,* an autoprotease that processes the Npro/C junction to release the structural proteins (Tratschin et al., 1998). Although N^pro is probably dispensable for viral replication (Tratschin et al., 1998), it has been shown that its deletion may affect virus spread and infection (Ruggli et al., 2009). This is consistent with the fact that Npro also possesses an interferon antagonist activity (Bauhofer et al., 2007; Doceul et al., 2008), that is independent of its protease function. *The second protein is the p7 protein.* P7 is a small hydrophobic charged protein of 6 to 7 kDa that oligomerizes and can generate ion channels into viral membrane (Harada et al., 2000). By manipulating membrane permeability for ions, this protein is essential for virus production and assembly (Gladue et al., 2012; Guo et al., 2013) but not for RNA replication. During translation, the host signal peptidase may release uncleaved E2-p7 fragments. However, this form is not essential for the viral particle production (Harada et al., 2000).

5. Binding and tropism

Flaviviruses have a very large tropism and can infect many cell types through the use of multiple receptors. Immune cells, liver, epi- or endo-thelial cells constitute preferential target for flaviviruses such as Dengue virus or Yellow Fever Virus (Back et al., 2013; Schoggins et al., 2012; Kramer et al., 2001). More particularly, intradermal dendritic cells (DC) are the first cells to be infected during the early step of infection (Pham et al., 2012; Qian et al., 2011).

Pestiviruses also infect a large variety of cells at the early stages of infection including epithelial cells, endothelial cells, lymphoreticular cells and macrophages. However, during persistent infection, the panel of target cells is highly enlarged. Viruses can be found in most of the tissues such as neurons, the gastrointestinal tract or peripheral blood mononuclear cells (Lindenbach et al., 2007).

It is known that Dengue virus primary attachment to dendritic cells is dependent on the cell surface expression of C-type lectin DC SIGN that binds glycans present within E glycoprotein (Tassaneetrithep et al., 2003; Pokidysheva et al., 2006; Alen et al., 2012). West Nile Virus is also known to use DC SIGN to infect DC but in a glycan-independent manner (Davis et al., 2006). Very recently, molecules from the TIM-TAM family have been identified to be critical for Dengue attachment to host target cell through binding with the phosphatidylserine present on particle surface (Meertens et al., 2012). Glycosaminoglycans are also known to be critical for flaviviruses initial attachment (Kroschewski et al., 2003). Other molecules have been proposed to constitute flaviviruses receptors such as integrin or CD14 but the cell entry receptor(s) required for Dengue E binding, endocytosis and fusion still remains to be identified.

After binding, flaviviruses are internalized into the cell by a clathrin-dependent endocytosis mechanism. Acidification of endosomal compartment triggers E dimers reorganization as trimer structures (Modis et al., 2004; Kielian et al., 2006; Modis et al., 2013). These conformational changes allow the exposure and insertion of the E fusion peptide into the endosomal membrane and finally, the fusion and the release of the viral genome into the cytosol. Importantly, the lipid composition of the membranes is determinant for the efficiency and the pH threshold of viral fusion (Stiasny et al., 2003; Stiasny et al., 2004).

As flaviviruses, pestiviruses entry is a multi-step process that involves initial attachment and binding steps, followed by clathrin-dependent endocytosis and low pH triggered membrane fusion. However, unlike flaviviruses, virus envelope glycoproteins must be primed to respond to low pH triggering (Krey et al., 2006). Both E2 and E^{rns} envelope glycoproteins are critical for virus initial attachment to

target cell but only E1 and E2 are involved in cell entry (Hulst et al., 1997; Wang et al., 2004). E^{rns} is known to bind to glycosaminoglycans whereas E2 represents the cell tropism binding protein through binding to specific cell receptor(s) (Tscherne et al., 2008; El Omari et al., 2013; Liang et al., 2003). For the BVDV pestivirus, this cell entry receptor has been identified to be the bovine CD46 molecule. Concerning E1, the recent characterization of BVDV E2 structure has demonstrated that E2 does not present any pattern characteristic of a fusion protein (El Omari et al., 2013). Thus, it has been hypothesized that the fusion function is harbored by E1. However, this assumption remains to be experimentally proven.

III. Hepaciviruses and Pegiviruses

When HCV has been classified in 1989 as a Flaviviridae, it was the only member of a new family, the hepaciviruses (Choo et al., 1989). However, few years later, several cases of human hepatitis were found as not caused by commonly known human hepatitis virus from A to E (Simons et al., 1995-2). The serum of a patient "GB" suffering from hepatitis were isolated and introduced in tamarins. Two new viruses were identified and cloned after serial passages in animals and classified as two new members of the Flaviviridae family. These viruses, more related to HCV than any other *Flaviviridae*, were named GBV-A and GBV-B (Muerhoff et al., 1995).

GBV-A is a long adapted new world monkey virus that has been likely acquired during passage in tamarin (Leary et al., 1996). However, this virus has not been detected in human blood despite some serologic reactivity and is not associated clearly with any disease. Origins of GBV-B are more mysterious as it has not been identified in nature and the only virus isolated comes from the GB patient. However, this virus can cause hepatitis in tamarin or owl monkeys in contrast to GBV-A (Buck et al, 2001; Landford et al., 2003). Chimpanzee cannot be infected suggesting that this virus may not cause hepatitis in higher primates and in humans.

Following the identification of these two viruses, a related third one designed as GBV-C has been identified in both chimpanzee and human samples (Simons et al., 1995-1). Although no clear correlation with human disease could be established,

serological reactivity to this virus is not rare in the human population as persistent infection can last several years (Alter et al., 1997; Stapleton et al., 2003). This virus is known to be hepatotropic but might be mainly lymphotropic *in vivo* (Fogeda et al., 2000). Considering its tropism and route of transmission (mainly parenteral and sexual), a high risk of co-infection with other hepatotropic viruses or with HIV exists (Ernst et al., 2013).

GBV viral particles present a low and heterogeneous buoyant density similar to Hepatitis C Virus (See Introduction - Chapter IV I.) and are associated with lipoproteins (Sato et al., 1996; Melvin et al., 1998). The RNA genome encodes for a long ORF flanked by a 5′ and a 3′ non-coding regions (Leary et al., 1996; Muerhoff et al., 1995). The translation is cap-independent as pestiviruses and is initiated by an IRES sequence. However, GBV-A/C and GBV-B present several differences. The 5′NCR of GBV-A/C is significantly larger than GBV-B. The GBV-A/C viruses do not have any poly (u/UC) stretch whereas GBV-B harbors a short poly (u/UC) (Lindenbach et al., 2007).

Concerning the viral proteins, the GBV-B structural proteins are composed of a capsid protein and two envelope glycoproteins, E1 and E2 similarly to HCV (Figure 8 and See Introduction – Chapter IV II.2). GBV-A/C do not encode a classical capsid sequence (Muerhoff et al., 1995) but several evidences demonstrated that basic-structural proteins are expressed in a non-classical way by these two viruses and may be the component of a viral capsid (Prince et al., 1996; Pavio et al., 2004). The entry process of these viruses is still poorly characterized.

All GBV viruses express non-structural proteins similarly to pestiviruses (except the N[pro] protein). GBV viruses harbor a p7-like protein (Figure 8) that presents partial similarities with HCV p7 (Ghibaudo et al., 2004). However, GBV-B p7-like protein called p13 has been shown to be need for virus infectivity *in vivo* (Takikawa et al., 2006). NS2-NS3 protein complex is a serine autoprotease complex that processes the NS2/NS3 junction (Figure 8) (Belyaev et al., 1998). NS3 harbors a protease activity at its C terminal and a helicase and NTPase activity at its N terminal junction (Muerhoff et al., 1995; Leary et al., 1999). NS3-NS4A protein complex is a protease complex in

charge of processing and releasing the GBV non-structural proteins (Sbardellati et al., 2000). The NS5B protein represents the RNA-dependent RNA polymerase (Figure 8) (Muerhoff et al., 1995; Leary et al., 1996).
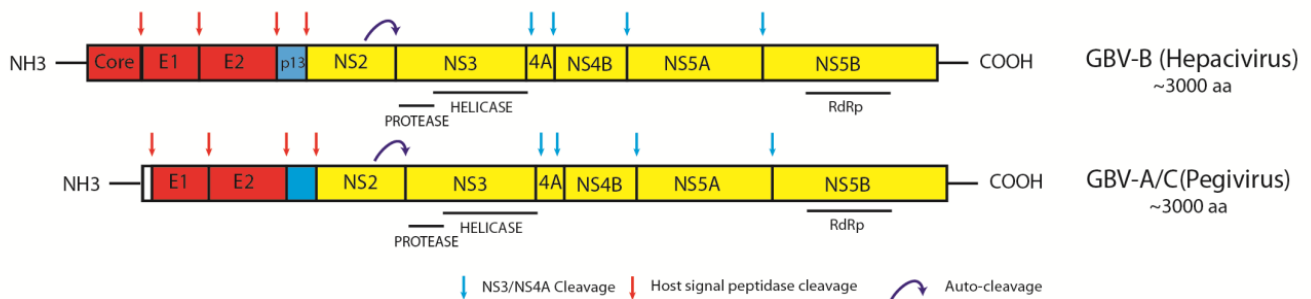
Figure 8. GBV-A/V (Pegivirus) and GBV-B (Hepacivirus) polyprotein organization and processing. Polyprotein lengths are indicated below the genus name. Red and yellow blocks represent respectively the structural and non-structural proteins. Polyprotein processing is indicated: Red and blue arrows indicates junctions processed respectively by the host signal peptidase and by the NS3/NS4A serine protease. Black arrow represents the processing of PrM by the furin. Curved arrow indicates the auto-processing of the NS2/NS3 junction by NS2. GBV-A/C p13 analogue is shown as a blue block in pegivirus polyprotein . RdRp, RNA-dependent RNA-polymerase.

Interestingly, GBV-B viruses present a higher degree of similarities with HCV than other GBV viruses. The HCV and GBV-B 5' IRES and 3' NCR present similar fold in contrast to GBV-A/C (Rijnbrand et al., 2004; Rijnbrand et al., 2005). Moreover, the presence of a capsid protein in the GBV-B and HCV genomes highlights the strong genetic relationship between these viruses (Muerhoff et al., 1995). In consequence, GBV-B virus has been classified into the hepaciviruses genus with HCV whereas the GBV-A/C viruses, that long remained unclassified, are now the constituent of a new Flaviviridae genus, the pegiviruses (Stapelton et al., 2011). More recently, another GBV virus has been isolated from a fugivorous bat (*Pteropus Giganteus*). This virus was identified as ancestral to GBV-A and GBV-C (Epstein et al., 2010) and did not seem to cause any hepatitis related diseases on collected bats.

CHAPTER II

The Hepatitis C Virus Infection
A worldwide liver disease

Hepatitis C Virus is a major cause of liver disease worldwide. Acute infection often leads to the installation of a chronic liver disease, and in rare cases, to the development of hepatocellular carcinoma. HCV high abilities to generate host neutralizing antibodies-escape mutants and restrict host immune response likely contribute to the installation of the chronic infection. Interestingly, several extrahepatic clinical disorders have been reported in HCV patients, suggesting that HCV may infect other cell types than hepatocytes. No vaccine is available and current treatments are still largely inefficient. The recent development of new direct acting agents (DAAs) seriously improved patient cares but the risk of appearance of resistant viruses needs to be taken into account.

In this chapter, we will first introduce the genetic diversity, the prevalence and potential origin of HCV infection. Then, in a second part, the hepatic and extrahepatic clinical manifestations that may occur during HCV infection will be reviewed. Finally, the host immune response features against HCV infection, as well as the current and future therapies will be described.

I.   Genetic diversity of the causative agent, the Hepatitis C Virus

The Hepatitis C Virus was classified into the *Flaviviridae* family due to its homology with the flaviviruses and pestiviruses in terms of genetic and polyprotein organization (Choo et al., 1991). However, HCV was found to be more related to pestiviruses than flaviviruses considering its non-structural proteins function and its cap-independent polyprotein translation that is cap-independent. Few years later, the identification of GBV viruses revealed the presence of HCV-highly related viruses that present a high degree of similarity with HCV (Muerhoff et al., 1995; Simons et al., 1995). One GBV virus, GBV-B, was classified as a member of the hepacivirus family whereas the others constitute now the closest *Flaviviridae* family related to the hepacivirus, the pegiviruses (Stapleton et al., 2011).

HCV presents a very large genetic diversity in comparison to other Flaviviridae such as flaviviruses (Figure 9). Indeed, its RNA-dependent RNA polymerase (NS5B) is highly error prone ($1,4$-$1,9 \times 10^{-3}$ substitutions per nucleotides and per year; Ogata et al., 1991) and do not have any 5′-3′ exonuclease proof reading activity (Steinhauer et al., 1992).

Figure 9. Phylogenetic tree of representative HCV coding sequences. Neighour joining tree was constructed using maximum composite likelihood nucleotides distances between coding regions using MEGA5 (Smith et al., 2013; Jackowiak et al., 2013; MEGA5 method: Tamura et al., 2011).

Thus, the properties of the NS5B protein have resulted to a large diversification of HCV into different genotypes and subtypes. HCV is now classified into 7 different genotypes (Figure 9, only the 6 major HCV genotypes are shown) (Simmonds, 2013; Smith et al., 2013). Each genotype is divided into different subtypes that may range from one (genotype 5) to thirteen (genotype 6). Accordingly to the viral region that is analyzed, HCV genotypes commonly harbor around 30% to 35% of sequence disparities whereas subtypes harbor between 15 to 30% of genetic differences (Figure 10) (Simmonds et al., 2005; Irshad et al., 2010). Particular protein regions are highly variable and submitted to genetic variation, e.g. the hyper variable region 1 (HVR1) located into the envelope glycoproteins E2. In contrast, other proteins are highly conserved among HCV variants such as the core proteins that allow nucleocapsid formation and the NS5B polymerase protein. Thus, HCV classification is essentially based on the genetic comparison of sequences encoding for such conserved and critical functions.

In a chronically infected patient, the HCV genetic diversity is also important and usually results on the diversification of particular subtype variants to an important number of new derived-variants (Figure 10) (Clementi et al., 2003; Pawlotsky, 2006).
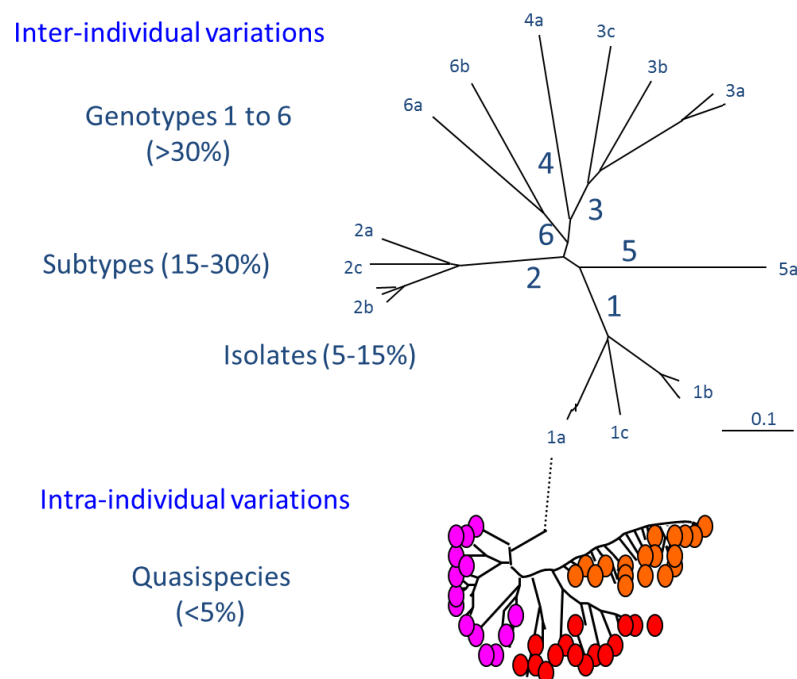
Figure 10. Intra-individual HCV variants (Quasi-species) in infected patients. Percentage of divergence among genotypes, subtypes, isolates and quasi-species are indicated.

These intra-individual variants are called quasi-species (Figure 10). In patient serum, these highly homologous strains reflect the virus adaptation to its host. This ability to generate an important number of genetic variants is a weapon of choice for HCV to adapt its cell or host tropism, as well as to evade host immune responses and generate resistance against anti-viral drugs. This genetic diversity is mainly concentrated within less conserved protein domains that are highly exposed to the host immune responses and to the neutralizing antibodies, such as the Hyper Variable Domain 1 (HVR1) of the HCV glycoprotein E2 (Buck et al., 1995; Mondelli et al., 2001).

II.   Prevalence and epidemiology

150 million people worldwide are known today to be chronically infected by the Hepatitis C Virus. According to the World Health Organization, 3 to 4 million people are infected each year and the number of deaths due to infection-related diseases is estimated around 350000. Although transmission through sexual intercourse remains rare, HCV infection mainly occurs through exposure with infected blood materials among which blood transfusion represents the leading cause (World Health Organization). Intravenous drug injections with contaminated syringes or parenteral transmission also represent important routes of transmission.

HCV prevalence is relatively low in Northern countries, reaching one to two percent of the population (Figure 11). However, the prevalence is higher in southern countries, notably in Southern Asia, Southern America and in Africa where the proportion of infected people can reach up to 10% of the population. Egypt is the country with the highest prevalence worldwide with up to 14% of infected people (Figure 11) (World Health Organization). However, this high rate of infection is not linked to a massive epidemic burden but rather to the use of contaminated syringes during a massive treatment campaign to eradicate schistosomiasis, a parasitic disease causing chronic illness.

Figure 11.   Global distribution of HCV genotypes in the world (WHO-2009).
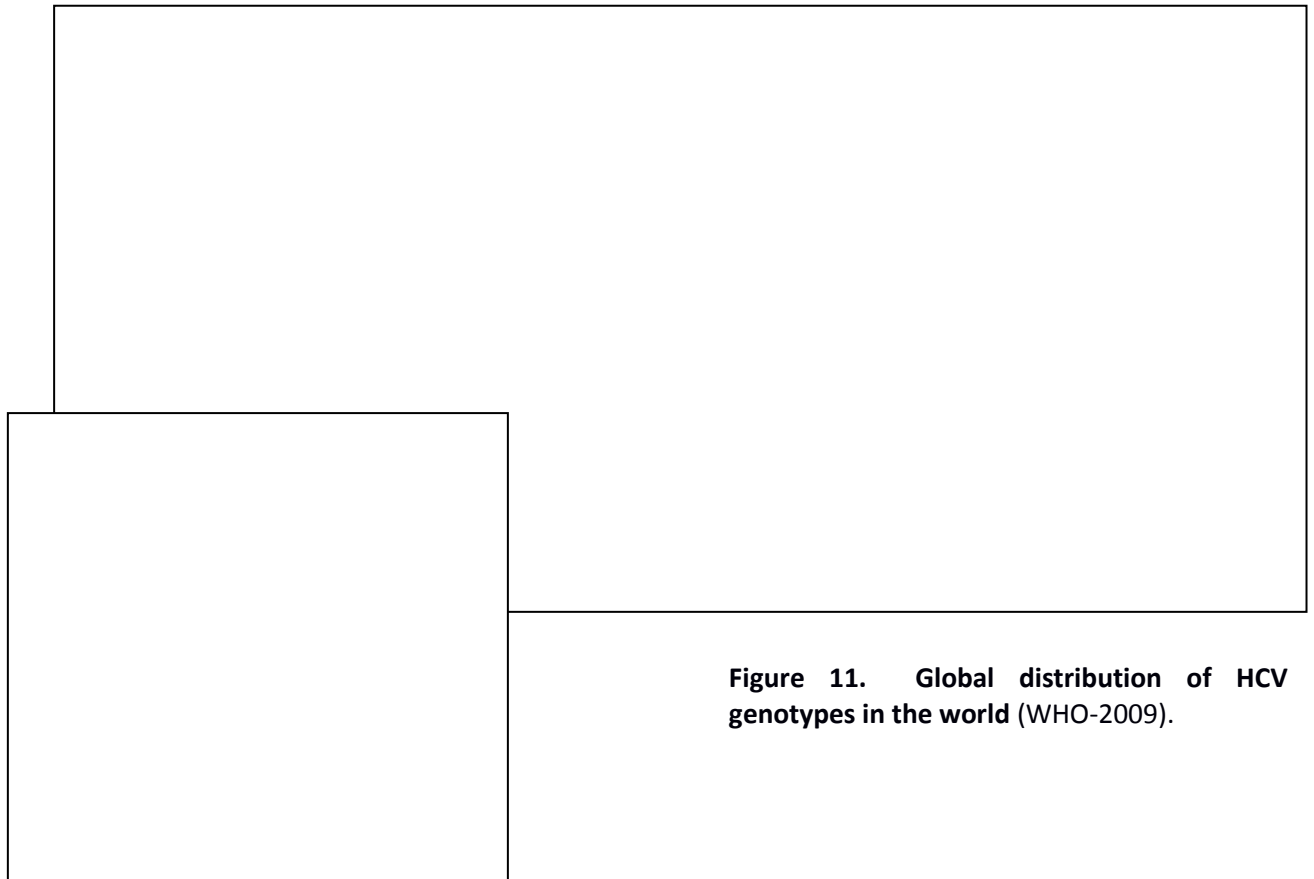
HCV genotypes and subtypes are un-equivalently distributed around the world (Figure 11) (World Health Organization, 2009). Genotype 1 is present on every populated continent where it represents the dominant genotype, at the exception of Africa where it is poorly settled. Genotypes 2 and 3 are also found in every populated continent but usually represent together less than 25% of the infection with the exception of the sub-Saharian Africa and Middle East where genotype 2 and genotype 3 respectively represent the dominant genotypes (Figure 11). Genotypes 4 and 5 are only present respectively in East Africa and South Africa where they represent the major genotype. Genotype 6 is only located in East Asia and represent 5 to 25% of infections cases. Altogether, these data seem to show that Africa presents important virus diversity in comparison to the other continents as genotypes 2, 4 and 5 are extremely dominant in this continent (Figure 11). Genotype 1 and 3 are present but represent a small proportion of infection cases. Thus, although the origins of HCV have not yet been established, it is interesting to point out the possibility that HCV emerged in Africa and that particular genotypes, which were minor in Africa

such as the genotype 1, then adapted and spread around the world to become dominant worldwide.

III. Origins and virus reservoirs

The origin of HCV infection in human is matter of debates. Two major hypotheses have been proposed to explain the emergence of this infection.

A first hypothesis suggests that HCV has been directly acquired by human and ancestors during evolutionary history (Simmonds, 2013; Jackowiack et al., 2013). Indeed, although several mammalian hepaciviruses have been identified, each seem specifically adapted to its host. For instance, HCV replication is highly dependent of human-specific factor such as the human liver-specific microRNA miR-122 (See Introduction – Chapter IV II.1).

In contrast, another hypothesis supports the idea that HCV infection has been acquired zoonotically. Consistently, a recent study of 1,258 bats blood samples collected in different countries all around the world permitted the identification of an important number of hepaciviruses and pegiviruses-like viruses in these species, highlighting the large biodiversity and worldwide prevalence of these viruses (Quan et al., 2013). Although hepaciviruses were previously detected only in primates, dogs and horses, pegiviruses were only found in primates and one study reported the infection of one particular species of bat (Epstein et al., 2010). However, authors showed that the hepaci- and pegi-viruses diversity is extremely larger than we previously thought (Quan et al., 2013), as they circulate in major lineages such as Chiroptera - that are present in most of the continent – (Figure 12). This high level of diversity suggests that mammals, and more specifically, bats may represent a basal and major reservoir for hepaci- and pegi-viruses. This assumption is supported by the fact that all the bats that were collected did not show any sign of diseases (Quan et al., 2013). Interestingly, viruses collected in Africa presented the highest genetic diversity, pointing out a possible African origin of these viruses (Figure 12). However, the fact that the African area-collecting samples were larger than the American and Asian ones need to be taken into account and may bias our view of the

genetic distribution of these viruses worldwide. Whether these viruses have spread and have been transmitted to other species during evolutionary history remain now to be elucidated.

Another recent study also identified new pegiviruses and hepaciviruses-like viruses in rodents (*Neotoma albigula* and *Peromyscus maniculatus* respectively) from the southwestern United States (Kapoor et al., 2013). These viruses presented a higher diversity than humans and primates viruses as well as unique genetic features. Interestingly, such findings may suggest the participation of rodents in the spread and evolutionary history of these 2 virus genera (Kapoor et al., 2013), representing a potential missing link between bats and primates/humans infection.
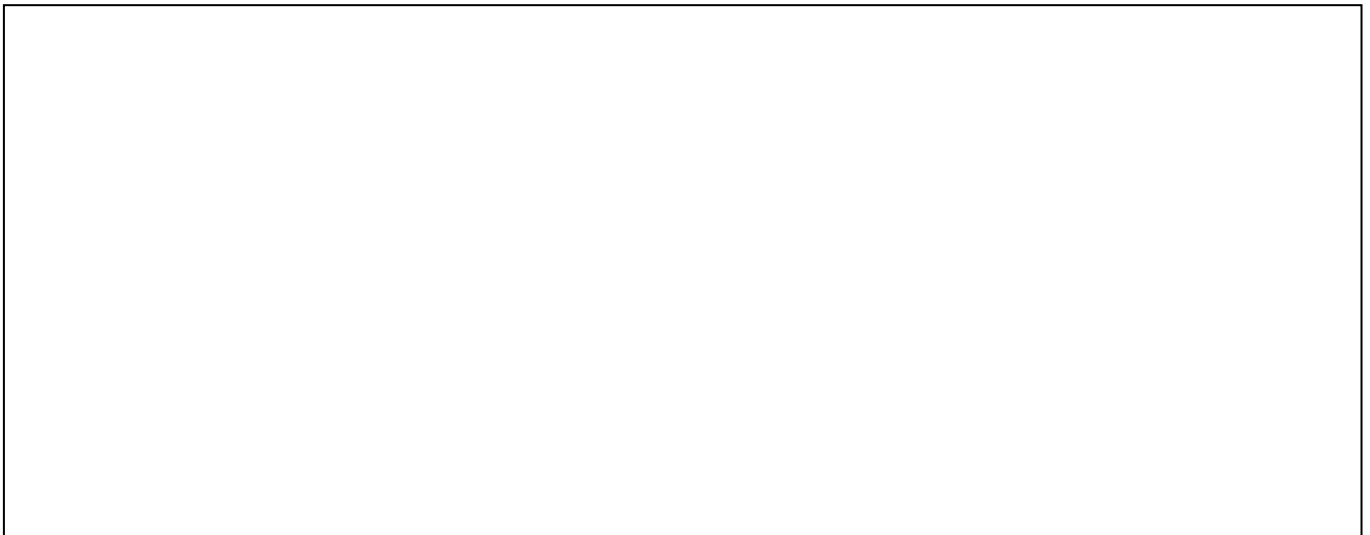
Figure 12. Geographic distribution of the bat-derived hepaciviruses and pegiviruses from the specimen collected. Proportion of bat-derived hepaciviruses and pegiviruses is represented in pie chart. Number of samples tested is indicated in parentheses (Quan et al., 2013).

IV. Course of infection

The hepatitis C virus infection is a long-term viral infection that can develop or persist during several decades in patients. Commonly, the first stages of infection correspond to an acute hepatitis syndrome that can evolve six months after infection into a chronic liver disease in 80% of the cases (Figure 13) (Alberti et al., 1999; Yamane et al., 2013). Serious liver damage including hepatic steatosis and fibrosis are observed in 20% of the patients (Figure 13) after a long and progressive disease

evolution that can last ten to twenty years. Hepatocarcinoma appears per year in 1-4% of the cirrhotic patient and constitutes the final stage of the infection disease (Figure 13) (Jeong et al., 2012). A liver transplantation is actually the only curative treatment for end-stage HCV patients although liver graft is often rapidly re-infected (Hsu et al., 2013).

1. Acute infection

Acute infection is often asymptomatic thus rendering the disease difficult to identify during the early stages of infection. Only 25% of the patients develop specific or un-specific symptoms that can be severe (Santantonio et al., 2008). However, the acute infection is rarely fulminant (Farci et al., 1996). HCV viral RNA can be detected in the patient blood 2 to 20 days after infection. 2 to 4 weeks after infection, the dramatic elevation of 10 to 20 times of the alanine aminotransferase (ALT) level in the blood often constitute the first sign clearly detectable of liver distress and deficiencies. This higher level of ALT may be linked sometimes to the appearance of synchronized fever-like syndromes, highlighting a potential virus infection (Santantonio et al., 2008).

Figure 13. HCV pathogenesis and course of infection. During HCV infection, acute infection progress in 80% of the cases toward a chronic liver disease that sometimes results in the development of cirrhosis and hepato-cellular carcinoma. See text for details. (Adapted from http://Hopkins-gi.nts.jhu.edu).

Then, seropositivity usually occurs after 7 to 8 weeks post infection and anti-HCV antibodies can be detected in patients (Post et al., 2004), allowing to clearly confirm - or counter- the infection by HCV. The acute phase of infection is usually treated with a 24 weeks treatment using pegylated interferon, demonstrated as being efficient to limit the chronic evolution of the disease (Grebely et al. 2011). The benefic effect of ribavirin as a combinative agent remains however matter of debates during this stage of disease.

20% of the infected patients manage to spontaneously clear viral infection during acute infection (Figure 13). Usually, virus clearance is observed in patients several years after initial infection through detection of anti-HCV antibodies in serum, despite the fact that viral RNA is undetectable (Bowen & Warren, 2005). Virus clearance is dependent on several viral and host genetic factors. For instance, polymorphisms in the IL-28B locus, which encodes for IFN-λ, have been shown to be associated with spontaneous and treatment-induced clearance of HCV (Ge et al., 2009; Rauch et al., 2010).

2. Chronic infection and end-stage pathogenesis

In contrast to spontaneous clearance, 80 to 85% of the patients present persistent viremia six months after infection (Figure 13) (Farci et al., 1996). The presence of high level of HCV RNA in patient blood after this period is considered as the sign that infection is evolving from the acute to a chronic phase.

As acute infection can be asymptomatic, transition to chronic infection can be silent and patients are often identified when chronic infection is installed. The causes of this transition are not well defined but are thought to be determined by the virus-host interactions and by the host genetic and physiologic properties. Appearance of particular HCV quasi-species able to evade the immune system has been proposed as a potential cause for chronic infection transition (Farci et al., 2000). Moreover, age, gender (male are more prompt to develop chronic infection than women), obesity, alcohol consumption or a co-infection with HIV or HBV may also represent important factors that can influence the evolution toward chronic infection (Grebely

et al., 2011).Chronic infection induces severe liver and hepatocytes dysfunctions such as fibrosis (formation of excess fibrous tissue in liver) or steatosis (Yoon et al., 2006) (abnormal lipid retentions in hepatocytes). Usually, fibrosis constitutes the first manifestation of future cirrhosis (Levrero et al., 2006).

HCV is the cause of 20 to 25% of the cirrhosis worldwide (Alter et al., 2007). 20 years after infection, 20% of the chronic patients develop cirrhosis (Figure 13), characterized by a global and irreversible liver disease through severe fibrosis, tissue disorganization and nodules formations (Levrero et al., 2006). Cirrhosis can be asymptomatic (compensated cirrhosis) and show no or few clinical manifestations. However, decompensation of cirrhosis leads to important clinical manifestations including severe hepatocellular insufficiencies, hypertension and a pre-cancerous state. In this form of cirrhosis, the survival rate for the next 5 years falls from 90 to 50% (Ascione et al., 2007). Interestingly, as the hepatitis C virus is a non cytophatic virus, the liver damages are mainly due to the reaction of the immune system against HCV, despite its ineffectiveness to eradicate viral infection (Heydtmann et al., 2001). Decompensated cirrhosis leads in 1 to 4% of the patients per year to hepatocellular carcinoma that constitutes the most advanced stage of HCV liver pathogenesis (Chen & Morgan, 2006). In this case, the only curative treatment for patients is the graft of a healthy liver. However, allograft re-infection is systematic in the few months or years following transplantation and clinical manifestations can re-reappear within 100 days. 5 years survival rate post transplantation is today estimated at 70% (Duclos-Vallee et al., 2009).

Mechanisms linking viral infection and the development of a liver cancerous-state are not yet clarified. Cellular stress and inflammation due to a severe immune response or potential interference of cellular pathways by viral proteins may represent some interesting route to explore (Levrero et al., 2006; Castello et al., 2010). In fact, the HCV core protein has been suggested to have an *in vivo* oncogenic activity through activation of intracellular signaling pathways and oxidative stress overproduction (Ahmad et al., 2011).

V. Extrahepatic manifestations

Hepatitis C infection has been widely associated with several extrahepatic disorders over the years, related to large variety of organs or tissues: epithelium, pancreas, thyroid, bone marrow, spleen, lymph node, biliary cells, lymphatic cells or even the brain (Figure 14) (Zignego et al., 2007). The important diversity of disorders observed during HCV infection point out the possibility that HCV infection is more likely a systemic disease than only a liver restricted-disease. However, the association between HCV and the majority of these disorders is not cleared. Although the relationship with some clinical manifestations is very close, other associations are only suspected, suggested or based on the observation of a single clinical case.

Strong evidence of association exists concerning the ability for HCV to infect neuroepithelio/endothelial cells and peripheral blood mononuclear cells (PBMCs). Hepatitis C infection is associated with cerebral disorders such as cognitive abnormalities or depression (Fletcher et al., 2012) but no evidences were clearly established to decipher whether these disorders were due to a global and systemic disease or directly to a viral infection.

Figure 14. HCV extrahepatic manifestations. Non-exhaustive list of the clinical manifestations observed in HCV infected patient and classified according to their degree of prevalence and association with HCV infection. (Adapted from Zignego et al., 2007).

A recent study demonstrated that HCV is in fact able to infect brain endothelial cells. Through the use of anti-receptors antibodies or the inhibition of the NS3 protease, this work demonstrated that these cells express functional HCV receptors and support viral entry and replication (Fletcher et al., 2012). Virus infection was shown to induce an important endothelial permeability as well as cellular apoptosis, pointing out important cellular abnormalities that could explain cerebral disorders.

PBMCs represent today the most documented extrahepatic site of infection. In fact, mixed cryoglobulinaemia (MC) has been shown to be highly associated with HCV infection (Misiani et al., 1992; Ferri et al., 1993) and represent the most current extrahepatic disorders observed in infected patients (Figure 14). MC is defined by the presence and accumulation of circulating immunocomplexes produced by a B cell lymphoproliferation. The association between MC and B cell lymphoproliferation has been observed and confirmed in HCV patients. Evidences of MC have been observed in 19 to more than 50% of the patients despite the fact that no clinical manifestations are usually observed due to the low level of cryoglobulins circulating in patient sera (Wong et al., 1996). However, 10 to 30% of the HCV patients that develop a MC may present a large variety of MC-related disorders such as weakness, arthralgias, kidney dysfunctions or severe peripheral neuropathy (notably characterized by axonal damage) that represent the most current complications (70 to 90% of the MC-related disorders) (Zignego et al., 2007). Other studies suggest the influence of MC on liver pathology and dysfunctions (Kayali et al., 2002) although it is difficult to determine clearly what the part of MC is in the global liver pathogenesis.

Several epidemiologic evidences support an important association between HCV infection and the occurrence of B cell non-Hodgkin's lymphoma (NHL) (Figure 14) (Matsuo et al., 2004; Moehlen et al., 2012; Peveling-Oberhag et al., 2013), notably in HCV low prevalence countries (Morton et al., 2004; Duberg et al., 2005). Consistently, regression of B cell NHL in HCV patients after anti-viral therapies has been previously observed (Hermine et al., 2002). Moreover, B-cell NHL lymphomas

commonly appeared in MC HCV-related patients that never developed any mononuclear lymphoproliferative disorders, suggesting that the virus may represent the potential cause of these disorders (Pioltelli et al., 1996).

MC is also associated with the development of NHL in HCV infected patients (Racenelli et al., 2001; Vallat et al., 2004). Consistently, MC has been shown to increase the risk of these patients to develop a NHL of 35 fold (Monti et al., 2005). B-cell NHLs appears in 8 to 10% of the MC HCV-related patients (Ferri et al., 1994; Ferri et al., 2004).

The panel of B-cell NHLs is very large and usually indolent for the patients (Zignego et al., 2007). Among this panel, the marginal zone lymphoma (MZL) represents the most current B-cell NHL observed (Luppi et al., 1998). Aggressive and diffuse lymphomas harboring large cells remain rare in HCV patients (Trepo et al., 1998).

All these evidences of PBMC's related-disorders strongly suggest that HCV is able to infect cell types such as B or T lymphocytes. Indeed, a large number of evidence from a decade of studies now indicates that particular HCV variants have the ability to infect B or T cells and that these cells may constitute a reservoir for HCV to evade the immune system and to neutralize responses (Ferri et al., 1993; Lerat et al., 1996; Morsica et al., 1999; Blackard et al., 2005; Pal et al., 2006; Pham et al., 2008; Durand et al., 2010). Indeed, the presence of the HCV RNA positive or negative strand has been observed in patient PBMC's as well as the expression of non-structural proteins (Cribier et al., 1995; Lerat et al., 1996; Morsica et al., 1999; Roque-Afonso et al., 2005; Di Liberto et al., 2006; Durand et al., 2010). The molecular (cellular and viral) determinants of HCV lymphotropism are reviewed in Introduction - Chapter VI.

An important link between HCV infection and autoimmune disorders has been also reported in HCV infected patients. Indeed, non-organ specific autoantibodies have been observed in HCV patient cohorts in a higher prevalence than in control cohorts (Vergani et al., 2013), reaching 70% in particular patient cohorts (Gregorio et al., 1998). Other studies also reported that presence of such antibodies is linked to a greater severity of liver damage during HCV infection (Cassani et al., 1997; Ferri et al., 2009). Consistently, several autoimmune endocrine syndromes have been

observed in high prevalence in HCV patients and reported to be related to HCV infection, such as thyroid autoimmune disorders (Marazuela et al., 1996; Deutsch et al., 1997; Vergani et al., 2013) and diabetes mellitus type 2 (Lecube et al., 2006; Zignego et al., 2007).

A large number of other disorders or diseases are observed in a high prevalence in HCV patients such as MC-related nephropathy that may lead to oedemas or renal failure (20 to 60% of the patients with MC-related HCV infection may develop such renal disorders), lichen planus (a muco-cutaneous auto immune disease resulting in the inflammation of the basal epithelium) or porphyria cutanea tarda (a metabolic disorders of heam biosynthesis that damage the skin) (Figure 14) (Zignego et al., 2007). Others disorders have been observed in HCV patients such as thyroid cancer or aortic artherosclerosis but their association with HCV infection remain to be confirmed.

VI. Immune response

1. Innate immune response

The establishment of HCV persistent infection is directly related to the ability of HCV to evade and restrict both the cellular innate anti-viral response and the HCV-specific adaptive immune response.

During HCV infection, hepatocytes respond to virus invasion by secreting type 1 interferons (IFN-α and IFN-β) that play a key role in the first steps of the host anti-viral responses against infection. IFNs induce the expression of several interferon-stimulated genes (ISGs) that restrict virus replication but also trigger the stimulation of a future HCV-specific adaptive immune response (Figure 15) (Schoggins et al., 2013). More precisely, during hepatocyte infection, the first triggered response against infection is the synthesis of IFN- β. Viral double stranded RNA (dsRNA) and the polyuridine motif of the HCV 3′ untranslated region (UTR) are recognized as pathogen-associated molecular pattern (PAMP) by two pattern recognition receptors (PRRs), respectively the Toll-like receptor 3 (TLR3) and the retinoic acid-inducible

gene I (RIG-I) (Figure 15) (Rehermann, 2009). PAMPs recognition by PRRs induces the activation of specific cellular pathways that lead to the synthesis and secretion of IFN- β. The binding of IFN- β to the IFN-α/β receptor on the membrane of surrounding cells then induces the activation of the intracellular JAK/STAT signaling pathways that leads to the synthesis of IFN-α and to the expression of several ISGs (Saito et al., 2008). ISGs restrict several cellular functions such as translation (Schoggins et al., 2011) or induce the degradation of RNA (Guo et al., 2004), thus leading to the impairment of viral translation and replication. However, as mentioned above, the virus has developed several mechanisms that counteract the cellular anti-viral responses. The NS3/4A protein complex notably cleaves and inactivates proteins involved downstream the RIG-I and TLR3 mediated-signaling pathways, thus leading to an impairment of the IFN-β production (Li et al., 2005). Core, NS5A and E2 are also known to interfere with the JAK/STAT signaling pathway and thus, affect the expression of the ISGs and the production of IFN-α (Lin et al., 2006; Polyak et al., 2001; Taylors et al., 1999). Importantly, this ability of HCV to constraint the cellular anti-viral control then severely impact on the host ability to trigger an efficient adaptive resp.onse, notably through IFN-α secretion. However, it has been demonstrated recently that hepatocytes have developed an unconventional mechanism to induce IFN-α secretion and stimulation of adaptive immune response. It has been shown that secretion of IFN-α by plasmacytoid dendritic cells, which are the major IFN-α secreting cell among PBMCs, does not require direct cellular recognition of infectious viral particles (Dreux et al., 2012). Indeed, infected hepatocytes are able to secrete viral-RNA containing exosomes that directly stimulate IFN-α secretion of plasmacytoid dendritic cells. This indicates that the immune system has also developed effective alternative mechanisms to bypass the inhibition of anti-viral response and stimulate an adaptive immune response.
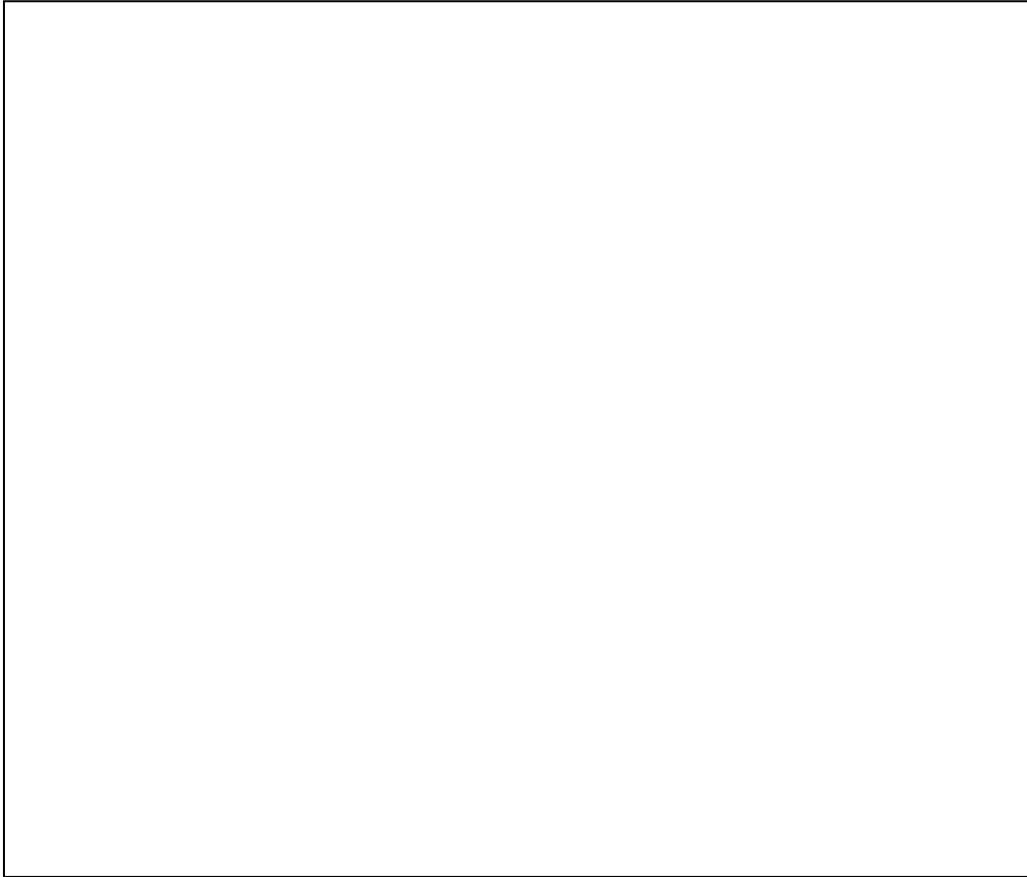
Figure 15. Innate sensing of HCV in the infected hepatocyte. Innate sensing is mediated by PKR (1), RIG-I (2) and TLR3 (3). These proteins recognize specific HCV features, including the dsRNA in the HCV IRES and the HCV poly U/UC. This recognition activates downstream signaling pathways that induce the expression of antiviral genes, IFN-β and other proinflammatory cytokines (4). AP-1, activator protein 1; CLDN1, claudin 1; IKKe, IkB kinase-e; LDLR, low-density lipoprotein receptor; OCLN; occludin; SR-BI, scavenger receptor class B member 1; TBK-1, TANK-binding kinase 1; TRAF3, TNF receptor–associated factor 3. GAG, glycosaminoglycans (Horner et al., 2013).

2. Adaptive immune response

a) Cellular adaptive immune response

During viral infection, Lymphocytes T CD8+ and CD4+ (or CD8+ and CD4+ T cells) mediate the cellular adaptive immune response. Pathogen recognition and antigen presentation by antigen-presenting cells (APCs) induce the clonal expansion of antigen-specific T cells and the initiation of a pathogen-specific T cell response. CD4+ T cells or T helper are non-cytotoxic T lymphocytes that regulate CD8+ T and B lymphocytes functions through secretion of interleukine 2 (IL2). In contrast, CD8+ T cells are cytotoxic lymphocytes that target specifically the infected cells and induce their apoptosis.

HCV-specific T cell responses usually appear 4 to 8 weeks after the beginning of the acute phase of infection. The initiation of the cellular adaptive response is correlated with a hepatic inflammatory events and a rise in serum alanine transaminase (ALT) (Figure 16) (Neumann-Haefelin et al., 2013). At this stage, a high viremia is already observed and then decreases simultaneously with T cells responses (Figure 16). However, the HCV-specific T cell responses are usually weak or unsustained, likely due to the viral control of innate antiviral mechanisms which contributes to impair the stimulation of the adaptive immune system (Bowen et al., 2005). This absence of a strong and significant T cell response usually results in the installation of a decreased and persistent viremia and in progression toward chronic infection (Figure 16).

In contrast, effective CD4+ helper and CD8+ cytotoxic T cells responses can persistently resolve virus infection (Figure 16). It has been shown in chimpanzee that the presence of memory CD8+ and CD4+ T cells is critical to prevent infection upon re-challenge (Grakoui et al., 2003). Moreover, in chronically infected patient, the appearance of T cells within the liver seems associated with a decrease in virus titer (Shin et al., 2006).

CD8+ T cells are present during the acute phase of infection and persist during chronic infection (Kaplan et al., 2007). These cells harbor during HCV infection a significant cytotoxicity against infected cells to impair viral spread. However, this T cell response is highly dependent of the CD4+ T cell response. Indeed, it has been shown that depletion of CD4+ T cell abrogates CD8+ T cell response in chimpanzee upon HCV re-challenge (Grakoui et al., 2003). Indeed, CD4+ T cells are important mediator of an effective adaptive response. An important and persistent HCV-specific CD4+ T cell response during acute infection is commonly associated with a future virus clearance then followed by a decrease of the CD4+ response. In contrast, an absent or transient response leads to chronic infection (Figure 16) (Diepolder et al., 1995; Gerlach et al., 1999).

Figure 16. Schematic representation of the cellular immune response during acute HCV infection. a, Viremia (red line) is present early and is followed by persistent infection. CD4+ and CD8+T-cell responses (black and green lines, respectively) and rises in serum transaminase (blue) are weak or even absent. b, Viremia may persist for several weeks in the absence of a significant cellular immune response. The delayed rise of CD4+ and CD8+T-cell responses is associated with transient control of viremia but following reduction of the CD4+T-cell response, viremia rebounds and infection become persistent. c, Although viremia arises early, the virus becomes undetectable in plasma following emergence of CD4+ and CD8+T-cell responses. A rebound in viremia may occur before final viral clearance (Bowen & Walker, 2005).
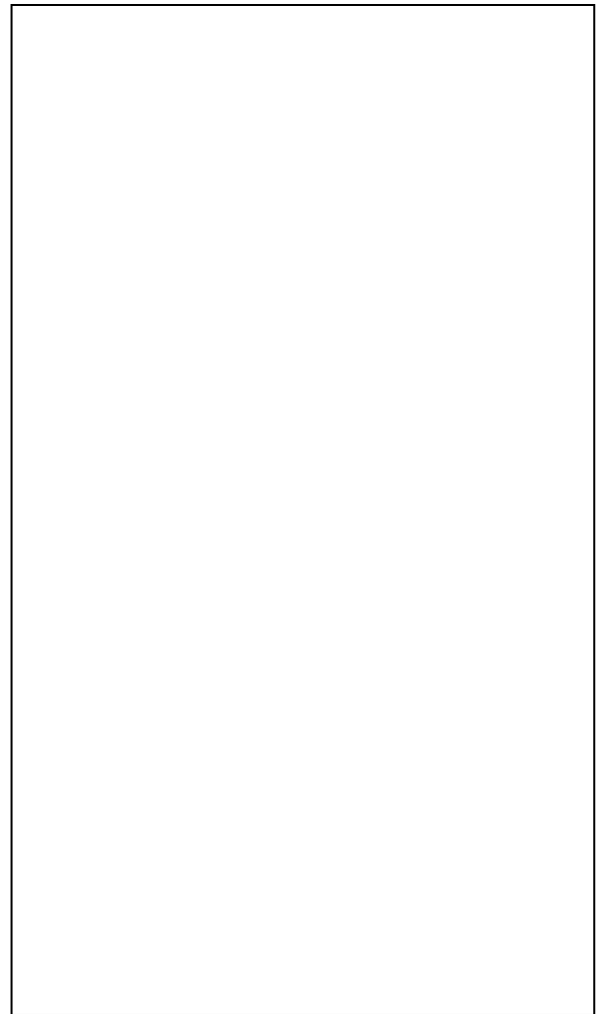
b) *Humoral adaptive immune response*

The second branch of the adaptive immune response is mediated by B lymphocytes (or B cells). After antigen recognition and presentation by APCs, the antigen-specific B cell subset proliferates and differentiates to plasmocytes that secrete antigen-specific neutralizing antibodies (Nt Abs). Antibodies play a key role during virus infection, by preventing virus entry through binding to surface antigens, or by inducing virus degradation through opsonisation.

The role of humoral immunity during HCV infection remains controversial. Studies have reported the ineffectiveness of HCV neutralizing antibodies to resolve HCV infection or to prevent virus re-infection in chimpanzees and humans (Yu et al., 2004). Indeed, the rapid appearance of neutralization-resistant HCV variants or the

epitope masking of envelope glycoproteins by lipoproteins may contribute to attenuate the role of Nt Abs during HCV infection.

However, other studies predicted an important role for Nt Abs in the delay or prevention of HCV infection. Patients with primary antibodies deficiencies were reported to present a higher susceptibility to develop chronic infection and a rapid end-stage liver disease, suggesting an important role for Nt Abs in the control of disease progression (Chapel et al., 2001). Anti-HCV immune globins (Igs; a substance made from human blood plasma and that contains antibodies) were shown to delay or prevent HCV infection in chimpanzee if inoculated before or simultaneously to challenge (Farci et al., 1994). Moreover, the development of the HCVpp system has permitted to assess the high neutralizing activities of serum from infected chimpanzees and humans (see Chapter III). Consistently, although the Igs have been considered safe during a long time, a commercial i.v. Ig (IGIV) product prepared from pooled plasma with no anti-HCV antibodies was reported to transmit HCV to recipients and thus became an interesting object of research. A study demonstrated that the absence of anti-HCV positive plasma donations in IGIV compromised the safety of Igs through the removal of anti-HCV Nt Abs (Yu et al., 2004). Authors concluded that the presence of these Nt Abs in some plasma pooled with other HCV-positive plasma likely contributed to the historic safety of IGIV.

More recently, studies analyzed the neutralizing responses in patient cohorts infected by a single source of viral inoculum. Indeed, even though the HCVpp system efficiently allowed the assessment of the neutralizing activities of patient sera, attempts to decipher the role of the neutralizing responses in patient during the acute phase of infection were limited by the large heterogeneity of the studied patient cohorts and of the HCV variants related to infection. Thus, the access to patient cohorts that have been infected with a homogenous viral inoculum represented an interesting opportunity to understand the role of Nt Abs during HCV infection.

 Strong neutralizing responses were observed during acute phase of infections and were correlated with resolution of infection (Lavillette et al., 2005; Pestka et al., 2007). This suggests that Nt Abs are important actors in the control of viral spread and viral clearance, as the strong neutralizing response were shown to disappear once the

infection were resolved. In contrast, neutralizing responses were absent during the acute phase of infection in patients who then developed a chronic infection.

The presence of Nt Abs in chronically infected patients highlights the existence of a tight balance between the host neutralizing response and the generation of escape variants (Von Hahn et al., 2007). Indeed, the selective pressure of the Nt Abs on E2 epitopes such as HVR1 likely contributes to the constant generation of HVR1/E2 quasi-species that can evade the host neutralizing response. This is consistent with studies suggesting that an important HVR1-mediated quasi species diversity is a relevant indicator of chronic infection (Farci et al., 2000).

VII. Therapies

Today, no vaccine is available yet against HCV. To overcome this limitation, therapeutic treatment has been rapidly settled in the early 90's. For 20 years, the most current treatment against HCV was bi-therapeutic, based on pegylated IFN-α and ribavirin. However, this treatment was unspecific, induced important side-effect in patient and its efficiency was restricted to particular HCV genotypes. Today, development of direct acting agents (DAAs) that target HCV non-structural proteins makes the current treatment evolve toward a tri-therapy. Despite the fact that these new specific anti-viral compounds significantly improve patient treatment, their use seems to rapidly show some limitations. Thus, there is still a need today to identify new therapeutic targets in the HCV life cycle to develop efficient multi-therapies.

1. Bi-therapy

IFN-α is a well-known anti-viral molecule. This molecule induces a general anti-viral state within infected and surrounding cells through inhibition of viral replication and virus infection respectively, through the expression of ISGs. Moreover, IFN-α enhances cellular immune responses, cellular toxicity and decreases viremia. It has been shown that chronically infected patients have a defect in IFN-α production,

which can be compensated by a treatment using exogenous INF-α (Feld et al., 2005). Ribavirin, a nucleotide analogue of guanosine commonly used against viral infection, was combined to IFN-α at the end of the 90's to improve patients treatment (Scheel et al., 2013). This molecule is known to have no an anti-viral effect when used in monotherapy despite an ability to impair the level of ALT. However, when combined to IFN-α, the effects of this molecule are synergistic to the IFN anti-viral effects (Scheel et al., 2013). Several modes of action have been proposed to explain the anti-viral effects of Ribavirin. For instance, it has been suggested that this molecule may unbalance the CD4+ T cell responses, decrease the cellular Guanosine Triphosphate (GTP) stock within infected cells or even block polyprotein translation initiation (Galmozzi et al., 2012). However, these mechanisms are still matter of debates. Very recently, an original mode of action for Ribavirin has been suggested to explain how Ribavirin could potentiate INF-α activity. A study reported that Ribavirin could enhance liver susceptibility to INF-α stimulation and increase INF-α-mediated activation of intrahepatic ISGs by lowering the baseline expression of these genes (Testoni et al., 2013).

IFN-α anti-viral effect efficiency has been improved by the addition of a polyethylene glycol to IFN, via a process called "pegylation". Pegylated IFN-α harbors an extended half-life, which, once combined with Ribavirin, improves patients treatment and frequencies of medication, and induces a sustainable viral response (SVR; undetectable viral RNA during the 6 months following the end of the treatment) in infected patients. Other improvements of IFN half-life have been attempted, notably by combining IFN-α with human albumin (Alb-IFN) (Zeuzem et al., 2008). However, despite improved IFN stability, the development of Alb-IFN has been stopped in 2010. Use of IFN-λ has also been proposed to reduce IFN-α side effect despite similar efficiencies (Schaefer et al., 2012).

Pegylated IFN-α/Ribavirin therapy dependent-SVR is highly dependent on the viral genotype. Genotypes 2 and 3 are known to be more sensitive to biotherapy and the SVR usually occurs after 6 months of treatment in 80% of the case (Chayama et al., 2011; Scheel et al., 2013). However, when patients are infected by the other genotypes

(1, 4, 5 and 6), SVR only occurs within the 12 months following beginning of the treatment and only in 40 to 50% of the case (Poynard et al., 2003). Moreover, the important side-effects induced by this unspecific treatment may result in patient contraindications or in the brutal arrest of patient treatment.

2. Current and futures therapies

Due to these important limitations in terms of treatment efficiencies or restrictions, the research of specific anti-viral agents able to generate effective SVR against a large panel of HCV genotypes has been extensively active over the last few years.

Very recently, in 2011, two new DAAs have been approved for treatment of HCV infection: the telaprevir and boceprevir. These molecules target the NS3/NS4A serine protease activity through binding of the NS3 active site and are the first specific antiviral compounds used in HCV therapy (Poordad et al., 2011; Scheel et al., 2013). These molecules have led to serious progress in patient treatments and their use often leads to virus clearance in patients that do not present a severe liver disease. For instance, in patients infected by HCV genotype 1, these molecules have improved the SVR from 40% to 70% in combination with Ribavirin and Pegylated IFN-α (Salloum et al., 2012). However, these compounds still raise some concerns. When use in a tritherapy setting, patient treatment is still associated with enhanced side effects and patient contraindications. Moreover, their use as monotherapy agents seem to rapidly induces the emergence of resistant viruses as it has been observed with the telaprevir (Reesink et al., 2006). Thus, despite their important efficiencies to treat HCV infection, the use of these molecules highly suggests the need for complementary molecules in order to design multitherapy strategies such as for HIV and to prevent the appearance of emerging resistance.

To this purpose, important numbers of other molecules targeting HCV non-structural proteins are currently in clinical trials such as specific NS5A or NS5B inhibitors (Figure 17) (Scheel et al., 2013; Varshney et al., 2012). New NS3 inhibitors are also developed in combination with Pegylated IFN-α and Ribavirin in order to reduce tritherapy side effects and improve treatment efficiency (Scheel et al., 2013;

Schaefer et al., 2012). Interestingly, combination of two DAAs targeting NS5A and NS3 in genotype 1b infected patient was shown to induce a SVR without treatment with IFN and/or Ribavirin (Chayama et al., 2012). Another strategy for HCV treatment may consist in the association of DAAs with small inhibitors of cellular factor critical for HCV entry, translation or assembly. Indeed, it has been shown that erlotinib or ezetinib, respectively inhibitor of EGFR (Lupberger et al., 2011) and NPC1L1 (Sainz et al., 2012) surface molecules, impaired virus entry within hepatocytes. Similarly, inhibitors that target cellular factors important for HCV replication such as miR-122 (See Introduction – Chapter IV II.1) or the cyclophilin A are currently in clinical trials (Figure 17) (Ploss et al., 2012; Scheel et al., 2013). The *Miravirsen*, a miR-122 antagonist currently in phase 2 (Figure 17), is a nucleic acid-modified DNA phosphorothioate antisense oligonucleotide that act by sequestering mature miR-122 in heteroduplex. This molecule has recently been shown to durably decrease HCV RNA levels in gt1 HCV infected patients in a dose dependent-manner (Janssen et al., 2013). No emergence of viral resistance was reported.
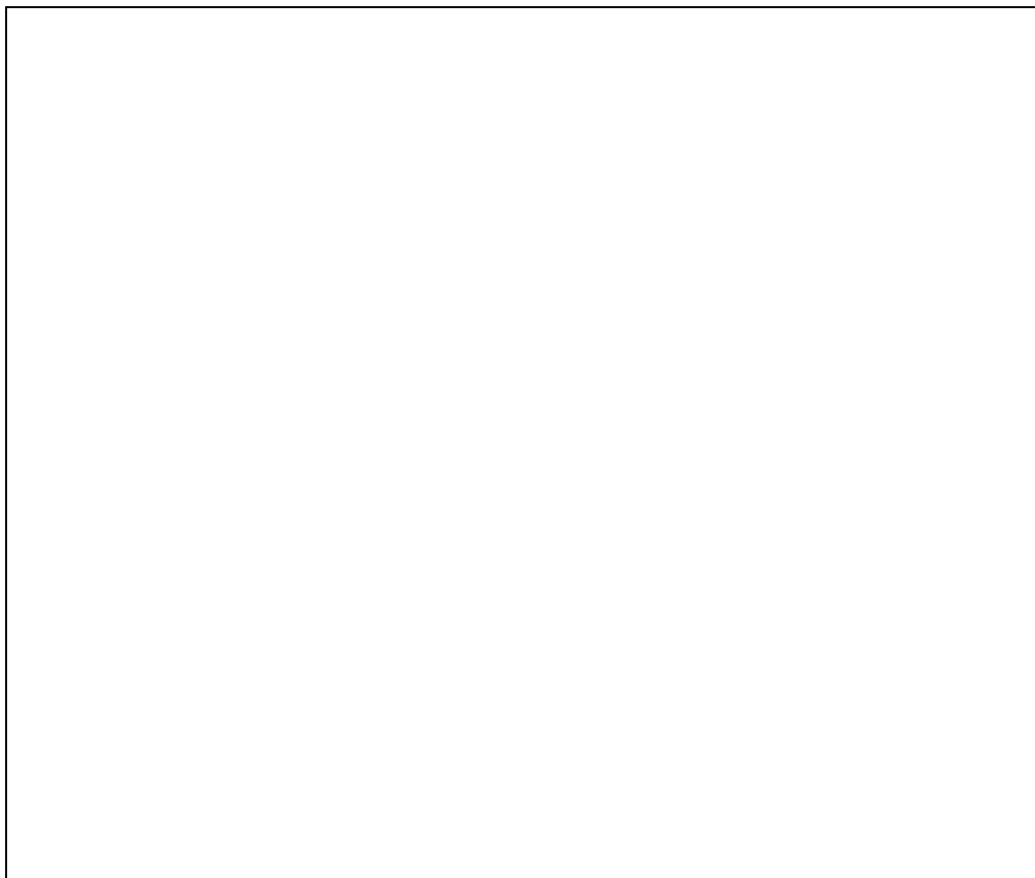
Figure 17. HCV inhibitors currently in clinical studies. The most promising compounds (in phases 2 and 3) of each class are presented. Occurrence of resistance, potency on several genotypes and development stage of each class inhibitor compounds are indicated (Horner et al., 2013).

Entry inhibitors targeting the biological function of HCV cellular receptors such as the HDL transfer activity of SR-BI (ITX-5061) are also under evaluation (Figure 17) (Syder et al., 2011). However, targeting functions of host cellular proteins as a synergistic approach for bi/tritherapy may severely increase the risk of serious side effects in infected patients through the deregulation of important cellular functions or pathways.

Today, the design of a safe and efficient treatment to fight HCV infection is highly dependent on the use of multitherapies targeting viral proteins functions. Combination of the current DAAs with other DAAs targeting viral protein functions during other steps of the viral life cycle may constitute relevant therapeutic strategies, similarly to those employed against HIV. As virus entry represents the first critical step of the virus life cycle, this process may represent a target of choice for an effective therapy. Thus, identification of conserved active sites or molecular mechanisms in viral proteins involved in this step may be crucial.

3. Vaccine

As for HIV, the generation of an efficient HCV vaccine remains a major challenge today to constraint the pandemic (Liang et al., 2013). An important limitation hampering the design of an efficient vaccine is the high genetic variability of the virus. The important evasion of the neutralizing response by quasi-species in patients is one hallmark of this high variability. Today, it thus remains challenging to identify critical conserved epitopes that can induce a strong, durable and efficient immune response without inducing any side effects. Several strategies are currently developed for this purpose. Injection of E1 and E2 proteins in chimpanzees was shown to stimulate T cells response and to induce production of neutralizing antibodies, demonstrating that the use of recombinant E1 E2 proteins may constitute a potential option for early prophylactic treatment during infection (Choo et al., 1994). Other strategies are based on the development of viral vectors harboring HCV proteins such as E1 and E2. Indeed, use of HCV pseudo-particles harboring onto their surface E1 and E2 envelope glycoproteins could induce a strong neutralizing

responses within mice and macaques (Garrone et al., 2011). In parallel, other strategies aim at developing therapeutical vaccines in order to induce a synergistic immune response during bi- or tritherapy. Indeed, several studies reported that the use of recombinant peptides or viral proteins was able to stimulate immune responses in infected patients (Klade et al., 2008; Habersetzer et al., 2011).

However, beyond studies aiming to design an efficient HCV vaccine, the continuous development of more and more efficient DAAs and the heterogeneous prevalence of HCV in the world render the development of such compounds uncertain at the industrial level. Indeed, in high prevalence countries, HCV is genetically very heterogeneous and its eradication required the development of complex vaccines able to target several HCV genotypes. In contrast, in low prevalence countries, HCV diversity is less heterogeneous but actual and future DAAs-related treatments are consider as promising options to efficiently cure infected patients.

CHAPTER III

Molecular tools and experimental systems

From the early beginning of its discovery, HCV has proven to be an arduous object of research and thus, has raised important technical challenges. Several experimental models have been developed all over the last two decades in order to improve our understanding of the virus life cycle, pathogenesis and virus-host interactions. Initially, HCV was only studied through the use of serum-derived HCV patients. Then, the development of sub-genomic replicons allowed the study of viral replication and the generation of soluble envelope glycoproteins helped to identify the first HCV cellular receptors. More robust infectious systems followed, through the use of HCV pseudoparticles or the development of HCV cell culture systems. Now, the development of alternative and relevant animal model to the chimpanzee is paving the way toward a better understanding of HCV infection *in vivo*. In this chapter, we will describe the experimental systems that have been developed to study all the aspect of the HCV life cycle and pathogenesis, from the HCV serum derived particle to the last transgenic mice model competent for HCV infection and replication.

I.   Serum derived HCV particles

The first attempt to characterize HCV infection and replication was based on the use of patient serum-derived HCV particles (HCVsp) *ex vivo.* Importantly, entry and replication of HCV were observed in hepatoma cell lines and in primary human hepatocytes as well as in B and T cell lines. However, the use of such model encountered an important number of difficulties and limitations raising the need of other experimental systems (Fournier et al, 1998). Indeed, no productive infection of any cell lines was observed with the HCVsp. The proportion of viruses produced and released from the infected cells was very low as well as the RNA replication which was barely monitored. Moreover, the high heterogeneity of HCV *in vivo* in term of particle density and lipoprotein composition make the interpretation of such experiments problematic. During the years that followed, these limitations stimulated efforts to develop new tools to better characterize HCV life cycle. Systems allowing the characterization of HCV replication, entry and finally, the whole virus life cycle progressively emerged and seriously improved our knowledge on HCV.

Experimental models and tools	Assays or studies					Limitations
	Receptor/ Antibody binding	Neutralization	Cell entry	Replication	Assembly Egress	
Soluble E1/sE2	✓	✗	✗	✗	✗	Studies restricted to glycoproteins
Sub-genomic Replicon	✗	✗	✗	✓	✗	Studies restricted to viral replication
HCV virus like particle (HCV-VLP)	✓	✗	✗	✗	✗	Non infectious virus
HCV pseudoparticles HCVpp	✓	✓	✓	✗	✗	Studies restricted to virus entry No lipoporteins association
HCVcell culture system (HCVcc)	✓	✓	✓	✓	✓	Studies restricted to a single strain JFH-1 Higher buyoant density than serum-derived particles
HCV primary hepatocytes grown virus (HCVpc)	✓	✓	✓	✓	✓	Difficulties in cell culture Difficulties in logistics and availabilities of cells

Figure 18. HCV experimental systems and tools. Overview of the different experimental systems used to study particular steps of the HCV life cycle or viral proteins characteristics. Abilities of systems to be competent for specific assays or studies are symbolized as a green symbol. Red crosses mean that the model is not suitable for the related assays or characteristics. Limitations of each model are mentioned.

II.   Soluble HCV envelope glycoproteins

As envelope glycoproteins represent the main mediator of viral attachment to target cell as well as they are ensure membrane fusion, their study is critical to understand virus entry-mechanisms. Moreover, as these proteins are in the front line against the host-immune responses, they represent a tool of choice to identify and characterize anti-envelope neutralizing antibodies (Figure 18). Studies of the two HCV envelope glycoproteins, E1 and E2, encountered several difficulties in the past as these proteins have been shown to easily misfold and form aggregates (Dubuisson et al., 2000). These difficulties have made challenging the crystallization of E1 and E2 whose structures still remain undiscovered.

Another important difficulty to study HCV envelope glycoproteins is linked to the ability of their transmembrane domains to highly constraint E1 and E2 into the endoplasmic reticulum membrane (Cocquerel et al., 1999), making their purification and characterization difficult to perform. Although it was difficult to circumvent the E1/E2 misfolding, the ER-retention problem has been bypassed by the removal of the transmembrane domain of E2 and its expression as a soluble protein (sE2) (Rosa et al., 1996; Cerino et al. 1997). Thus, the production and use of this protein allowed several advances in our understanding of HCV entry as this protein was able to bind hepatoma cell lines (Figure 18). Soluble E2 allowed the identification of two major HCV cell entry receptors, the tetraspanin (CD81) (Pileri et al, 1998) and the Scavenger Receptor B1 (SR-BI) (Scarselli et al, 2002). The use of sE2 protein also offered the first time the possibility to assess of the neutralizing properties of patient-derived antibodies (Figure 18) through their ability to inhibit sE2 binding to hepatoma cell line (Rosa et al., 1996).

Despite all these major advances, it has been demonstrated that the correct folding of E1 and E2 is tightly linked to their association as heterodimeric complexes (Owsianka et al. 2001; Cocquerel et al. 2003). Thus, the development of experimental systems studying E1E2 as a full heterodimer complex incorporated onto viral particles might constitute a more relevant model for E1E2 functional studies.

III. The first HCV Virus Like Particles

In order to do so, the first models using viral like particles incorporating HCV envelope glycoproteins onto their surface was based on the use of vesicular stomatitis virus (VSV) pseudotyped with modified HCV envelope (Lagging et al. 1998; Matsuura et al., 2001). To avoid ER retention and allow incorporation of E1E2 at the plasma membrane, E1 and E2 transmembrane domains and cytoplasmic tails were replaced by the ones of the VSV glycoprotein G (VSVg) (Takikawa et al. 2000). Although efficient incorporation and viral particles production could be observed, viral particles were poorly or not infectious at all, underlining the limitation to use heterologous transmembrane domains that probably affect E1E2 heterodimerization and functionality (Buonocore et al., 2002).

Another useful system for the study of E1E2 complexes incorporated onto viral particles is the virus like particles (Figure 18) (VLPs) (Baumert et al, 1998). This model is based on the self-assembly of viral particles that cannot replicate and has been used for a large variety of viruses. After transfection of vectors encoding for the HCV core protein and the HCV envelope glycoproteins, HCV self-assembled particles could be purified from the cytoplasm of insect or mammalian cells but no particles were released into the cell supernatant, likely due to their retention into intracellular compartment. Interestingly, purified VLPs presented morphological similarities with patient derived HCV particles (Baumert et al, 1998), demonstrating the relevance of such model to study HCV Core function and virus morphology (Blanchard et al, 2002, Roingeard et al., 2004). This model also appeared as a promising tool to develop the first potential vaccine strategies against HCV, as the VLPs represented an interesting vector to expose E1E2 glycoproteins to host immune response (Figure 18). Similarities between the antigenic properties of VLPs and those of patient-derived HCV particles strengthened this idea (Baumert et al. 1999; Lechmann et al., 2001; Steinmann et al., 2004.). Despite all of the fundamental informations on virus structure and Core functions the VLPs brought to the community, the use of these particles were limited to attachment studies in a viral entry context (Figure 18). Thus, the need of a HCV-like particle system able to

recapitulate entry in hepatocyte and allowing the monitoring of infection (through encapsidation of a marker gene) was still urgently needed.

IV. HCV replicons

In 1999, an important technical breakthrough opened the way to the characterization of HCV replication. Lohmann et al. established cell lines constitutively expressing and replicating a subgenomic HCV RNA derived from a Con1 cDNA isolated from the liver of a chronically infected patient with a genotype 1b strain (Lohmann et al, 1999). The term *sub* was employed to define the replicon as only the HCV non-structural protein-encoding sequence was used to generate the replicons. Indeed, it was observed that the RNA replication do not require the presence of structural proteins for several other *Flaviviridae* (Khromykh & Westaway et al., 1997; Behrens et al. 1998). Two main subgenomic replicons were thus constructed. One expressed the non-structural proteins from NS2 to NS5B whereas the other, shorter, only expressed the HCV proteins from NS3 to NS5B (Figure 19). This model thus constituted the first model of functional HCV replicon allowing the study of HCV replication.

More precisely, the constructed replicons were bicistronic. On one hand, the 5′ non-coding region of HCV was replaced by the selectable markergene coding for the neomycin phosphotransferase that confers resistance to the cytotoxic G418 drug. On the other hand, an IRES element from encephalomyocarditis virus (EMCV) was inserted upstream the HCV non-structural protein-encoding sequences to ensure translation of non-structural proteins. The HCV 3′ non-coding region was conserved downstream the HCV non-structural protein-encoding sequence (Figure 19).
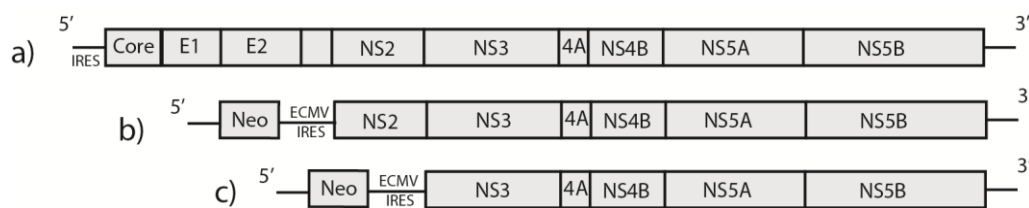
Figure 19. Schematic representation of HCV replicons. a) HCV genomic organization. b) subgenomic replicon NS2-NS5B. c) Subgenomic replicon NS3-NS5B. Neo, Neomycine phosphotransferase; ECMV, Encephalomyocarditis virus.

Huh7 cell lines persistently expressing HCV replicon were generated through transfection of the subgenomic cDNA, and then placed under selection with G418. HCV replication could be easily assessed and quantified, with up to 1000 to 5000 positive sense HCV RNA copies per cell (Lohmann et al, 1999). Moreover, studies that followed identified an important number of adaptive mutations acquired by the replicon after serial passages of the cell lines expressing the HCV replicon. These mutations mainly impacted on replication efficiency of the replication, by dramatically increasing its ability to replicate (Blight et al., 2000, Pietschmann et al., 2001). Precise identification of conserved mutations identified that the central region of NS5A, the C-terminal region of NS3 and two particular positions in NS4 constituted a preferential spot for adaptive mutations (Lohmann et al, 2003), thus constituting precious information for the potential generation of a non-selectable subgenomic replicon that allows highly efficient HCV replication.

Because HCV replicons offered for the first time a chance to decipher the HCV replication (Figure 18), this model highlighted opportunities to develop and test anti-viral drugs targeting HCV replication and non-structural proteins. However, as this model only focused on replication and so far, does not allow the study of other HCV life cycle steps (Figure 18), the characterization of other stage of the HCV life cycle still remained challenging. Especially, generation of new HCV experimental systems, based on a real virus-cell infection context was of crucial need. The development of HCV-like particles infecting hepatoma cell lines and targeted by neutralizing antibodies opened the way to a new panel of fundamental discoveries and therapeutical opportunities.

V.   The HCV pseudoparticles

In 2003, the generation of the first HCV infection systems open the way to major advances in our understanding of the HCV entry process. Indeed, this system allowed for the first time to generate HCV-like particles harboring complete and functional HCV envelope glycoproteins incorporated onto retroviral or lentiviral core

(Bartosch et al., 2003; Drummer et al., 2003; Hsu et al., 2003). These HCV-like particles, named as HCV pseudoparticles (HCVpp) were able to infect hepatoma cell lines *in vitro* by recapitulating HCV viral entry. This system was based on the transfection of HEK293T cells with three expression vectors respectively encoding for an E1E2 HCV envelope, a retro- or lenti-viral core and a packaging-competent retrovirus genome containing a marker gene as the Green Fluorescent Protein (GFP) or the Luciferase (Figure 20) (Bartosch et al., 2003). These marker genes allowed for the first time to monitor and quantify HCV infection through flow cytometry or luminometer technologies. Although the presence of a marker gene and so far the absence of any replication system did not permit particles propagation in cell culture and allowed only one single round of infection, this system provided the advantage to be easily manipulated in a biosafety level 2 laboratory which is not the case for HCV replicative virus.

As E1E2 are known to be incorporated and recruited at the ER-plasma membrane by neoformed viral particles in HCV infected cells, the HCVpp model was not able to recapitulate such assembly process (Figure 18). Retroviral (or lentiviral) cores are known to recruit their envelope glycoproteins at the plasma membrane and not at the ER-membrane. Thus, it has been suggested that the over expression of E1E2 allows a small portion of these proteins to be incorporated at the plasma membrane and to be recruited by the retroviral core (Bartosch et al., 2003).

The HCV pseudoparticles system allowed effective production of viral particles, with up to 10^4-10^5 infectious units per ml (Bartosch et al., 2003). These particles were able to infect efficiently Huh7 hepatoma cell lines but also primary human hepatocytes, which confirmed the restricted hepatotropism of HCV (Bartosch et al., 2003; Lavillette et al., 2005) and the critical role of the HCV envelope glycoproteins during HCV cell entry. As these particles precisely mimic virus entry and the early stages of infection (Figure 18), they offered for the first time a large panel of new possibilities (Bartosch et al., 2003; Lavillette et al., 2005).

E1 and E2 precise functions have been assessed. A large number of mutagenesis analyses have been performed on these proteins and a collection of residues or functional domains involved in folding, heterodimerization, binding or viral fusion

was identified (Drummer & Poumbourios, 2004; Ciczora et al., 2005; Drummer et al., 2006; Ciczora et al., 2007; Drummer et al., 2007; Lavillette et al., 2007; Owsianka et al., 2006; Rothwangl et al., 2008; Rothwangl & Rong, 2009; Albecka et al., 2011).

Development of new methods for the identification and characterization of E1E2 neutralizing antibodies have been developed (Figure 18). Effectiveness of particular anti-E2 antibodies have been confirmed and used in a large number of assays such as neutralization assays, western blotting or binding assays (Bartosch et al., 2003; Lavillette et al., 2005; Lavillette et al., 2007). Moreover, HCVpps harboring E1E2 envelope from the six major genotypes were successfully generated, allowing the test of potential neutralizing activities of antibodies or anti-viral drugs targeting entry on a large panel of viral type and subtypes (Lavillette et al., 2005).
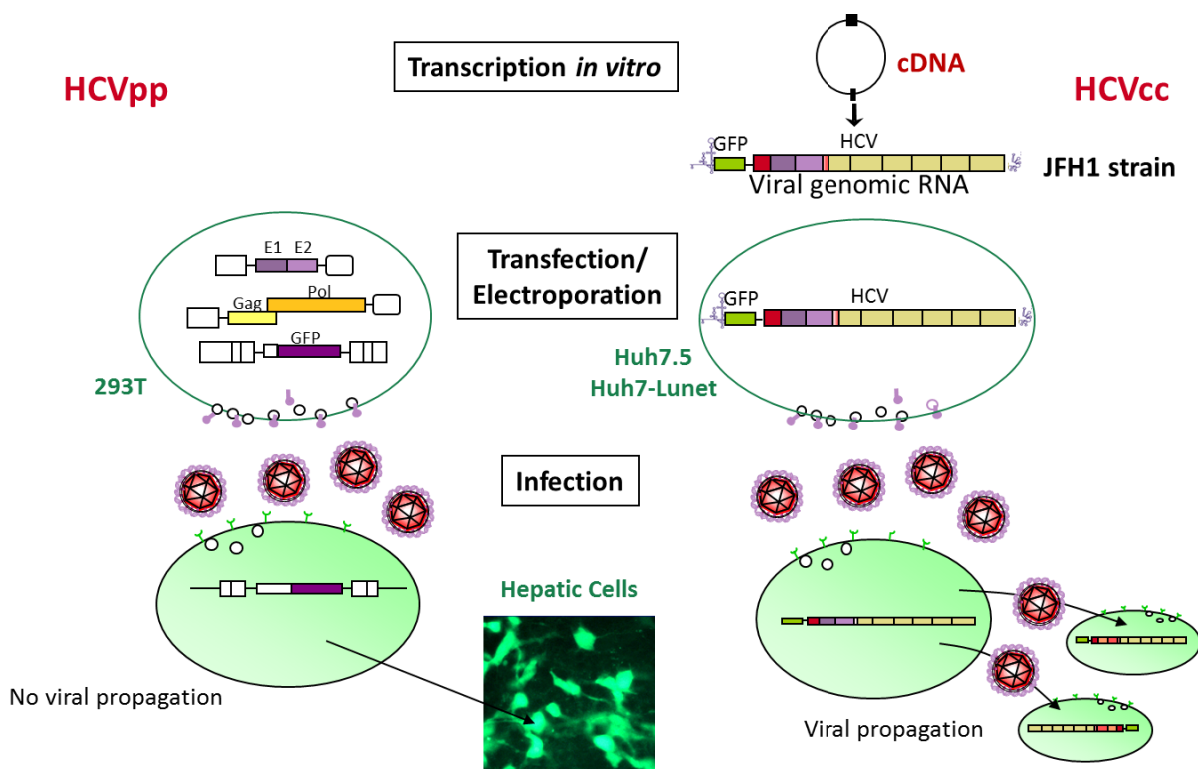
Figure 20. HCV pp and HCVcc model. (a) HEK293T are co-transfected with three expression vectors encoding for the E1E2 polyprotein, a retroviral core and a packaging-competent retrovirus-derived genome encoding for a marker gene (here, the GFP). Produced viral particles (HCVpp) are harvested and used to infect naïve Huh7. Viral entry is analyzed by flow cytometry through quantification of the GFP signal in infected cells. (b) Huh7.5 are electroporated with a full length (or chimeric) JFH-1 viral RNA. Electroporated cells produced authentic cell culture grown virus (HCVcc) that can infect and replicate within naïve Huh7.5.

The HCV pseudoparticles systems also allowed the confirmation of the importance for entry of the tetraspanin CD81 and of the Scavenger receptor B1 (Drummer et al., 2006; Bartosch et al., 2003; Bartosch et al., 2005). Moreover, it also pointed out that these two receptors were not sufficient to recapitulate HCV entry and that probably more receptors and entry factors remained to be discovered. This model has been critical during the years that followed, by constituting a useful tool to identify the last two major remaining HCV cell entry receptors, the cell surface tight junction Claudin-1 and Occludin (Evans et al., 2007; Ploss et al., 2009).

Finally, the HCV pseudoparticles constituted also an interesting system to investigate the extrahepatotropism of HCV and its ability to infect other cell types in infected patients.

Although the HCVpp system allowed the identification and characterization of the interaction between the viral particles and the lipoproteins, which are important for cell entry and virus fusion, these particles are produced without any associated-lipids as they are assembled in HEK293T cells. As it has been demonstrated that patient derived particles are associated with lipoproteins and that these compounds play an important role in virus attachment and entry, the HCVpp model only allows the study of the later stage of virus entry. The study of the complete entry process as well as of the other stages of infection thus highly the need of a more advanced experimental system, using a HCV clone able to replicate and propagate *in vitro*.

VI. The HCVcc system

In 2003, a study described the ability of an HCV RNA derived from a genotype 2 HCV subgenomic replicon that was able to efficiently replicate in cell culture without any adaptive mutation (Kato et al., 2003). This replicon, called JFH-1 for Japanese Fulminant Hepatitis 1, was isolated from a Japanese patient that cleared the virus after having developed a fulminant hepatitis (Kato et al., 2001). In 2005, the development of a cell cultured system (HCVcc) based on the transfection into highly permissive Huh7 derived cell clones of a full-length RNA genome deriving from the

JFH-1 replicon led to the production of infectious viral particles both *in vitro* and *in vivo* (Figure 20) (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The development of this model constituted a major achievement and has offered for the first time the possibility to the study the complete HCV life cycle (Figure 18).

The first studies confirmed the observations made with the HCVpp system. HCVcc particles presented a specific tropism for hepatocytes and viral entry was dependent of the previously identified cell entry receptors (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). HCVcc replication could be inhibited by IFN-α, highlighting the potential of this model to easily test anti-viral molecules. Moreover, entry could be inhibited by anti CD81 or SR-B1 antibodies as well as by anti-E2 antibodies. Interestingly, the observation that HCVcc particles are associated with lipoproteins components offered the possibility to study the early step of virus attachment in contrast to the HCVpp system. The HCVcc model unveiled a complex entry process involving both lipoproteins and viral glycoproteins that act in a concerted action to allow virus initial attachment and subsequent binding. Beyond the fact that entry and replication could be now studied in the HCV life cycle context, the HCVcc system allowed for the first time to study virus assembly and egress (Figure 18), offering new possibilities in terms of therapeutic strategies.

Over the years, the HCVcc model has been highly improved. Serial passages of the JFH-1 virus permitted to select adaptive mutations that efficiently improved virus production and virus titers (Kaul et al., 2007; Yi et al., 2007). Up to now, due to these improvements, the viral production has raised by up to 10^4 to 10^5 infectious particles per ml. Moreover, the development of highly permissive cell line clones deriving from Huh7 has been critical for the robustness of the system and to reach efficient virus production. The Huh7.5 (Lindenbach et al., 2005) or Huh7.5.1 (Zhong et al., 2005) clone were notably selected for their high ability to replicate HCV subgenomic replicons and harbor a defect in their interferon triggering system through mutational inactivation of RIG-I.

In order to counteract the limitation of the use of a single viral strain, numerous attempts have been made to generate new HCVcc genome. Several chimeric JFH-1

genomes has been developed and allowed the study of the structural proteins as well as p7 and NS2 of other HCV types (mainly 1a and 2a) or sub-types in the context of the JFH-1 genome (Lindenbach et al., 2005; Pietschmann et al., 2006).

Very recently, full length HCV gt1a (Li et al., 2012) and gt2b (Ramirez et al., 2013) infectious culture systems has been developed. These viruses were able to replicate efficiently in cell culture, allowing for the first time to analyze and compare specificities of HCV types or sub-types in a full life cycle context In the future, increase this panel of full length HCVcc viruses would be essential to efficiently assess of the potential of anti-viral compounds *in vitro* and *in vivo.*

VII. The HCVpc model

Despite the efficient replication of HCVcc in Huh7 derived cell clone-cultures, important concerns have been raised by the use of these cell lines. The infection of chimeric mice or chimpanzee clearly demonstrated that the buoyant densities of HCVcc particles are lower in animal models than in Huh7 cell culture system (Lindenbach et al., 2006). Indeed, Huh7 derived cell lines present some defect in their lipid metabolism that do not allow the correct assessment of particle association with lipoprotein. Thus, as lipoproteins play a critical role for virus assembly and during the early stage of entry, the optimal study of these stages of virus life cycle pointed out the need of a more relevant virus cell culture system closer from the expensive animal models. Other concerns were also raised by some particular features of Huh7 derived cell lines such as several genes deregulations, a defective immune response and an abnormal proliferation (Yokoo et al., 2004; Nagao et al., 2005).

A response to these concerns has been proposed in 2010 by the long term culture of primary human hepatocytes (PHH) that were able to replicate HCVcc at an efficient level. These cultures presented all the cellular marker of human hepatocytes and constituted so far a relevant cell culture model (HCVpc) able to recapitulate liver architecture (Banaudha et al., 2010; Ploss et al., 2010; Podevin et al., 2010).

The first attempt to develop such model was based on the culture of differentiated and non-dividing human primary hepatocytes infected by serum derived HCV particles (Ito et al., 1996). Although HCV particles were able to replicate and infect naive cell lines, replication was hardly detectable through RT-PCR and infectious titers were very low (Fournier et al., 1998). Moreover, the reproducibility and relevance of the observation made were highly limited by the high heterogeneity of the serum derived HCV particles. The development of the HCVcc system in 2005 offered a new opportunity to study the HCV life cycle and HCV properties in a more relevant biological context and cellular architecture with a well characterized virus.

Observations made with the HCVpc model were consistent with the ones obtained with the HCVcc system. Virus entry could be blocked with anti CD81 and anti SR-BI antibodies as well as with anti-E2 antibodies (Banaudha et al., 2010; Ploss et al., 2010). However, HCVpc present a lower buoyant density than HCVcc particles, consistently with the buoyant density of serum derived HCV particles. Interestingly, HCVpc particles located into the VLDL fraction were highly associated with ApoB whereas ApoB was undetectable in the HCVcc VLDL fraction demonstrating that Huh derived cell clones are unable to secrete authentic VLDL (Podevin et al., 2010).

Today, this model represents the most relevant HCV cell culture system available. However, important limitations are raised by the donor dependent-genetic variability of the human hepatocytes that may affect the reproducibility of the observations or introduce experimental bias.

VIII.   HCV animal models

1. Natural animal models

The development of suitable animal model to study HCV infection is today of critical need. As no vaccine is yet to be available, a complete understanding of the virus interaction with its host is required to develop efficient preventive and therapeutic strategies. To this purpose, the animal model constitutes the only experimental

system that both allow the study of the virus life cycle as well as the host immune response to infection.

Chimpanzee and a non-rodent small mammal, the *Tupaia belangeri*, represents today the only naturally HCV permissive animal models that support HCV infection and allow the study of the host immune responses.

Although Chimpanzee infection is less pathogenic than in humans (weaker viremia and no fibrosis or cirrhosis) this model has been critical to understand particular immune responses mechanisms against HCV and to evaluate the potential of vaccine candidate (Landford et al., 2001; Bukh, 2004; Bowen & Walker, 2005; Houghton et al., 2011). Moreover, this model has also been useful to confirm the ability of particular neutralizing antibodies to protect animals against infection. However, the limited number of animal used in the cohort due to their high costs, ethical considerations and the absence of a similar course of infection compared to humans point out the need of other complementary and efficient animal models (Landford et al., 2001; Buck et al., 2001; Harrington, 2012).

The *Tupaia belangeri* has been shown to be naturally susceptible to HCV infection as the primary Tupaia hepatocytes are able to support HCV infection (Amako et al., 2010; Tong et al., 2011). However, infection *in vivo* appears as very weak despite observations of liver injuries and Core expression within the liver. Indeed, HCV RNA was undetectable within animal sera as well as anti-HCV antibodies (Amako et al., 2010; Yang et al., 2013). As these animals are rare (and so far expensive), are provided only small animal cohorts for infection studies, which develop an attenuated infection. Thus, their use in infection studies raise the same concerns as Chimpanzees do, and highlight the need of alternative animal models more suitable for large and relevant studies.

2. Humanized animal models

Mice and rats are commonly used as animal models in laboratories and for clinical studies. However, these animals do not support HCV infection and thus cannot be

naturally used as animal model (Dorner et al., 2011). Studies have shown that this restriction does not occur during viral assembly or egress but rather during entry and virus replication (Long et al., 2011). Thus, the use of rodent as HCV animal model is tightly dependent of their potential humanization, either through the xenograft of human liver cells (simultaneously or not with human immune cells) or via transgenesis (Figure 21).

a) *Xenograft-derived humanized mice model*

A model of humanized liver mice generated by xenograft is the uPA-SCID model. This model uses immunodeficient SCID mice that overexpress a lethal transgene in their hepatocytes, the urokinase-type plasmino-gen activator (uPA) (Mailly et al., 2013; Billerbeck et al., 2013). In this system, the rapid destruction of mice hepatocytes is not controlled. The engraftment of human hepatocytes into the liver mice is thus only possible during the first weeks of life (Meulemann et al., 2005; Vanwolleghem et al., 2010). It has been shown that these mice are able to support very efficiently both HCVcc and serum derived HCV particles infection, that can be sustained up to 10 months (Mercer et al., 2001; Vanwolleghem et al., 2010). This model also permitted to certify *in vivo* of the inhibitory effect against HCV infection of anti-receptors antibodies (CD81 and SR-B1) (Meulemann et al., 2008; Meulemann et al., 2012; Lacek et al., 2012), small antiviral compounds (Lupberger et al., 2011; Sainz et al., 2012) or of the recently developed direct acting agents (DAAs) (Vanwollegehem et al., 2007; Shi et al., 2013). A study also recently demonstrated by using this model that particular DAAs combinations are efficient against HCV infection in a genotype-dependent manner (Shi et al., 2013).

More recently, another engrafted-humanized liver mice model, the Fah$^{-/-}$ Rag2$^{-/-}$ γ-c$^{-/-}$, has been developed. These mice are immunodeficient through homozygote mutations into the Rag2 recombinase gene and into the common γ-chain of the interleukin receptors (Mailly et al., 2013; Billerbeck et al., 2013).
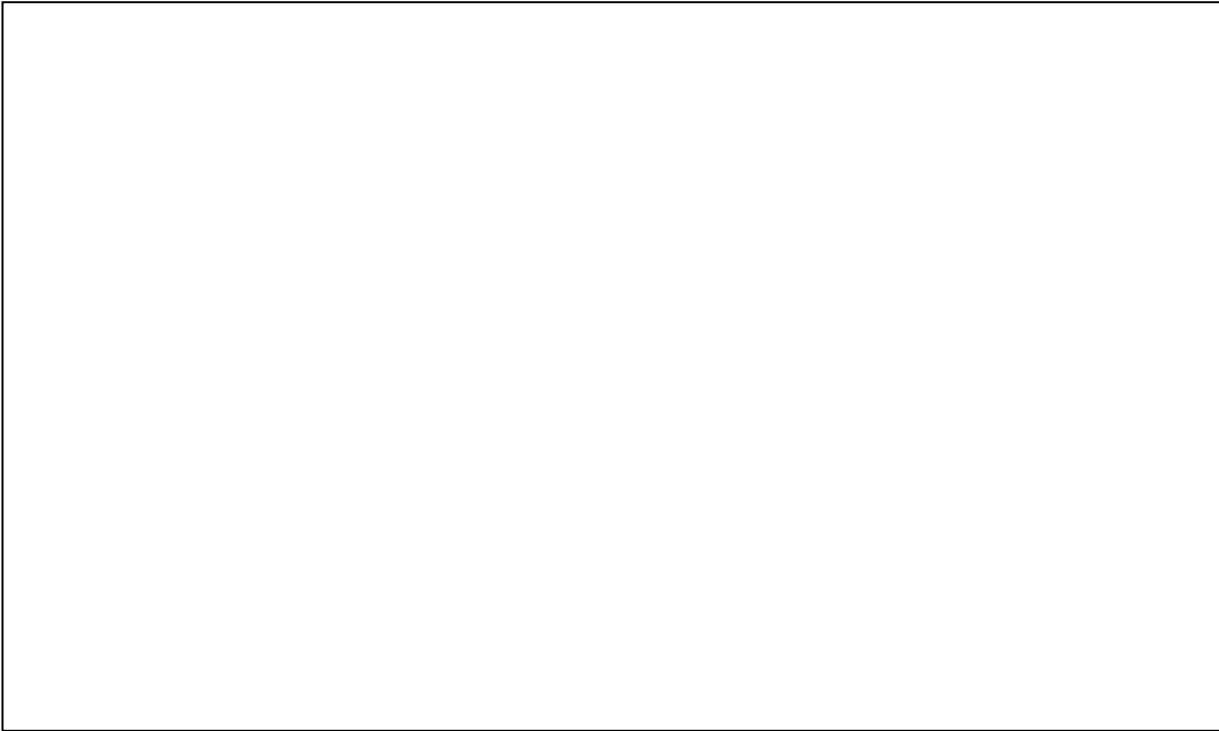
Figure 21. Current adopted approaches for the development of mice models to study HCV infection. Xenotransplantation of human cells (left; hepatocytes or immune cells), genetic humanization through the expression of specific human factor (middle) or HCV adaptation to mouse cells (right) represent the three major strategies. HLA transgenic mice can be crossed with the different models in order to improve immune responses (Mailly et al., 2013).

Moreover, they present a defect in their tyrosine metabolism as they do not produce the tyrosine catabolic enzyme fumarylacetoacetate hydrolase (Fah), which results in the accumulation of toxic metabolites that induce liver degeneration (Grompe et al., 1993; Grompe et al., 1995). This degeneration allows the engraftment of human hepatocytes into the liver mice and the development of a humanized liver (Bissig et al., 2007). Interestingly, this model allows the control of the liver degeneration by using the 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) that interfere with the production of toxic metabolites and prevent hepatocytes degradation (Grompe et al., 1993; Grompe et al., 1995). Thus, in contrast to the uPA-SCID model, this efficient control system makes it possible to engraft liver mice at any time during their adult life. Up to now, one study showed that these mice efficiently support infection by HCVcc (genotype 2a or 1a-b/2a chimeric virus) and by serum derived-HCV particles deriving from a clinical isolate of gt 1a(Bissig et al., 2010). Thus, this model represents a relevant and promising tool to assess the efficiency of anti-viral compounds targeting HCV.

Up to now, the uPA SCID model has been more intensively used than the FRG model but the latter presents important advantages (as the control of the liver degeneration) that can contribute to its development for fundamental and clinical studies within the next years. However, as these two models do not harbor any functional immune system, relevant assessment of virus-host interactions and evaluation of potential vaccine candidates remain hard to achieve

To overcome this limitation, others models have been developed, such as the AFC8-huHSC/Hep mice based on the Balb/c Rag2$^{-/-}$ γ-c$^{-/-}$ (BRG) mice model. These mice overexpress a fusion protein that activates apoptosis pathway and induces cell death after injection of a molecular compound (Pajvani et al., 2005; Washburn et al., 2011). Transplantation of new born mice with human progenitor cells and CD34+ hematopoietic stem cells isolated from the same donor allowed the development of chimeric mice with both a humanized liver and immune system. These mice were able to support serum derived HCV particles infection and a specific T cell response against HCV could be observed. However, no B cell responses could be observed and no viral particles could be detected in mice serum (Mailly et al., 2013).

b) Transgenic humanized mice model

In 2011, a study reported that the adenoviral transduction of Rosa26-Fluc mice with vectors encoding for the HCV entry factors CD81 and OCLN, the two entry factors that mediate HCV host tropism (See Chapter V – II. 3.) led to viral entry of a bicistronic HCVcc Jc1 virus into mice hepatocytes *in vivo*. This virus expresses a Cre (HCV-Cre) recombinase able to activate a Luciferase reporter into the Rosa26-Fluc genome (Dorner et al., 2011). Viral entry was detectable through *in vivo* bioluminescence in 1 to 2% of the hepatocytes and was inhibited by anti-CD81 antibodies or anti-E2 antibodies demonstrating the relevance of this system to recapitulate virus entry *in vivo*. However, HCV-Cre was not able to replicate in mice hepatocytes and no de novo viral particles were released in serum likely due to the innate and adaptive mice immune responses.

To overcome this restriction, another study from the same authors reported very recently the development of a new Rosa26-Fluc system permissive for the entire HCV life cycle (Dorner et al., 2013). In this study, mice expressing the 4 HCV entry factors were crossed with mice carrying genetic disruptions into several molecular receptors involved in innate and adaptive immunity. Infection of the transgenic mice disrupted for IRF1, IRF7, IFN-αβR and STAT1 with HCV-Cre led to a marked increase of Luciferase signal in mice hepatocytes (Dorner et al., 2013). Severe decrease of the Luciferase signal after infection of mice both deficient for the expression of cyclophilin A (a cellular factor essential for RNA replication) and the expression of STAT1 indicated that abrogation of immune response allowed HCV-Cre replication in these transgenic mice. Persistent replication and presence of viral proteins were confirmed by using transgenic mice expressing a blue fluorescent protein able to be translocated into the nucleus after cleavage by the NS3-4A protease complex (Dorner et al., 2013). Viral particles isolated from mice serum were infectious *in vitro* despite very low viral titers, demonstrating that these mice were able to produce de novo HCV particles and recapitulate the entire HCV life cycle (Dorner et al., 2013). Despite the defect in innate immune responses, some immunological features could be observed in Stat1 deficient mice such as the infiltration within the liver of NK cells and IFN-γ-producing CD8+ T cells, consistently with previous observations made in humans.

This new model constitutes a major advance in the field of HCV animal model, allowing for the first time to recapitulate the complete HCV life cycle in both an infectious and immune context.

However, the high level of transgenes expressed by these mice as well as the potential interfering immune response against adenoviral transduction may introduce several bias in the data obtained with this model about HCV immune responses. Moreover, low infectious titer of mouse serum-derived HCV particles observed *in vitro* and the absence of detectable viral RNA after 90 days indicate that this model is not able to induce persistent infection and to recapitulate HCV pathogenesis.

Altogether, these restrictions highlight the need to improve this model. The isolation and use of an adapted viral variant able to highly replicate in these mice (Figure 21) may constitute an interesting improvement, even though this variant would not be able to recapitulate the extreme genetic diversity of HCV.

CHAPTER IV

Virus life cycle and molecular virology

Hepatitis C Virus is an unusual virus in the *Flaviviridae* family. Viral particles are structurally heterogeneous which hampers a relevant characterization of the virus structure. Due to its particular association with lipoprotein components in patient serum, HCV also harbors a buoyant density that is lower than most of the known viruses. This ability of HCV to hijack the host lipid metabolism is critical for its entry and assembly into hepatocytes. This association makes the relationship between the virus and its host more complex, and thus, restrains the development of relevant experimental systems. Although HCV presents a high degree of genetic and functional similarities with pesti- or pegiviruses, the role of some of its proteins is still poorly defined. Accordingly, several steps of the HCV life cycle remain to be precisely understood such as virus entry or assembly. In this chapter, we will first introduce the major virus particle structural features. Then, we will review the virus structural and non-structural proteins. Finally, the steps of the HCV life cycle will be summarized: virus entry, protein translation, RNA replication, virus assembly and egress.

I. Viral particle structure

HCV is an enveloped virus of 40-80 nm in diameter, heterogeneous in terms of morphology and that does not display any clear form of symmetry (Figure 22A) (Bradley et al., 1985; Gastaminza et al., 2010; Merz et al., 2011; Catanese et al., 2013). For these reasons, no clear model of particle structure is actually available, thus placing the HCV structural features in strong contrast with the well-defined flaviviruses particles structures. Similarly, the arrangement of the structural proteins onto the virus surface remains mysterious. HCV particles harbor onto their membrane two envelope glycoproteins, E1 and E2, which mediate virus attachment

Figure 22. HCV particle structure and lipoprotein association. **A**. Representative electron micrographs of HCVcc particles captured on a magnetic bead recognizing the tagged E2 envelope glycoprotein. Scale bars represent 50 nm (Catanese et al., 2013).**B**. Schematic representation of a lipo-viro-particle **C**. Models of HCV-lipoprotein association. A two particle model proposes the existence of transient interaction between viral particle and lipoprotein (left). A single particle model proposes that lipoprotein and HCV particle share a common envelope and form lipo-viral hybrid particles (right) (Lindenbach et al., 2013).

However, their structures and organization onto viral particles as well as their conformation rearrangements during virus binding and membrane fusion have not been elucidated yet. As other Flaviviridae, the viral envelope surrounds a nucleocapsid that is composed by

> A buoyant density is the measure of the tendency of a substance to float in some other substance. The buoyant density of molecules in suspension in a fluid is determined by density-gradient

dimeric complexes of the structural basic protein Core (equivalent to the flaviviruses and pestiviruses Capsid protein). The nucleocapsid contains a single copy of the viral genome, a positive stranded RNA (Figure 22B) that is released into the cytosol after virus internalization and membrane fusion (Figure 22A).

Unlike flaviviruses and pestiviruses, hepaciviruses and pegiviruses particles present a heterogeneous and very low buoyant density (Figure 23). Indeed, the buoyant density (see box) of the HCV most infectious particles usually ranged around densities that are lower than 1.10 g per ml (1.03-1.10 g/ml), which is significantly lower than flaviviruses (1.20-1.23 g/ml) and pestiviruses (1.12-1.15 g/ml) (Lindenbach et al., 2005; Lindenbach et al., 2013). This particular buoyant density is due to the very distinct lipid composition of HCV particles that associates with serum lipoprotein.

> Lipids are transported in the blood as lipoproteins. Lipoproteins are composed of a hydrophobic lipid core containing mainly triacyglycerols (TG) and cholesteryl esters (CE). The core is surrounded by a phospholipid monolayer that contains unesterified cholesterol and particular proteins called apolipoproteins. There is five class of lipoproteins according to their density and electrophilic mobility: the Chylomicrons, the Very Low Density Lipoproteins (VLDL), the Low Density Lipoproteins (LDL) the Intermediate Density Lipoproteins (IDL), and the High Density Lipoproteins (HDL).
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>
>
> (Image from http://www.wasanlab.com/research.html)
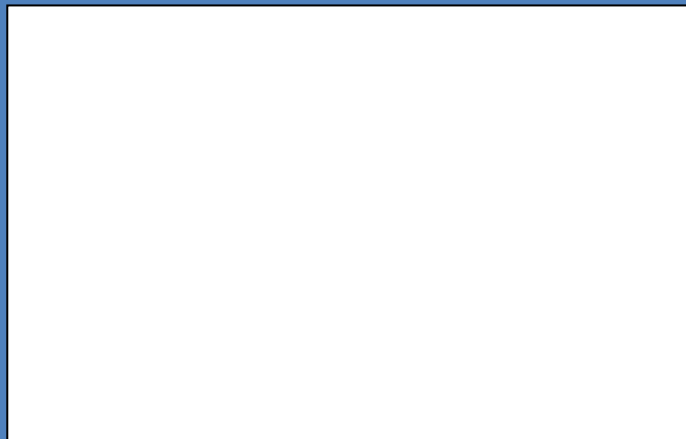
Indeed, serum derived HCV particles have been found to be associated with particular lipoproteins components such as apolipoprotein B-48 and -100 (apoB-48, B-100), apolipoprotein C-I (apoC-I) and apolipoprotein E (apoE) that are the components of low density lipoproteins (LDL) or very low density lipoproteins (VLDL) (Thomssen et al., 1992; Lindenbach et al., 2013). Consistently, HCVcc particles have been found to harbor a lipid composition similar to the very low- and low density-lipoprotein.

These particles are associated with the lipoproteins component apoC-I and apoE as suggested by immunoprecipitation assays with anti ApoE and ApoC-I antibodies (Kono et al., 2003; Nielsen et al., 2006; Diaz et al., 2006; Felmlee et al., 2010). However, their association with apoB remains unclear (Chang et al., 2007; Merz et al., 2010, Catanese et al., 2013). HCV particles of medium and high density are associated respectively with high density lipoproteins (HDL) or immunoglobulins (Igs) but these associations affect their infectivity, underlining the critical role of LDL and VLDL for virus infectivity (Hijikata et al., 1993; Nielsen et al., 2006).
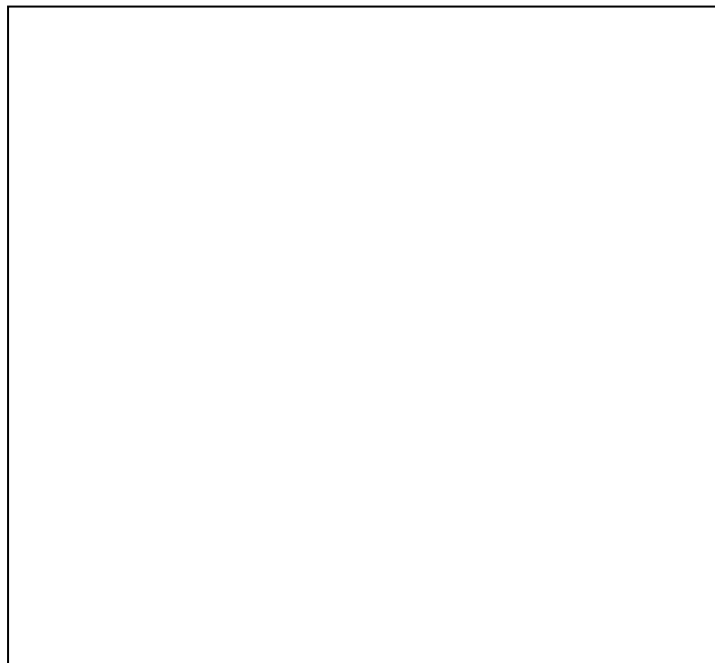
Figure 23. Comparison of the buoyant density of several virus genus, family and serum lipoproteins. HDL, high-density lipoprotein; HCV, hepatitis C virus; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein. *Sparklines represent the buoyant density ranges across a linear scale of 0.93 g per ml (left) to 1.27 g per ml (right) (Adapted from Lindenbach et al., 2013).

Altogether, these information have contributed to the qualification of the HCV particles as lipo-viro particles (LVPs) (Figure 22B, 22C, 23) (Bartenschlager et al., 2011; Lindenbach et al., 2013). This unique property offers to the HCV particle a panel of original features. Among them, the ability of HCV to be recognized by a panel of lipoprotein receptor molecules such as the LDL receptor (LDL-r) is critical for the initial attachment of the viral particles onto hepatocytes (Chang et al., 2007, Owen et al., 2009, Hishiki et al., 2010). This unique association with lipoproteins may also constitute a molecular barrier that protects the envelope glycoproteins epitopes against host neutralizing responses.

However, the nature of the interactions between HCV envelope glycoproteins and lipoproteins remains to be identified and their characterization could represent interesting informations to understand LVPs architecture. Indeed, elucidate whether the LVPs are the result of transient associations between viral particles and lipoproteins or represent lipo-viro hybrid particles with a unique envelope remain today challenging (Figure 22C).

II.   Genome and viral proteins

The Hepatitis C Virus genome is a single positive strand RNA that encodes for a large polyprotein containing viral structural and non-structural proteins (Figure 24). Such as the other *Flaviviridae*, a long ORF is flanked by 2 non coding regions at the 5′ and 3′ extremities of the viral RNA.

The ORF encodes for a polyprotein of around 3000 amino acids cleaved co- and post-translationnaly into 11 structural and non-structural proteins by cellular or viral proteases (Figure 24) (Moradpour et al., 2006). HCV polyprotein organization, such as its genomic organization, is closer to pestiviruses than flaviviruses. As GBV-B, the HCV N terminal part of the polyprotein contains the 3 structural proteins: Core, E1 and E2, then followed on the ORF by p7 non-structural proteins (p7, NS2-3-NS4A/B-NS5A/B). The 11th protein, the F protein, is the result of a reading frame shift in the capsid-encoding sequence. During translation, C/E1, E1/E2, E2/p7 and p7/NS2 junctions are processed an ER resident-enzyme, the host signal peptidase (Figure 24). Then, as pestiviruses, NS2 processes its junction with NS3 and the NS3/NS4A serine

protease complex processes the remaining junctions to release the non-structural proteins NS4A, NS4B, NS5A and NS5B (Figure 24) (Moradpour et al., 2006).
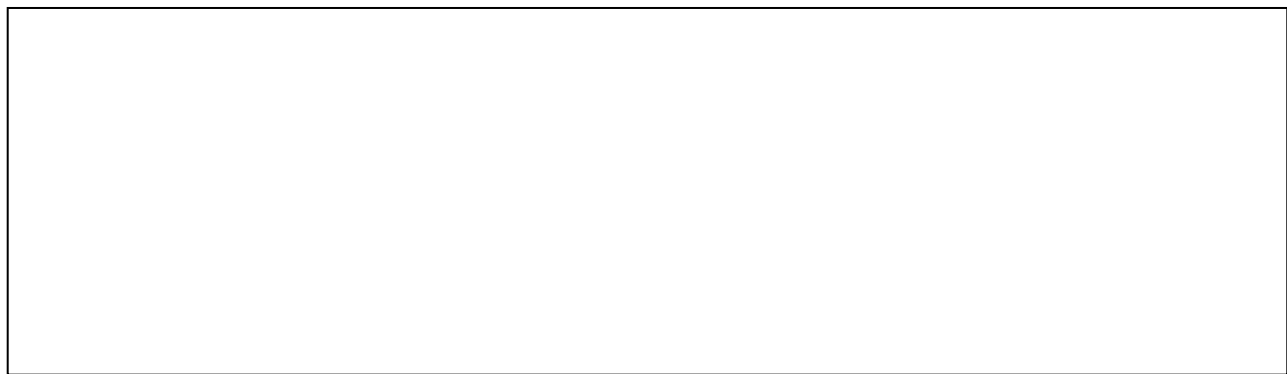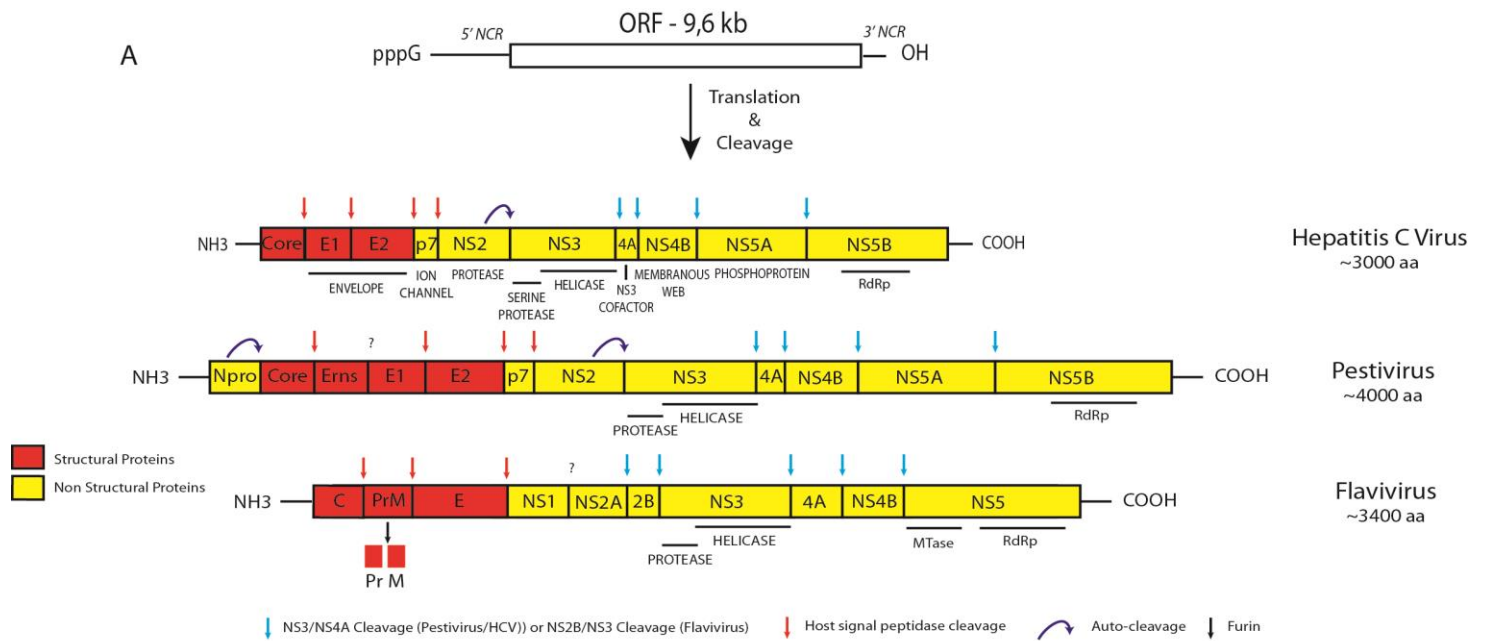
Figure 24. HCV polyprotein organization and comparison with flavivirus and pestivirus polyprotein. A. HCV polyprotein is produced after translation of an ORF of 9,6kb. Polyprotein lengths are indicated below the genus name. Red and yellow blocks represent respectively the structural and non-structural proteins. Roles of each structural and nonstructural HCV protein are indicated below the HCV polyprotein. Polyprotein processing is indicated: Red and blue arrows indicate junctions processed respectively by the host signal peptidase and by the NS3/NS4A or NS2/NS3 serine protease. Black arrow represents the processing of PrM by the furin. Curved arrow indicates the auto-processing of the NS2/NS3 junction by NS2. RdRp, RNA-dependent RNA-polymerase. **B.** Structure and ER membrane association of the different HCV proteins (Adapted from Moradpour et al., 2006).

1. RNA non coding regions

Such as other *Flaviviridae*, the 5′ and 3′ NCR contain structural features that are conserved among genotypes. However, the HCV 5′NCR with a 341 nucleotides (nt) sequence is longer than the 5′NCR pestiviruses (Figure 25) and shorter than the 5′NCR of GBV viruses (Lindenbach et al., 2007). The HCV 5′ NCR contains 4 structural domains (I to IV). Domains I and II are essential for RNA replication (nt 1 to 115) even though the entire NCR is required to ensure correct RNA synthesis (Figure 25) (Kim et al., 2002). HCV polyprotein translation is similar to pesti- and pegiviruses. The translation is cap-independent and requires an internal ribosome entry site (IRES) sequence that is structurally and functionally similar to the pesti- and pegiviruses one (Figure 25) (Puglisi et al., 2001; Reusken et al., 2003). In the HCV RNA, the IRES is constituted by the domains II to IV of the 5′NCR (nt 44 to 341) as well as by the beginning of the polyprotein encoding sequence (Figure 25). As pestiviruses, the HCV IRES is critical to recruit the ribosomal subunit 40S and for the initiation of translation that requires similar initiation factors for both genera (eIF4 A, B, F) (Spahn et al., 2001). Liver-specific miR-122 has been shown to enhance translation and replication (Jopling et al., 2005; Fukuhara & Matsuura, 2013) and contributes to the restricted tissue tropism of HCV. It has been suggested that miR-122 binding to 5′ HCV NCR, in concert with Ago2 (a catalytic component of the RNA-induced silencing complex RISC), stabilizes viral RNA and reduces its decay (Wilson et al., 2011; Shimakami et al., 2012; Fukuhara & Matsuura, 2013). Consistently, the high degree of conservation of the 5′NCR also provides a good target for anti-viral therapies using RNA interference strategies.

The 3′NCR contains 200 to 235 nt and is divided in 3 distinct regions. The first domain is a short and variable domain (VR) of ~40 nt followed by a short polyuridine/polypyrimidine (polyU/UC) tract that must be at least of 26 nt length (Figure 25) (Lindenbach et al., 2007). The third domain is 98 nt highly conserved domain named 3′X-tail that contains three hairpin structures essential for RNA replication (Figure 25) (Kolykhalov et al., 2000; Yi & Lemon, 2003). Indeed, an interaction between the 3′X-tail domain and NS5B has been observed. The entire

3'NCR region is also critical for translation, through the stimulation of the 5' NCR IRES function. Interestingly, the 3'NCR of GBV-B is longer than HCV but harbor region of structural homologies with the HCV X-tail. Moreover, hepaciviruses (HCV and GBV-B) are the only *Flaviviridae* that harbor a polyU/UC tract in their 3'NCR (Lindenbach et al., 2007).
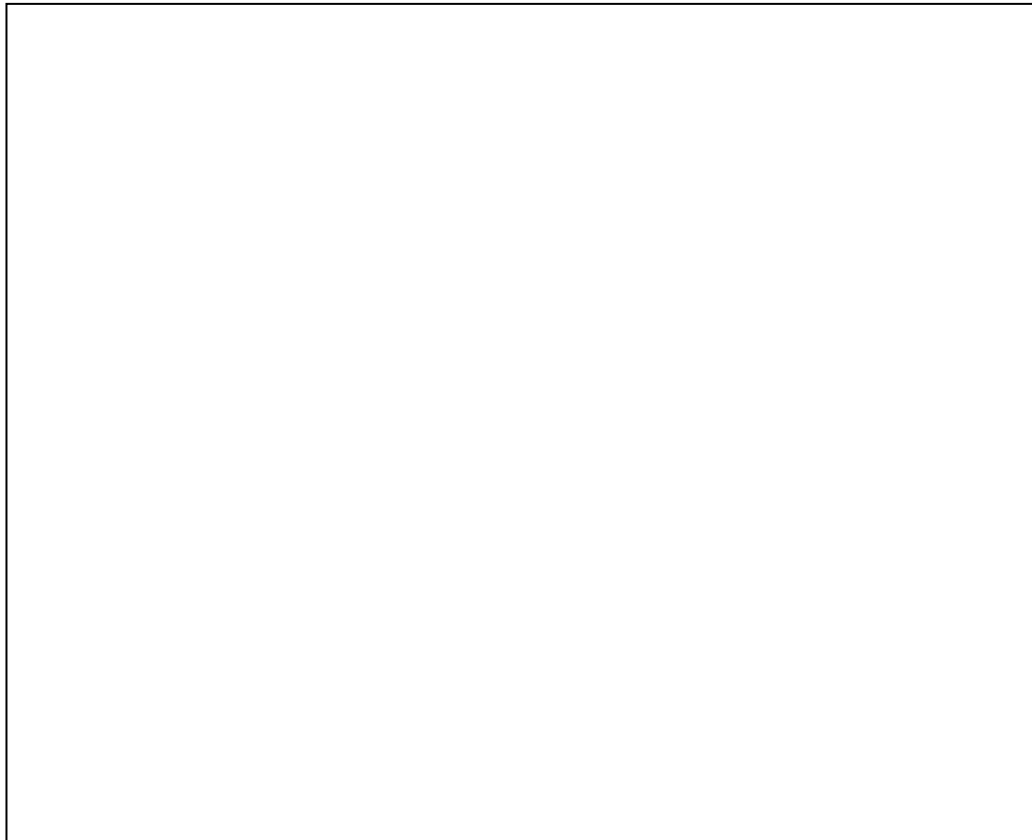
Figure 25. Genomic organization of HCV viral RNA and comparison with flavivirus and pestivirus viral RNA. ORF lengths are indicated for each virus genus. Below the ORF, Non Coding regions (NCR) are highlighted and structural NCR features, hairpins loops and domains are indicated. ST, Stem loop; CS or RCS, Conserved sequences; VR, variable region (Adapted from Lindenbach et al., 2007).

2. Structural proteins

a) Core protein

The Core protein (C) is the structural protein that composes the virus nucleocapsid. This protein, that is highly basic and conserved among genotypes, is initially produced as an immature form of 23 kDa after the processing of the Core/E1 junction by the signal peptidase. Then, its C terminal anchoring domain is cleaved by

the signal peptide peptidase to allow the release a mature form of the Core protein of approximately 21 kDa (Okamoto et al., 2008). Before the processing, this C terminal domain is notably required for the translocation of E1 into the ER lumen. Core protein is composed of two distinct domains qualified as D1 and D2. D1 is constituted by the first 118 amino acids of the protein which are mainly hydrophilic. This domain is involved in nucleocapsid assembly and interacts with the 5'NCR of the viral RNA (Klein et al., 2005), underlining its critical role for virus assembly. In contrast, the rest of the protein (domain 2) is hydrophobic. The properties of this domain confer to the Core the ability to associate with cellular membranes of lipid droplets, association that is critical for viral particle assembly (Rouille et al., 2006; Bartenschlager et al., 2011; Lindenbach et al., 2013). In parallel, the domain 2 is also a structural chaperone for the correct conformation of the domain 1. Several evidences also showed that the role of the Core is not strictly limited to a structural role via the alteration of the metabolism of lipids or the regulation of several cellular pathways involved on liver pathogenesis (Jackel-Cram et al., 2007).

b) E1 and E2 envelope glycoproteins

The two others HCV structural proteins, the envelope glycoproteins E1 and E2 are incorporated onto the viral surface. These two proteins that are critical for virus entry through initial attachment, binding to specific cellular receptors and fusion are presented in Introduction – Chapter V I.

3. Non-structural protein

a) P7 protein

As pesti- and pegi-viruses, HCV expresses a small hydrophobic protein (called p7 for HCV and pestiviruses, p13 for GBV-B) that is released from the polyprotein by the signal peptidase. This protein is composed of two hydrophobic transmembranous domains linked together by a small cytoplasmic domain. Similarly to pestiviruses p7, HCV p7 associates as multimeric complexes to form ion channels essential for viral particles production and release (Bentham et al., 2013). Recent evidence using p7 mutants showed that this protein is critical for the final steps of viral assembly. Its

activity modulates core unloading from site of virus assembly as well as capsid maturation, capsid incorporation of viral RNA and capsid envelopment (Gentzsch et al., 2013). Moreover, it has been also demonstrated that p7 is involved in the early steps of virus assembly via its dialog with the non-structural protein NS2 that governs the recruitment of Core protein on virus assembly sites (Boson et al., 2011). Similarly to pestiviruses, inefficient cleavage commonly occurs and results in accumulation of E2-p7-NS2 or E2-p7 precursors (Lindenbach et al., 2007). Although these precursors do not impact on virus production or infection, it has been shown that the inhibition of the p7 processing is detrimental for virus production (Shanmugam et al., 2013) by inhibiting NS2 recruitment on assembly site and NS2 interactions with other non-structural proteins such as NS3.

b) NS2

NS2 is a 23 kDa non-structural membranous protein. Although its structure is not known, the most recent topology model indicates that this protein probably harbors three transmembranous domains and a cytosolic cysteine protease domain (Jirasko et al., 2008). As pestiviruses and GBV viruses, this protease domain associates with the N terminal part of NS3 that acts as a cofactor for the processing of the NS2/NS3 junction (Grakoui et al., 1993). This cleavage has been shown to be essential for RNA replication and virus infectivity (Welbourn et al., 2005; Jones et al., 2007). NS2 is also critical for viral particle production as mutations in NS2 abrogate late steps of virus assembly (Yi et al., 2009; De la Fuente et al., 2013). NS2 appears as a global mediator of this process, through the recruitment of a lot of viral proteins such as p7, E2 and NS3 to assembly sites (Jirasko et al., 2010; Boson et al., 2011).

c) NS3/NS4A

NS3 is a 70 kDa protein with two major functions identical to pestiviruses NS3. The N terminal domain of the protein encodes for a serine protease activity whereas the C terminal domain encodes for a helicase and NTPase activity.

Similarly to pestiviruses and GBV viruses, NS3 associates with NS4A that anchored the complex onto the ER membrane to form a competent serine protease complex.

This complex cleaves the NS3/NS4A, the NS4A/NS4B, the NS4B/NS5A and the NS5A/NS5B junction to release the non-structural proteins from NS3 to NS5B (Moradpour et al., 2007). Interestingly, the GBV-B serine protease activity shares substrate specificity with the HCV serine protease and can be inhibited by inhibitors targeting the HCV NS3/NS4A complex (Lindenbach et al., 2007). The serine protease activity of the NS3/NS4A complex is also known to interfere with the cellular innate responses by processing proteins involved in RIG-I and TLR-3 signaling pathways (Meylan et al., 2005; Cheng et al., 2006). As mentioned below, NS3 is also associated as a co-factor to NS2 to allow the processing of the NS2/NS3 junction and the release of NS2.

Additionally to its protease function, NS3 is essential for RNA replication (Lam & Frick, 2006). Although the monomeric form of NS3 is able to bind RNA, the dimeric form, via the helicase activity, is involved in RNA unwinding (Lindenbach et al., 2007). This function is dependent of the NTPase activity that hydrolyzes ATP to generate energy (Gu et al., 2010). Indeed, it has been shown that NS3 conformation is modified after ATP hydrolysis. Moreover, the C terminal domain of NS4A also modulates the helicase activity of NS3 as mutations in this domain dramatically affect helicase activity (Beran et al., 2007). NS4A, that is very similar to pestivirus and GBV virus NS4A, is also involved in the replication process independently of NS3, via the hyperphosphorylation of NS5A (Lindenbach et al., 2007).

NS3 is also important for virus assembly. Indeed, mutations in the helicase domain that enhance RNA replication may also impair virus assembly, indicating that NS3 orchestrates both functions in a tight way (Ma et al., 2008; Chatel-Chaix et al., 2011). It has been suggested that the NS3-NS4A helicase activity would be involved in RNA packaging during nucleocapsid assembly (Lindenbach et al., 2013).

d) NS4B

NS4B is an ER anchored hydrophobic membrane protein of 27 kDa that is processed by the NS3/NS4A protease complex. This protein, which is very similar in size and composition to the pestivirus and GBV virus NS4B, harbors four transmembranes

domains flanked by two cytoplasmic N and C terminal domains. These two terminal domains are organized as alpha-helical structures and are critical for the ER localization of NS4B even though the N terminal domain can be translocated into the ER lumen and form a 5th transmembrane domain (Lundin et al., 2003; Gouttenoire et al., 2009). NS4B protein is critical for RNA replication. This protein is able to oligomerize and interacts with other viral proteins to alter intracellular membrane. This membrane rearrangement forms the "membranous web", an important membranous complex that is associated with the RNA replication site and critical for viral replication (Lohmann et al., 2013). Moreover, the terminal domains of NS4B both harbor important functions for replication. Although its N terminal domain possesses a NTPase activity that generate energy essential for replication, its C terminal domain would be important for NS5A hyperphosphorylation (Jones et al., 2009). Similarly to NS3, NS4B plays also a key role during virus assembly independently of its function in RNA replication (Jones et al., 2009) but is also able to inhibit intracellular innate responses by interfering with the RIG-I mediated signaling pathway (Nitta et al., 2012).

e) NS5A

NS5A is a phosphoprotein incorporated onto the ER membrane. This protein is involved in RNA replication through binding of viral RNA into the membranous web. Moreover, it is also involved in virus assembly where it can associate with the core protein in close proximity of the lipid droplets and the virus assembly sites (Miyanari et al., 2007). The N terminal part of this protein is highly conserved and involved in RNA replication (Elazar et al., 2003; Penin et al., 2004) whereas the C terminal part harbors a nuclear localization signal highlighting that NS5A may regulate particular genes expression (Satoh et al., 2000).

NS5A is divided into 3 functional domains. The domain 1 harbors an unconventional zinc binding motif highly conserved in hepaciviruses and pestiviruses. This motif is composed of four cysteins that are critical for RNA replication, underlining the fact that NS5A may be considered as a zinc mettaloprotein (Tellinghuisen et al., 2004; Tellinghuisen et al., 2005). The others domains are also critical for virus particles

production and replication. The domain 2 harbors critical residues for RNA replication (Tellinghuisen et al., 2008) and the domain 3 is likely important for virus assembly through interaction with Core during virus assembly (Masaki et al., 2008).

Two major form of this protein co-exist: a phosphorylated form of 56 kDa and a hyperphosphorylated form of 58 kDa (Grakoui et al., 1993). The phosphorylation is performed by the casein kinase II similarly to pestiviruses or by other viral proteins such as NS3, NS4A or NS4 (Kim et al., 1999; Lindenbach et al., 2007). The phosphorylation of NS5A orchestrates the balance between RNA replication and virus assembly (Evans, 2004; Masaki et al., 2008; Tellinghuisen et al., 2008). Although the hyperphosphorylation impairs RNA replication, it may enhance in the same time virus assembly. This property of phosphoprotein also allows NS5A to interfere with the interferon host response via the dephosphorylation of STAT-1 (Lan et al., 2007).

f) NS5B

NS5B is the HCV RNA dependent RNA polymerase. This protein of 68 kDa is inserted into the ER membrane via a unique alpha helical transmembrane domain incorporated into the ER membrane (Moradpour et al., 2007). A large cytoplasmic domain of around 500 amino acids constitutes a highly conserved enzymatic domain involved in RNA synthesis (Bressanelli et al., 1999). However, this enzyme has a million times lower fidelity than prokaryotic or eukaryotic RNA polymerase as it does not have any exonuclease or proof reading activity (Lindenbach et al., 2007).

Like many other nucleotides polymerase, NS5B can be compared to a "right hand" with three distinct domains: a finger, a thumb and a palm (Bressanelli et al., 1999). Contacts between the finger and the thumb domains form a gap allowing the RNA strand to access to the palm. This domain, that line up with dTTPs, constitutes the active site of the polymerase and synthesizes new RNA strands. It has been shown that mutations that influence the tight interactions and contacts between the thumb and fingers domains may significantly increase replication efficiency and thus, virus production (Schmitt et al., 2011). Several viral or cellular proteins regulate the activity of NS5B as NS5A or the cellular cyclophilins that facilitate the NS5B-RNA interactions (Shirota et al., 2002; Watashi et al., 2005).

III. HCV Life Cycle

Hepatocytes, the major cell type within the liver, represent the main reservoir of HCV *in vivo.* After attachment to the hepatocyte cell surface through recognition of a large panel of specific molecules or receptors, HCV is internalized into the hepatocyte through clathrin-mediated endocytosis. Fusion between the viral membrane and the endosomal membrane releases the viral RNA into the cytosol (Figure 26 a,b). RNA is then translated as a large polyprotein which is cleaved into several structural and non-structural proteins (Figure 26 c). Viral replication and viral particles assembly occur in ER-derived membranes in the cytoplasm as many positive-strand RNA viruses (Figure 26, d). Association of structural proteins and neo-synthetized positive RNA strand in ER derived membranes allows the assembly and production of new viral particles that are then secreted via the VLDL secretory pathway (Figure 26 e,f).

Figure 26. HCV life cycle. Following virus internalization and membrane fusion (a), HCV viral RNA is released into the cytosol (b) where it is translated into a large polyprotein. HCV polyprotein is processed co- and post-translationnaly by several cellular or viral proteases (c). Genome replication occurs at ER-membrane associated replication complexes (called membranous web) where minus strand RNA are synthesized and used as a template for the production of new positive strand RNAs (d). Virus assembly takes place in the ER, likely on cytosolic lipid droplets (cLDs) and is mediated by several HCV non-structural proteins (e). After nucleocapsid assembly, viral particles are released through the secretory pathway (f), where they acquire their buoyant density (Adapted from Popescu & Dubuisson, 2009).

1. Attachment, internalization and fusion

This is the first step of the virus life cycle. Virus initial attachment to hepatocytes is mediated by different surface molecules such as the glycosaminoglycans or the low-density lipoproteins receptor (LDL-r). Then, glycoprotein-envelope mediated binding allows the attachment of the viral particles to specific cellular receptors such as the scavenger receptor B-I (SR-BI), the tetraspanin CD81 and the tight junction molecules Claudin-1 and Occludin (Lindenbach et al., 2013). The virus particle is then internalized through a clathrin-dependent endocytosis mechanism and fusion between the virus membrane and the endosomal membrane allows the release of the viral RNA into the cytosol. As HCV entry is the main topic of this thesis, this step of the HCV life cycle is precisely described in Introduction – Chapter V II.

2. Polyprotein translation

As other positive-strand RNA virus, HCV RNA is directly used by the cellular translation machinery as a template for the synthesis of the polyprotein (Niepmann, 2009). As mentioned in the II.1., translation is cap-independent and mediated through an IRES sequence located in the HCV RNA 5' NCR (Shi et al., 2006). Translation is initiated by the association of IRES with the 40S ribosomal subunit (Spahn et al., 2001). IRES-4S complex recruits the eukaryotic initiation factor-3 (eIF3) and the eIF2-GTP-initiator tRNA complex to form a larger 48S-intermediate ribosomal complex (Ji et al., 2004). Hydrolysis of GTP is followed by the recruitment of the 60S ribosomal subunit which converts the 48S-intermediate into a functional 80S ribosome, able to initiate protein translation (Ji et al., 2004). Translation produces a large polyprotein co- and post- transcriptionally cleaved into several structural and non-structural proteins by several cellular and viral proteases. Polyprotein processing is detailed in section II.

3. Genome replication

HCV replication is mediated by several non-structural proteins, from NS3 to NS5B. The critical roles for viral replication are ensured by NS3 that unwinds viral RNA, and by NS5B that ensure the synthesis of new strands of viral RNA (Ishido et al.,

1998; Lohmann et al., 1999; Lam et al., 2006). Replication occurs in replication complexes formed by altered ER-derived membranes, viral non-structural proteins and replicating viral RNA (Lohmann et al., 2013). Alteration of ER-membranes is mainly due to the action of NS4B membrane protein. This protein induces the formation of a large network of ER-derived membranes called the "membranous web". This structure has been observed both *in vitro* and *in vivo* in infected chimpanzees (Egger et al., 2002; Rouille et al., 2006). Positive stranded viral RNA is used as a template by the replication machinery to generate minus stranded RNA that remains base paired to the positive strand (Ali et al., 2002). This replicative form (RF) is then directly use by the replication machinery to produce newly-synthetized positive strand viral RNA (Lohmann et al., 2013). These RNA can then be packaged into neo-forming viral particles, translated or used as a new template to generate a replicative form. The role of NS5A in replication is still poorly described, but its phosphorylation level has been shown to regulate viral replication (See section II.3.e)

4. Viral particle assembly and release

Virus particle production and release is a multi-step process involving the nucleocapsid formation, the envelopment of the viral particle, its maturation and its release through the VLDL secretory pathway. Despite great advances in our understanding of the HCV assembly through the use of the HCVcc system, several steps of this process remain elusive or matter of debates.

It has been previously observed that after homodimerization, Core traffics and accumulates onto the surface of cytosolic lipid droplets (cLDs) (Moradpour et al., 1996; Miyanari et al., 2007). It has been shown that this core trafficking is dependent onthe activity of the diacylglycerol O-acetyltransferase 1 (DGAT-1) as its inhibition impacts

Lipid droplets (LDs) are cellular organelles rich in lipid. They constitute an important reservoir for neutral lipid such as triacyglycerols (TGs) and cholesteryl esters (CEs). Altogether, these lipids form a neutral core that is surrounded by a phospholipid monolayer (From Krahmer et al, 2009).
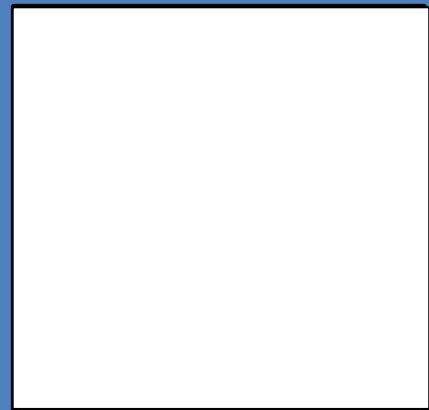
virus assembly (Boulant et al., 2007; Miyanari et al., 2007; Herker et al., 2010). Interaction between cLD-associated Core and NS5A is then thought to be an initiation signal for Core viral RNA packaging. As viral RNA replication occurs within ER-derived membranes, several models have been proposed to explain how the viral RNA could be encapsidated by cLD-residing Core (Bartenschlager et al., 2011).

A model of nucleocapsid assembly suggests that viral RNA association to core-resident cLDs would occur in smooth ER (sER) derived-membranes (Figure 27 a). Core-charged nascent cLDs located in the rough ER (rER) would traffic toward the sER likely through microtubules. cLDs would then interact with the ER derived-membrane replication complex via attachment with particular tethering proteins (Figure 27 a) (Bartenschlager et al., 2011). ER-resident NS5A would transfer viral RNA from the ER replication complex to the cLDs-associated core to initiate nucleocapsid formation. Core is thought to be slowly transferred as nucleocapsid from cLDs to luminal lipid droplets (luLDs) (Figure 27 a). Nucleocapsids are then released into the ER lumen through budding of luLDs. During this process, it has been suggested that p7 and NS2 recruits the envelope glycoproteins E1E2 and the NS3-4A protein complex into the assembly site (Lindenbach et al., 2013). Interaction between NS3-4A and NS2-p7 would allow the relocalisation of Core from the cLDs to the ER-derived membrane where nucleocapsid assembly takes place.

Alternatively, a second model of nucleocapsid formation suggests that virus assembly could be initiated directly on the surface of nascent cLDs in rER (Figure 27 b). Core-associated cLDs could charge viral RNA/NS5A complex in the rER and traffic toward specialized ER derived membrane of the smooth ER (sER). Then, viral RNA would be transferred by NS5A into cLDs-associated core to initiate the formation of newly-synthetized nucleocapsids (Figure 27 b) (Bartenschlager et al., 2011). Nucleocapsids would be released similarly to the first model.

However, the localization of core on cLDs has been recently questioned. Recent studies showed that Core of high-titer HCVcc viruses poorly accumulates on cLDs during assembly and are rather present on ER membranes (Shavinskaya et al., 2007; Boson et al., 2011).
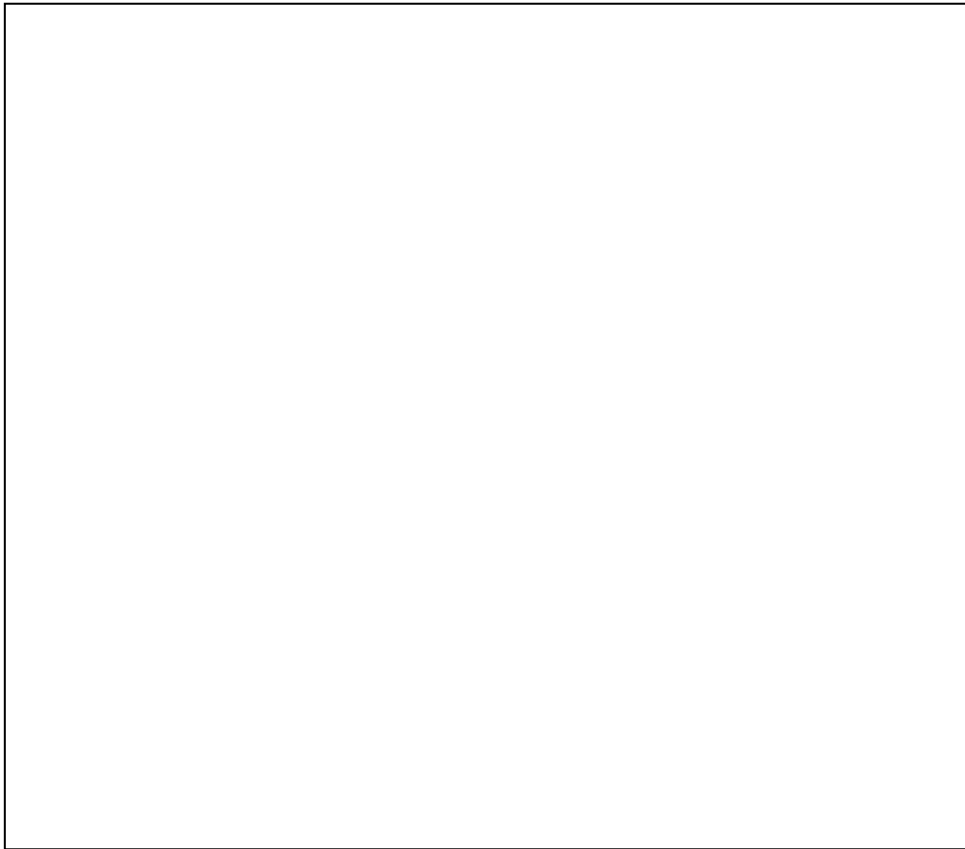
Figure 27. Models of nucleocapsid formation during virus assembly. HCV nucleocapsid formation is thought to initiate at the surface of cytosolic lipid droplets (cLDs) (a) or at the ER membrane (b). MT, microtubules; cLD, cytosolic lipid droplets; luLD, luminal lipid droplets (Bartenschlager et al., 2011). For details, see text.

In contrast, Core of low-titer HCVcc viruses has been shown to accumulate on cLDs. Long term culture of these viruses showed a progressive evolution of its Core location phenotype toward a Core accumulation on ER membranes, associated with an improved virus titer. These studies demonstrated thus that an efficient viral particle production and virus spread is associated with an accumulation of Core at the ER membranes, suggesting that cLDs could not represent a major site for nucleocapsid formation as previously suggested. Moreover, as the core localization on ER membrane was shown to be dependent on p7, it has been suggested that this protein mediates the initiation of assembly by delocalizing Core from the cLDs to the ER membrane.

In regards of all these potential models of nucleocapsid formation, a possibility would be that cLDs serve as a retention system for Core during low assembly processes such as for low-titer HCVcc virus. The Core-charged cLDs trafficking

toward ER membranes or the rapid Core relocalisation from cLDs to ER membrane by p7 may represent the initiation signal for nucleocapsid assembly.

After nucleocapsid formation, viral particles mature through the VLDL secretory pathway prior to their secretion. Consistently, viral particles have been shown to acquire their low buoyant density during egress (Gastaminza et al., 2006; Gastaminza et al., 2008). As Huh7 hepatoma cell lines display a deficiency in their VLDL pathway, HCVcc particles present however a higher buoyant density than serum-derived viral particles (Lindenbach et al., 2013).

Envelopment and maturation of viral particles occur in lipid rich microdomains, located in ER membrane specialized sites where luLDs bud after nucleocapsid formation (Bartenschlager et al., 2011; Lindenbach et al., 2013). Indeed, it has been shown that HCV viral particles production can be blocked by inhibitor of microsomal triglyceride transfer proteins (MTP), involved in the transport of lipids into the ER lumen (Huang et al., 2007).

During viral particle maturation, nascent luDLs acquire several exchangeable apolipoproteins such as ApoE and ApoC. ApoE is critical for virus assembly, maturation and release. As detailed in Introduction-Chapter V II.1.a.iii, previous studies showed that downregulation of ApoE seriously affected viral particles production and that anti ApoE antibodies were able to inhibit virus entry (Chang et al., 2007; Jiang et al., 2009; Hishiki et al., 2009). Moreover, ApoE-NS5A interaction has been shown to be an important mediator of viral particle assembly (Benga et al., 2010).

However, the role of ApoB in viral particles maturation remains controversial. HCVcc particles have been shown to not circulate with ApoB although this apolipoprotein is an essential component of VLDL (Jiang et al., 2009; Coller et al., 2012). Studies also reported that several cell lines were able to produce HCVcc particles in an ApoE but not ApoB dependent manner (Long et al., 2011; Da Costa et al., 2013). Consistently, it has been suggested that HCV viral particles maturation could depend of ApoE containing microdomains rather than on VLDL particles (Lindenbach et al., 2013).

CHAPTER V

HCV Entry

Entry is a critical step for virus infection. Through attachment, internalization and fusion of the viral particles with cellular membranes, this process ensures the release of the viral genome into the host cell cytosol and the initiation of virus replication. This complex process is mediated by several interactions and molecular dialogs between viral structural components and different cellular mediators. The lipoproteins associated to the viral particles are thought to initiate the attachment of the viral particle to the hepatocyte cell surface. Then, the two HCV envelope glycoproteins E1 and E2 likely interact specifically and subsequently with several cell surface receptors, leading to the internalization of the viral particle and to the fusion of the viral membrane with the endosomal membrane. Over the years, the identification of the large number of HCV entry receptors and cell factors required for entry has led to a better understanding of the narrow tropism of HCV for the liver. Now, the kinetics of interaction between viral particles and the cellular receptors needs to be more precisely characterized. The roles of E1 and E2 during these processes are not well understood. Despite the fact that E2 has been identified to be the major HCV binding protein, the precise role of E1 during entry and its interrelation with E2 are not known. Indeed, the HCV fusion protein and how fusion occurs still remain elusive. In this chapter, we will first present the HCV envelope glycoproteins. Their biogenesis, folding and potential role will be reviewed. Then, the cellular mediators of HCV entry will be introduced and their identification and roles during entry will be described. Finally, virus internalization process and the ability of HCV to spread via cell-to-cell transfer will be summarized.

I.   HCV envelope glycoproteins

1. Translation

HCV E1 and E2 glycoproteins are two highly N-glycosylated type I transmembrane proteins of respectively 190 aa (31 kDa) and 365 aa (70 kDa), each one harboring a C terminal transmembrane domain contiguous to an N terminal ectodomain (Lavie et al., 2007). Thus, HCV E1 and E2 harbor an amino acid length similar to the one of pestiviruses E1 and E2. Dengue E protein remains larger with 500 aa although PrM and M are smaller than HCV and pestivirus E1 with respectively 166 and 75 aa (Lindenbach et al., 2007). After translation, the structural proteins are processed by the ER signal peptidase (Figure 28). Unlike pestiviruses, E1 is the first protein to be completely processed through cleavage in the C terminal domain of Core and in the transmembrane domain of E1. E2 is then released through processing of the E2/p7 junction via cleavage in the transmembrane domain of E2 (Figure 28).
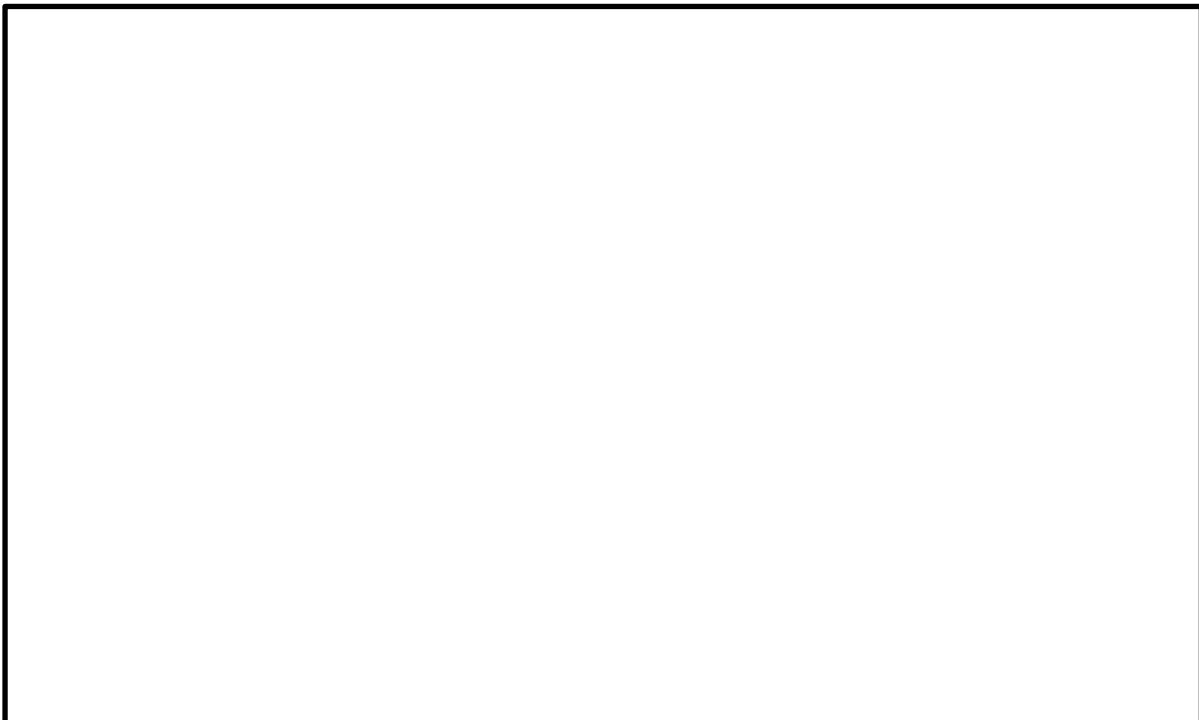
Figure 28. Schematic representation of E1E2 processing and folding. E1/E2 and E2/p7 junction is cleaved by the host signal peptidase, allowing transmembrane rearrangements, E1E2 folding, and heterodimer maturation.

The processing of the E1/E2 junction has been shown to be particularly important for a stable insertion of the E2 transmembrane domain into the ER membrane (Op de Beeck et al., 2001; Cocquerel et al., 2001). Inefficient cleavages may occur, and induce the production of uncleaved E2-p7-NS2 or E2-p7 fragments that are thought to be dispensable for virus assembly and infectivity (Jones et al., 2007). However, it has been suggested that the release of E2-p7 fragment may contribute to regulate the role of p7 in infected cells (Brazolli et al., 2005). After polyprotein cleavage, Core, E1 and E2 are inserted into the ER membrane through a peptide translocation signal located at their C terminal (Cocquerel et al., 2000; Op de Beeck et al., 2001; Cocquerel et al., 2002; Rouille et al., 2006). Although E1 and E2 ectodomains are exposed in the ER lumen, Core ectodomain is present in the cytosol. As pestiviruses, the signal peptide peptidase then cleaves the C terminal anchoring domain of Core to release mature forms of the protein (Lindenbach et al., 2007).

2. Maturation

a) Transmembrane rearrangements and heterodimerization

Right after polyprotein processing, transmembrane domains of E1 and E2 are matured. As other *Flaviviridae,* these regions are structured by two stretches of hydrophobic residues (the second containing the ER peptide translocation signal) separated by a small stretch junction of positively charged residues (Figure 29) (Cocquerel et al., 2002). During translocation into the ER and before protein cleavage, the two stretches form a hairpin structure into the ER membrane, with the positively charged stretch facing the cytosol although the 2 transmembrane terminal domains are exposed into the ER lumen (Figure 29). Cleavage of the E1/E2 and E2/p7 junctions induces E1 and E2 transmembrane rearrangements: the second hydrophobic stretch unfolds toward the cytosol to form a single membrane spanning domain with the first hydrophobic stretch (Figure 29) (Cocquerel et al., 2002). The positively charged properties of the stretch junction are conserved among genotypes and critical for the dynamics of this process. The rearrangement of the

transmembrane domains allows E1 and E2 to associate as heterodimer complex, in a slow process stabilized by noncovalent interactions.

Figure 29. E1E2 transmembrane structural rearrangements during polyprotein translation and processing. After polyprotein translation, the two transmembrane stretch of E1 form a hairpin structure (1). Cleavage of the E1/E2 junction induces the translocation of the second hydrophobic stretch toward the cytosol to form a single transmembrane domain (2). E2 transmembrane undergo similar rearrangements after translation (3, 4) (Cocquerel et al., 2002).

A major common point between pestiviruses and HCV envelope glycoproteins is that E1 or E2 do not undergo proteolytic cleavage during their maturation, in contrast to flaviviruses envelope glycoprotein M (Lindenbach et al., 2007). This suggests that E1 and E2 are expressed directly as functional proteins that only require proper heterodimerization and glycosylation. It has been shown in several studies that the functional conformation of both E1 and E2 is dependent on the presence of the other glycoprotein (Michalak et al., 1997; Patel et al., 2001; Cocquerel et al., 2003; Brazolli et al., 2005; Wahid et al., 2013), and thus, on a complete heterodimer complex. During heterodimer formation, intramolecular disulfide bridges are formed within E1 and E2 (Brazolli et al., 2005). These bridges shape the heterodimer complex: E1 is thought to be the first protein to obtain a functional conformation, which would be then required for E2 correct conformation and heterodimerization. E2 harbors 18 cysteins that form 9 disulfide bridges. These bridges have been shown to be essential for CD81 binding and for Abs recognition. Indeed, abrogation of disulfide bridges has been shown to impair E2 epitope

neutralization by monoclonal Abs (Fenouillet et al., 2008). Interestingly, it has also been shown that E1 and E2 are linked at the surface of HCVcc particles by important intermolecular disulfide bridges, which is not the case at the HCVpp surface (Vieyres et al., 2010). These differences may be explained by the fact that the E1E2 HCVpp are not assembled within the ER, but in multivesicular bodies (Sandrin et al., 2005) that reach the plasma membrane. No E2 homodimers are incorporated onto the viral particle surface in contrast to pestiviruses E2, which may question the potential role of such homodimer in pestiviruses.

b) Glycosylation

Unlike flaviviruses, E1 and E2 glycoproteins are heavily glycosylated where glycans mass account for nearly half of the total protein mass. Indeed, despite a larger protein sequence (500 aa), flaviviruses E display a lower mass (53 kDa). HCV E1 and E2 possess respectively 5 and 11 glycosylation sites that only harbor N-linked high mannose type oligosaccharides (Figure 30) (Goffard et al., 2003; Zhang et al., 2004; Lindenbach et al., 2007). The majority of these sites are highly conserved among genotypes. Pestiviruses E1 and E2 are less glycosylated than HCV E1 and E2, with respectively 2 to 3 and 4 to 6 N-glycosylation sites (Lindenbach et al., 2007).

Figure 30. E1E2 N-linked glycans and transmembrane domain critical residues. Position of N-linked glycans are indicated as an N. Glycans involved in HCVpp entry or E1E2 folding are indicated in black squares and grey circles respectively. Black boxes represent E2 epitopes recognize by antibodies. E1E2 Transmembrane domains (TMD) are highlighted. Arrows indicates critical residues for E1E2 heterodimerization. HVR1, Hyper Variable Region 1 (Lavie et al., 2007).

ER resident E1E2 are mainly glycosylated by high-mannose type glycans. However, complex glycans have been found associated with HCVcc E2 glycoproteins, suggesting that high type mannoses were maturated in the Golgi apparatus (Vieyres et al., 2010). Thus, this indicates that HCVpp E1E2 are not fully glycosylated and that HCVcc E1E2 likely undergo maturation in the Golgi apparatus. However, all the high mannose type glycans are not transformed in the Golgi, as HCVcc E2 seems to harbor on the surface of the particle both high mannose type glycans and complex glycans (Vieyres et al., 2010).

The HCV N-glycans have multi-functional roles. By using the HCVcc system, a study showed that a N-glycosylation site in E1 (aa 196) is critical for both E1E2 heterodimerization and viral particles secretion (Figure 30) (Helle et al., 2010). Moreover, several sites have been identified in E2 to be involved in E2 folding, virus entry or egress (Goffard et al., 2005; Helle et al., 2007). N-glycosylation sites also appear to be important for both viral particles binding to cellular receptors (such as CD81), and protection of conserved epitopes against Nt Abs (Helle et al., 2007; Helle et al., 2009). Thus, N-glycans may represent interesting therapeutic targets to inhibit virus entry.

3. Structure

a) E1E2 heterodimeric structure is not resolved

Understanding how E1 or E2 are structured and organized at the particle surface is highly hampered by the lack of heterodimer crystallographic structures. Indeed, as the functional conformation of E1 and E2 is highly dependent on the other glycoprotein (Michalak et al., 1997; Patel et al., 2001; Cocquerel et al., 2003; Brazolli et al., 2005; Wahid et al., 2013), a relevant functional assessment of the E1 and E2 structure requires the analysis of the heterodimer as a unique protein entity. Thus, a global structure of E1E2 complex is still awaited, and needed. Beyond the fact that this finding would highlight new structural targets for anti-viral drugs design, these data would also constitute precious information on how E1 and E2 structurally behave during entry, and more particularly during fusion.

Up to now, only some E2 domains have been structurally resolved in complex with particular antibodies (Krey et al., 2013). Neither E1 or E2 have been crystallized yet. Notably, the crystallization of E2 is highly hampered by several intrinsic properties of this protein. E2 has an important ability to form aggregates in solution, its high level of N-glycosylation and the presence of several flexible loops within the structure seriously impacted the production of analyzable E2 crystal structure. Even though their independently crystalized structures would not have a great functional significance, they may however point out interesting epitopes that may represent interesting therapeutic targets.

b) The Krey E2 working model

Alternatively, an E2 structural model has been proposed in 2010 allowing us for the first time to consider a potential E2 structure (Krey et al., 2010). This model was designed first, by correlating residues and domain organization of alphavirus E1 fusion protein (whose structure is similar to flavivirus E) with HCV E2, and second, by assessing the potential of the different E2 conserved cysteins to establish disulfide bridges together. The obtained structure was likely similar to other class II fusion proteins, such as harbored by flaviviruses or alphaviruses (Figure 31) (Krey et al., 2010). The structure was divided into 5 domains: three major domains constituted the core (Domain I, II and III) of the protein and a heptad repeat region anchored the core to a last domain, the transmembrane.

Alphaviruses are single stranded RNA viruses that belong to the *Togaviridae* family. 27 viruses are classified in the alphavirus genus such as the Chickungunya virus or the Semliki Forest Virus. Alphaviruses harbor a large species tropism and are mainly transmitted through mosquitoes.

A central domain (domain I or DI) is composed of two inter-facing beta sheets domains, each constituted by 4 β-strands, preceded by an N-terminal extension. This N-terminal region, called hyper variable region 1 (HVR1) is a 27 aa region that is highly variable and constitute an immune barrier to protect conserved epitopes located in other domains of E2 (Figure 31) (Krey et al., 2010). HCV E2 is composed of two hypervariable regions that are mainly unstructured, which placed them in contrast to those of alpha- and flavi-viruses. According to *Krey et al*, this is consistent

with the fact that, in contrast to alpha- and flavi-viruses, HCV induces a chronic disease. Indeed, chronic infection requires that domains exposed to immune pressure harbor a high flexibility of adaptation to protect E2 most critical and conserved domains.

This is notably why HVR1 is considered as an "immunological decoy": the immune response against the highly exposed HVR1 domain stimulates the quasi-species evasion, without being able to clear viral infection. The first β-sheet domain, exposed at the front of the protein (DI front sheet), is disjointed by the insertion of a loop between the second and third β-strands of the top sheet, determined as the domain II of E2 (E2DII) (Figure 31) (Krey et al., 2010). The second β-sheet domain positioned backward (DI backward sheet) the front sheet does not possess any loop insertions. However, alpha- and flavi-viruses fusion protein domains II are composed of 2 loops, one inserted in the top sheet (at the same location as E2DII) and a second one inserted in the bottom sheet (Figure 31, 32), between the second and the third beta strands (Krey et al., 2010). In HCV E2, the absence of such insertion at this position is thought to be compensated by the presence of a glycan, shown to be important for the correct folding of E2 (Figure 31, 32) (Krey et al., 2010). Thus, E2 DII appears to be very distinct from the ones of the alpha- and flavi-viruses. The domain is smaller, and probably more disordered in contrast to the alpha- and flavi-viruses ones where the backward loop is stabilizing and structuring the second one (Figure 31, 32).

In alpha- and flaviviruses, the E2 DII harbors the fusion peptide, a segment of non-charged residues highly conserved. A putative highly conserved region (502-520) has been identified in HCV E2 DII (Figure 31; red circle in DII) and may represent such fusion peptide. The domain III possesses an Ig-like structure, and is linked to the domain I by the Inter Genotype Variable Region (IgVR). This region, a short segment of 11 residues connected to the C terminal of the domain I and to the N terminal of the domain III (DIII), is essential for alpha- and flaviviruses to allow the fold back of the DIII toward the DI-DII core during fusion-induced conformational changes (Figure 31) (Krey et al., 2010).
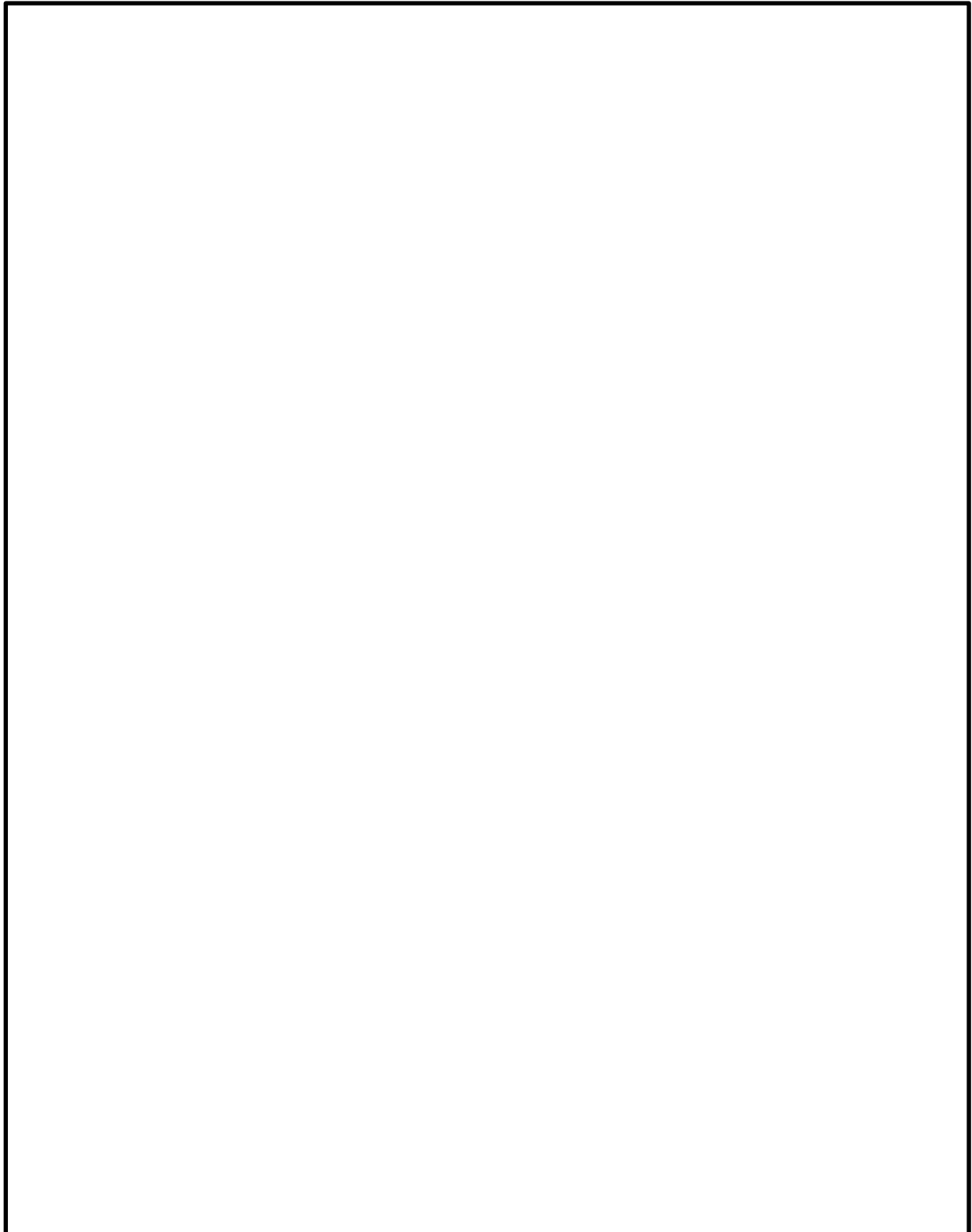
Figure 31. Tertiary structure model of HCV E2. Structure is presented as amino acids sequence (**A**) or as schematic diagram of the different E2 domains (HVR1, DI, DII, IgVR, DIII, Stem) (**B**). Disulfide bonds are indicated as black lines linking cystein residues. DI front sheet is colored in red and DI backward sheet is colored in pink. DII, DIII and Stem region are respectively colored in yellow, blue and grey. Unstructured regions HVR1 and IgVR are colored in brown. A dotted yellow curved lines backward DII symbolized the missing DII loop. Residues encircled in red in DII represent the putative E2 fusion peptide. Green circle represent the glycosylation sites. D, Domain; HVR1, Hyper Variable Region 1; IgVR, Inter genotypic Variable Region (Krey et al., 2010). **C.** Linear representation of the E2 DII domain organization. Domains are colored similarly to A and B. Amino acid positions of domain junctions are indicated for HCV envelope of genotype 1 (gt1) and 2 (gt2).

Connected to the C terminal of the domain III, the heptad repeat region (or stem) is a relatively conserved domain containing a single loop (Krey et al., 2010). In alpha- and flaviviruses, this region is essential for the fusogenic conformational changes, moving at the exterior of the core trimer to pull the transmembrane domain toward the host membrane.
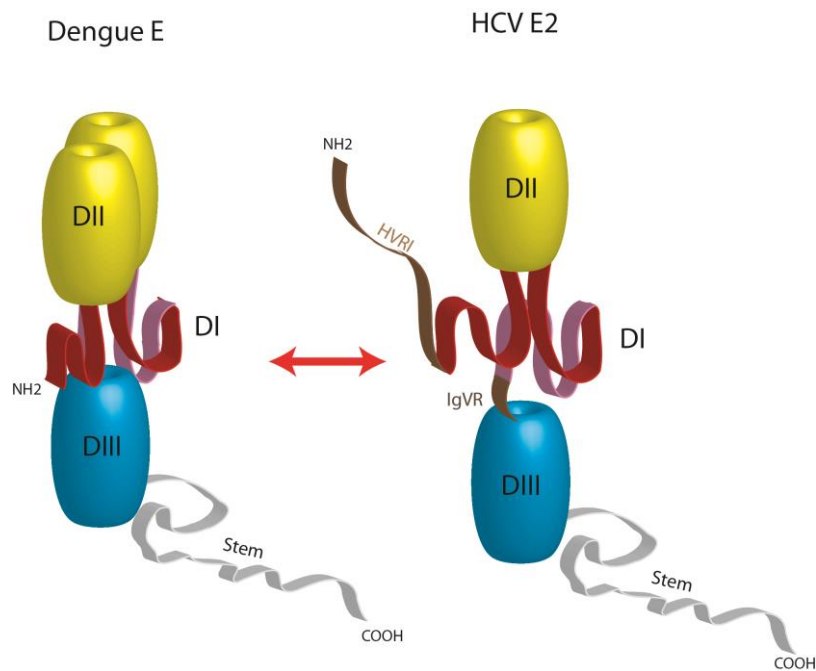
Figure 32. Tridimensional representation of the tertiary organization of Dengue E and HCV E2. HCV E2 structure is based on the *Krey* E2 structure model. Dengue E tertiary structure present a second insertion of a DII loop into its backward β-sheet. Dengue E do not harbor unstructured regions such as HVR1 or IgVR.

c) The structure of BVDV E2 and its implication for HCV E2

Structure of pestiviruses has long remained uncharacterized. However, very recently, the structure of BVDV E2 has been resolved. Unlike Dengue E, E2 appeared to be mainly composed of beta strands and organized quite linearly through its amino acids sequence (El Omari et al., 2013; Li et al., 2013). The protein was divided in 4 domains, DA to DD (Figure 33A) where the first one corresponded to the distal membrane domain. None of these domains presented a fusion loop and the structure was poorly responsive to low pH, highlighting its unlikelihood to be the BVDV fusion protein. Indeed, E2 distal domain DA and DB that were likely exposed on the particle surface presented Ig like fold structures consistent with a potential

attachment function of these domains (Figure 33A) (El Omari et al., 2013; Li et al., 2013). This was also supported by the fact that a soluble form of E2 from another Pestivirus, CSFV, has been shown to inhibit viral entry of both BVDV and CSFV highlighting a common cell entry receptor for pestiviruses (Hulst et al., 1997). In parallel, DA appeared to rearrange after low pH induction, likely due to the presence of a highly conserved histidine (El Omari et al., 2013). Thus, authors hypothesized that the distal domain DA of the attachment protein E2 covered the fusion peptide located at the distal domain of E1. During fusion, low pH environment would disorder DA and unmask the E1 fusion peptide as for alphaviruses (Figure 33B). Authors argue that this structure places the current HCV model in strong contrast with BVDV E2, suggesting that HCV E1 could harbor the fusion function.
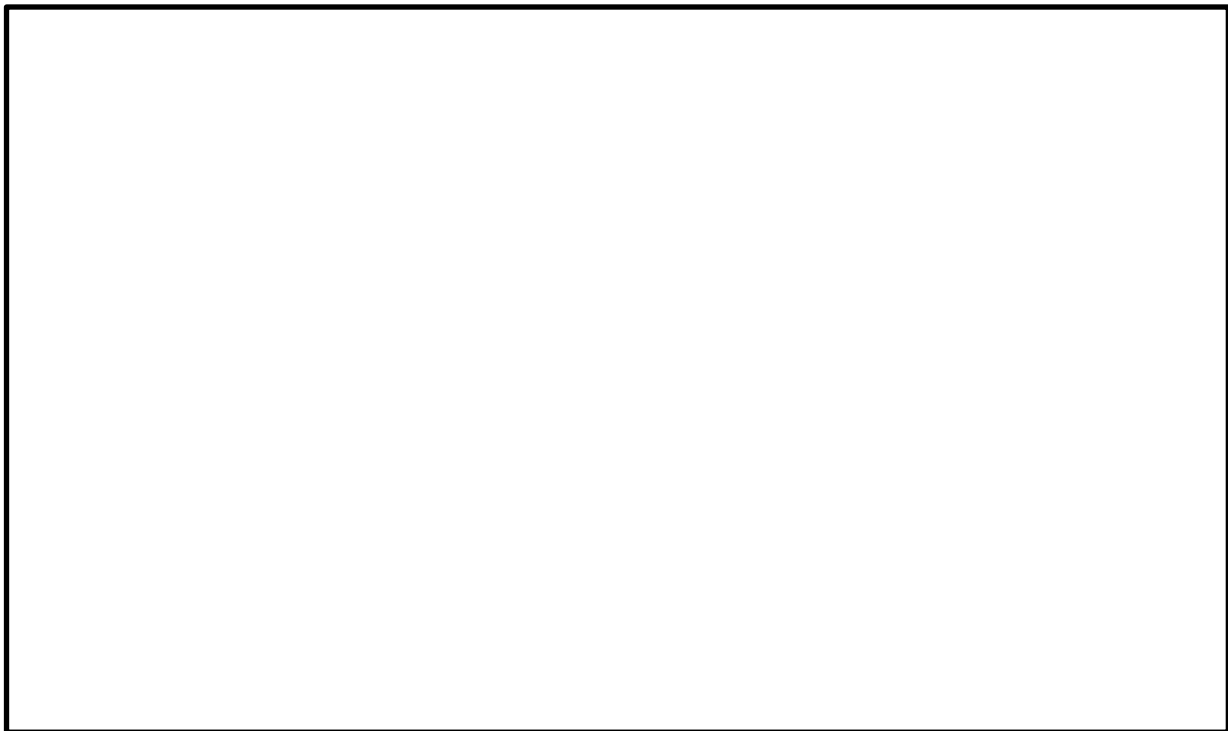
Figure 33. Pestivirus E2 structure and Pestivirus fusion model. A. Crystallographic structure of a BVDV E2 monomer. BVDV E2 is a β-chain linear structure that harbors four domains organized linearly through the polypeptide chain. DA (purple) and DB (green) do not harbor any fusion loop or hydrophobic patch. DA contains a conserved histidine that disorder DA after low pH induction. DC (yellow) and DD (red) do not match to any known structure. DD is involved in homodimerization through stabilization by a disulfide bridge. **B.** Pestivirus fusion model. E2 binding to a cell receptor is thought to induce viral particle endocytosis. Binding to a second receptor primes viral fusion and low pH drop disorder E2 DA which exposes the E1 N terminal fusion peptide. E1 fusion peptide is projected into the host endosomal membrane and the entire heterodimer is thought to mediate membrane merging (El Omari et al., 2013).

4. Roles

a) Heterodimerization

Transmembrane domains (TMB), small regions (~25-30 amino acids) located at C terminal of E1 and E2, have a critical role in E1E2 heterodimerization. Heterodimerization is abrogated after deletion of E2 transmembrane (Cocquerel et al., 2000; Drummer & Poumbourios, 2004; Op De Beeck et al., 2000). E2 transmembrane is important for proper E2 folding (Patel et al., 2001) which allows E1E2 heterodimerization and incorporation onto viral particles (Op De Beeck et al., 2004). Several studies have identified critical amino acids involved in this process, including the charged central residues that determine E1E2 cellular localization (Cocquerel et al., 2002; Ciczora et al., 2005; Ciczora et al., 2007). E1 and E2 ectodomain residues involved in heterodimerization are not fully identified. Indeed, the relevant identification of such motifs is hampered by the fact that site-directed mutagenesis in conserved domains often lead to an unproper E1E2 folding or heterodimerization (Ciczora et al., 2005; Ciczora et al., 2007; Drummer et al., 2006; Drummer et al., 2007; Drummer & Poumbourios, 2004; Lavillette et al., 2007; Owsianka et al., 2006; Rothwangl et al., 2008; Rothwangl & Rong, 2009). However, particular motifs in the N terminal (415-500; (Yi et al., 1997)) and C terminal domains (524-660 ; (Patel et al., 1999; Patel et al., 2000, Patel et al., 2001) within the ectodomain of E2 have been suggested to be involved in this process. WHY motifs are well known to be involved in protein-protein interaction. Studies showed that the W489HY was critical for E1E2 heterodimerization (Yi et al., 1997; Lavillette et al., 2007; Rothwangl et al., 2008) although a second motif W616HY appeared less critical (Lavillette et al., 2007; Rothwangl et al., 2008).

b) Attachment and binding

E2 ectodomain functions during virus entry have been intensively studied. Experiments using antibodies targeting E2 epitopes, or studying entry phenotype of E2 mutants with both the HCVpp and HCVcc systems have been conducted. Despite the fact that the majority of E1E2 mutants often presented a defect in folding and

heterodimerization, a large number of studies have played an important role in the identification of important domains and residues of HCV E2 involved in cell entry. As flaviviruses E and pestiviruses E2, HCV E2 is involved in receptor recognition and binding at the cell surface. HCV E2 has been demonstrated to specifically bind two particular cell surface molecules expressed at hepatocyte surface, the scavenger receptor B1 (SR-B1) and the tetraspanin CD81.

Assays using soluble E2 protein, the soluble large extracellular loop of CD81 (CD81 LEL) or neutralizing antibodies inhibiting CD81 binding permitted to precisely identify domains and residues in E2 that are involved in this interaction (Flint et al., 1999; Patel et al., 2000; Owisianka et al., 2001; Clayton et al., 2002; Roccasecca et al., 2003; Owisianka et al., 2006; Drummer et al., 2006; Rothwangl et al., 2008; Witteveldt et al., 2009; Drummer et al., 2012). Although it is known that the proper E2 conformation is dependent on E1, soluble E2 has been shown to bind CD81, highlighting that the folding of the CD81 receptor binding domain in E2 does not require E1 to acquire its functionality (Pileri et al., 1998; Patel et al., 2000; Roccasecca et al., 2003). Three major regions in E2 were identified to be involved in this interaction: one is located in DI (480-493) and the two other regions are located in DIII (528-535 and 544-551 (Flint et al., 1999; Owsianka et al., 2001; Clayton et al., 2002). Moreover, several other single residues have been identified to be involved in this interaction, mainly located in the domain I (Drummer et al., 2006; Owsianka et al., 2006). Overall, the residues and domains involved in CD81 binding are likely consistent with the proposed tertiary organization of E2.

In parallel, E2 HVR1 has also been shown to mediate attachment of viral particle to the cell surface and to be important for cell entry. Indeed, deletion of HVR1 and antibodies targeting this domain dramatically decrease cell entry (Scarselli et al., 2002, Bartosch et al., 2003; Catanese et al., 2007; Bankwitz et al., 2010, Dao thi et al., 2012). Despite its important genetic variability among genotypes, the basic properties of this region are quite conserved and seem to play an important role in entry

(Callens et al., 2005; Guan et al., 2012) as infectivity of viral particles has been shown to correlate with the quantity of basic residues present in HVR1 (Callens et al., 2005). Indeed, as detailed in section, HVR1 mediates attachment of the viral particle to SR-BI. Deletion of HVR1 renders anti-SRB1 antibodies inefficient against viral infection and abrogates SR-B1 binding (Bankwitz et al., 2010). Moreover, HVR1 seems to be an important shielding domain for conserved epitopes of E2 as deletion of HVR1 dramatically increases the accessibility of the CD81 binding domain for antibodies (Bankwitz et al., 2010). Accordingly, it has been shown that deletion of HVR1 in sE2 increases binding to CD81 (Scarcelli et al., 2002; Roccassecca et al., 2003). As HCVcc particles do not bind to CD81 (Evans et al., 2007), this observation is consistent with the idea that E2 CD81 and SR-B1 receptor binding domains are intimately linked. In the context of HCVcc, HCV CD81 binding could thus require a priming step, potentially dependent on E2 binding to SR-B1. Altogether, these observations indicate that HCV E2 is likely a complex binding protein, suggesting that E2 could harbor a more complex structure than the linear pestiviruses E2 structure.

In contrast, the role of E1 during entry and binding is still poorly characterized. Indeed, there is actually no evidence that E1 is an attachment protein. However, several residues have been identified in this protein to regulate heterodimer binding (Russel et al., 2009). However, it is likely that these residues regulate heterodimer conformation and E2 receptor binding domains (RBDs) accessibility rather than E1 potential RBD conformation. Consistently, a study showed recently that the E1 conserved cysteins appear as important determinant for E2 binding to CD81, by probably controlling the E1E2 heterodimerization during assembly (Wahid et al., 2013).

c) Fusion
i. Class of fusion protein

After viral particle internalization, the release of the viral genome requires the fusion between virus membrane and host cell membrane. This mechanism can be generalized as a process including i) conformational changes of the fusion protein, ii)

insertion of a fusion peptide into the host membrane, iii) lipid mixing (hemifusion) and iv) fusion of the two membranes. Despite this general process, viruses harbor a large diversity of fusion mechanism, through different fusion protein structures, conformational changes or cellular fusion sites (Kielian et al., 2006). Indeed, fusion can occur at the plasma membrane, and thus, only require a prior attachment step. In contrast, viruses can also fuse with endosomal membrane after internalization. In this context, an acidification of the endosomal compartment induces the fusion protein conformational changes required for membrane merging.

Class I fusion proteins. Fusion proteins are commonly divided into three distinct classes. Class I proteins are mainly composed of alpha helical structures and harbor a fusion peptide located at the N terminal of the protein (Figure 34). They are assembled as trimeric structures at the virus surface, both in their pre- and post-fusion conformational states (Kielian et al., 2006). During assembly, a major protein precursor is cleaved into two distinct proteins. One of these proteins harbors a receptor binding domain and is responsible for virus attachment. The second protein, usually constrained by the attachment protein onto the virus surface, is responsible for virus fusion (Kielian et al., 2006). Class I fusion-dependent conformational changes can be initiated by several mechanisms. First, attachment of the binding protein to a cellular receptor can induce drastic structural rearrangements of the binding and fusion protein that prime virus fusion.
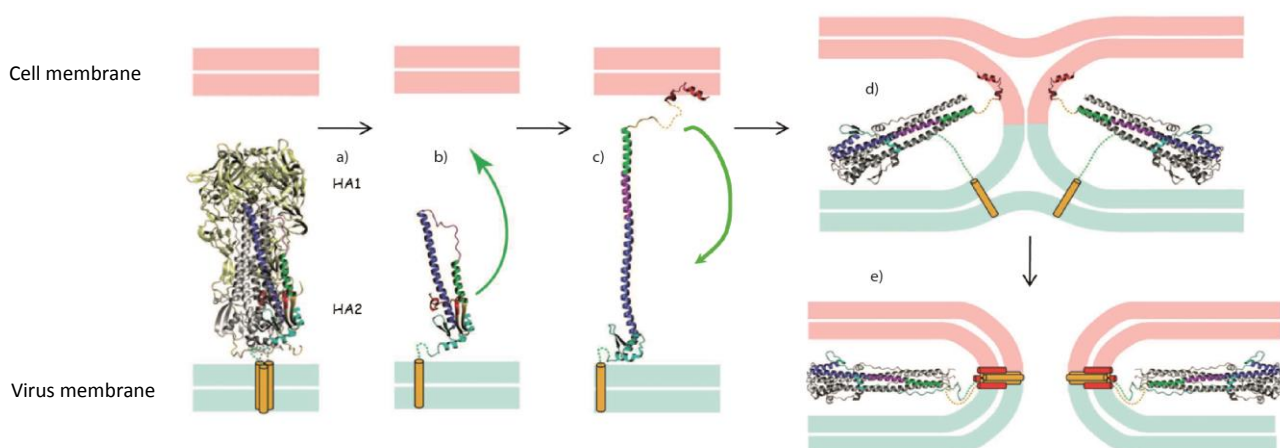
Figure 34. Class I fusion protein fusogenic conformational changes. Acidification of endosomal compartment allows HA1 dissociation from HA2 (a) and the projection of the HA2 N terminal fusion peptide toward the host endosomal membrane (b). Subsequent structural rearrangements of HA2 reverse the viral-membrane proximal segment (c), thus allowing the protein to fold over (d). The formation of a stable six helix bundle structure then allows membrane merging (e). For details, see text.

This is notably the case for HIV, where gp120 attachment to CD4 induces protein conformational changes, binding to a co-receptor and structural rearrangement of the fusion protein gp41 (Blumenthal et al., 2012). The second mechanism of induction is pH dependent. In this mechanism, fusion is dependent on the acidification of endosomal compartment. Protonation of charged residues within envelope glycoproteins induces drastic conformational changes that dissociate the binding and fusion proteins and expose the fusion peptide. For example, influenza harbors two envelope glycoprotein HA1 and HA2 that are processed from a single precursor during virus assembly (Figure 34). After HA2 binding to cellular receptor and virus internalization, an endosomal acidification dissociates HA1 and HA2 (Kielian et al., 2006). Dissociation allows the projection and the insertion of the HA2 fusion peptide in the endosomal membrane, thus corresponding to a pre-hairpin intermediate state (Figure 34 b,c). Structural reorganization of a HA2 α-helical structure allows the protein to reverse its viral-membrane proximal segment and fold over to form a stable six helix bundle structure (Figure 34, d). The formation of this structure then induces a membrane lipid mixing, followed by fusion of the viral membrane (hemifusion) and the host endosomal membrane (Figure 34, e).

Other mechanisms of induction of class-I dependent fusion have been observed, as those that require both cell surface attachment and binding to initiate virus fusion or those that involved endosomal cellular proteases that cleave envelope glycoproteins at low pH (Kielian et al., 2006).

   Class II fusion proteins. Class II fusion proteins are harbored by members of the *Flaviviviridae* and *Togaviridae* family. In contrast with class I fusion proteins, these proteins are mainly composed of β-sheets secondary structures (Kielian, 2006; Modis, 2013). Their fusion peptide is located into an internal loop usually denominated as domain II (DII) of the fusion protein. In their pre-fusion state, class II fusion proteins are dimeric structures lying down parallel to the lipid bilayer whereas in their post fusion state, they protrude as trimers onto the viral particle surface (Figure 35A a)). During assembly, Class II fusion proteins are not processed from a precursor protein (Kielian, 2006; Modis, 2013). However, fusion proteins are expressed with companion proteins that are proteolytically processed by cellular enzymes. Cleaved forms are

expressed with the fusion protein at the surface of the viral particle, and can either have a particular role to play during cell entry (i.e. attachment protein; alphaviruses) or not (i.e. structural chaperone; flaviviruses). In the last case, companion proteins may be required for proper fusion protein conformation. Commonly, all the class II fusion proteins require a low pH induction to change their conformation, insert their fusion peptide and induce membrane merging (Kielian, 2006; Modis, 2013). However, the range of sensitivity to low pH may change among class II fusion proteins. For flaviviruses, homodimers are arranged as head to tail anti-parallel structure, where DI and DIII of an E protein protect the fusion peptide located in DII of the second E protein. Acidification of endosomal compartment induces drastic homodimers conformational changes (Modis et al., 2004; Kielian, 2006; Modis, 2013). Homodimers rotate and extend to form trimeric structures that insert their fusion peptides into the endosomal membrane (Figure 35A b,c)). Fold over of several trimeric structures (Figure 35B) is required to induce membranes bridging, lipid mixing and fusion (Figure 35A d). In contrast to class I fusion proteins, no six helix bundle structure is observed in class II fusion proteins.

Figure 35. Schematic representation of Dengue E-mediated fusion. **A**. Dengue E dimers lie down parallel to the viral membrane (a). Endosomal acidification induces the drastic conformational changes: E dimers protrude onto the viral surface and DII fusion peptides become exposed (b). Homodimers rotate and extend to form trimeric structure and fusion peptides are projected into the host endosomal membrane. E trimers fold over through DIII fold-back, thus inducing lipid mixing and membrane fusion. **B**. Structural rearrangement of Dengue E fold over during fusion. Pre-fusion conformation (a) and post-fusion conformation (b) following DIII-fold back. DI, red, DII, yellow, DIII, blue. (Modis et al, 2004).

Class III fusion proteins. Class III fusion proteins have been recently identified in particular viruses such as the herpes simplex virus 1 (HSV-1) or the Vesicular Stomatitis Virus (VSV). These proteins notably present similarities with both class I and class II fusion proteins (Backovic et al., 2011). Indeed, they harbor a pre-fusion trimeric structure with a central domain mainly composed of α-helical. Fusion peptide is divided into two domains, located in two fusion loops composed of β-sheets (Backovic et al., 2011). Commonly, acidification and/or attachment to viral receptors are thought to initiate Class III fusion glycoprotein conformational changes. During VSV fusion, following pH acidification, the VSV fusion glycoprotein G dramatically extends and unfolds its viral membrane-proximal fusion loops toward the cell membrane (Figure 36). This movement allows the insertion of the G conformational fusion peptide into the cell membrane. G then folds over thus inducing lipid mixing and membrane fusion (Backovic et al., 2011) (Figure 36). Low pH-induced conformational changes of VSV-G (and of gB from HSV-1) are known to be reversible (Backovic et al., 2011). Moreover, G fusion peptide is sensitive to low pH at the pre-fusion state, which is a unique example in the viral world.
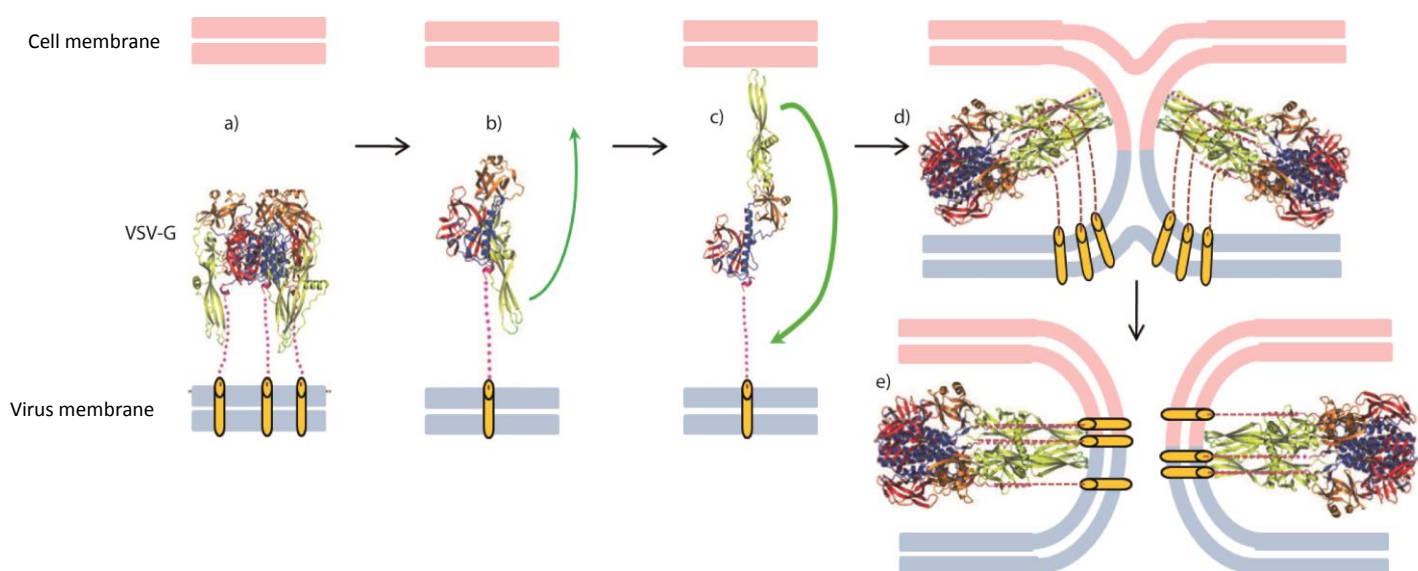
Figure 36. Class III fusion protein fusogenic conformational changes. a) VSV-G glycoproteins are assembled as homotrimer in pre-fusion conformational state. b) Representation of a VSV-G monomer at pre-fusion state. Following pH acidification, VSV-G monomer viral membrane-proximal fusion loops extends toward cell membrane. c) Fusion peptide is inserted into cell membrane and G monomer dramatically folds over to rearrange as post-fusion conformation state. This structural rearrangement allows lipid mixing (d) and fusion (e).

ii.   The elusive HCV fusion mechanism

The HCV fusion remains an elusive phenomenon. The HCV fusion protein(s) is still undefined and the precise mechanisms that induce and mediate virus fusion are not known (Figure 37). HCVpp fusion liposomes assays previously showed that HCV fusion is dependent on low pH induction and can occur without the presence of cellular receptor (Lavillette et al., 2006). Similarly to class III fusion protein, HCV exposure to low pH is reversible. Several studies showed that exposure of cell-bound virus to low pH did not affect viral particle infectivity, highlighting the existence of a molecular trigger that would prime HCV for fusion (Blanchard et al., 2006; Tscherne et al., 2006). Consistently, another study demonstrated that fusion is enhanced when HCV E2 is primed with CD81 LEL prior to fusion, which may suggest that HCV binding to CD81 constitutes a priming step for proper fusion (Sharma et al., 2011). This is an interesting point of similarity with pestiviruses, whose fusion also requires a priming step.

As HCV belongs to the Flaviviridae family, it has been suggested that HCV could harbor a class II fusion protein (Krey et al., 2010). Thus, similarly to E, HCV E2 has been proposed to harbor both a binding and fusion function (Figure 37). Based on the fact that *Flaviviridae* fusion peptides share a very conserved sequence, rich in glycine and hydrophobic amino acids, and are located in fusion loop mainly structured by disulfide bridges, two regions in E2 have been identified to be critical for virus fusion, one in DI (416-430) and one in DIII (600-620) (Figure 37) (Drummer et al., 2006; Lavillette et al., 2007; Rothwangl et al., 2008). The proposed E2 tertiary organization has been constructed based on sequence similarities of HCV E2 with class II fusion proteins as well as on literature reports (Krey et al., 2010). The structure obtained by *Krey et al* successfully resumed literature findings and class II fusion protein features, suggesting that HCV E2 is potentially a class II fusion protein. Indeed, a highly conserved and hydrophobic domain rich in glycine was shown to be located in DII, consistently with the fusion peptide location in E glycoprotein. Moreover, a group notably suggested that the stem region of E2 could

play a role during fusogenic conformational changes, as similarly observed for flaviviruses E (Albecka et al., 2011). Altogether, these observations place HCV E2 in strong contrast with pestivirus E2, where no potential fusion peptide has been observed.

However, the HCV fusion seems more complex than it first appeared as several studies reported that E1 is also tightly involved in this process. A potential fusion peptide-like region in E1 has been suggested to be involved in viral fusion (276-286) (Drummer et al., 2007; Lavillette et al., 2007) as well as a transmembrane proximal region (330-347) (Drummer et al., 2007; Perez-Berna et al., 2008). Interestingly, it has been shown that peptides deriving from these two regions are able to destabilize liposome *in vitro* (Perez-Berna et al., 2006).
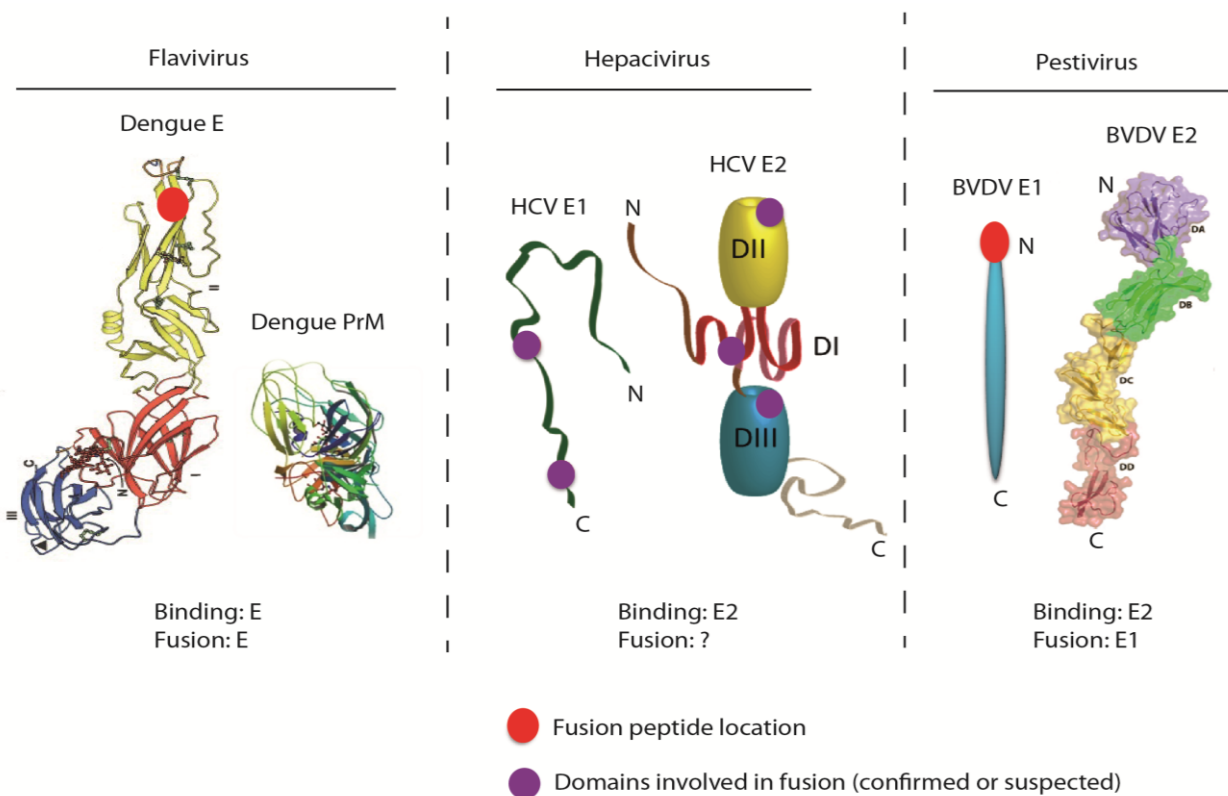
Figure 37. Overview of the HCV, flavivirus and pestivirus fusion-related glycoprotein domains. HCV E2 tridimensional structure is based on the *Krey* E2 structure model. HCV E1 tridimensional structure is arbitrary. E2 protein segments involved (or suspected to be) in virus fusion are highlighted by purple rounded areas. Purple area located in HCV E2 represents the fusion-peptide protein segment identified by *Krey et al*. Dengue (flavivirus) and BVDV envelope glycoproteins are showed to the left and to the right respectively of the HCV envelope glycoproteins. Dengue E fusion peptide is highlighted in red. BVDV E1 structure is arbitrary and likely harbors a fusion peptide at its N terminal, marked by a red rounded area. Proteins harboring the binding and/or the fusion function(s) are indicated for each genus.

Other studies also showed that E1 (and E2) transmembrane domains could be involved in envelope glycoproteins conformational changes required for HCVpp fusion (Ciczora et al., 2005; Ciczora et al., 2007; Op De Beeck et al., 2000). Thus, as pestiviruses E1 and HCV E1 have a similar size and have been suggested to be both involved in virus fusion (El Omari et al., 2013), the contrast previously stated between pestiviruses and HCV do not appear as strong as it was any more, suggesting that the HCV fusion is likely a very original process. HCV fusion related-domains are summarized in Figure 37 with the ones of flavi- and pesti-viruses.

In consequence, several hypotheses can be drawn regarding the HCV fusion process. First, E1 and E2 could both harbor a fusion peptide. As E2 DII only holds a single loop in contrast to flaviviruses E (Krey et al., 2010), this loop could be complemented by E1 during fusion. E1 would have a critical function during entry, consistently with the fact that this protein is not proteolytically processed during maturation as pestiviruses E1 (Lindenbach et al., 2007).

A second hypothesis could be that only E2 harbors the HCV fusion peptide. The HCV fusion peptide could be located either in DII or in several domains to form a conformational fusion peptide. Low pH induction could unmask or structure fusion peptide respectively through particular conformational changes. Indeed, it has been shown that pH treatment induces the exposure of new E2 epitopes (Op de Beeck et al., 2004).

Moreover, another study also showed that the protonation of histidine 445 in E2 DII is able to modulate fusion, suggesting that the protonation of this residue may represent a molecular switch for virus fusion (Boo et al., 2012). In this model, the involvement of E1 during fusion would be likely linked to its influence on the general E1E2 fusogenic conformational changes. This model is consistent with some particularities of the pestiviruses fusion model where the entire heterodimer is required to fold over the fusion protein and induce membrane merging (El Omari et al., 2013).

A third hypothesis would be that only E1 harbors a fusion peptide, similarly to pestiviruses. In this model, E2 could mask with its N terminal domain the E1 fusion peptide as it has been suggested for E2 BVDV-1 (El Omari et al., 2013). Protonation of a histidine residue after low pH induction such as the histidine 445 (Boo et al., 2012) could unmask the E1 fusion peptide and induce membrane fusion.

II. Entry Mechanism

1. Interaction with cell membranes and host factors

a) Initial attachment factors

i. Lectins

Classic C type lectin receptors (CLR) are membrane anchored proteins that act as adhesion molecules or as pathogen recognition receptors (PRR) (Zhang et al., 2013). These molecules are essential for effective recognition of pathogens by the immune system through their ability to interact with mannose type carbohydrates via their C terminal carbohydrates domain (CRD) (Mitchell et al., 2001). Among the CLRs, DC-SIGN (dendritic cell specific intercellular adhesion molecule-3-grabbing non-integrin) and its homolog L-SIGN (liver and lymph node specific-SIGN) have been shown to be specifically able to bind sE2, HCVpp as well as serum derived HCV particles (Gardner et al., 2003; Lozach et al., 2004). Competitive binding using mannan or antibodies targeting the CRD demonstrated the specificity of this interaction (Lozach et al., 2003).

DC-SIGN is mainly expressed on dendritic cells and macrophages and mediates the adhesion with T lymphocytes. Thus, in addition to their role of cellular adhesion molecules, the CLRs also induce via their capture, internalization and elimination of pathogens as well as a subsequent antigen presentation that is required for the stimulation of lymphocytes (Bartosch et al., 2006; Zhang et al., 2013). In contrast, the L-SIGN molecule is more constrained within the liver. This molecule is highly expressed on liver sinusoidal endothelial cells (LSEC) that are microvascular endothelial cells resident within the liver. LSECs harbor a unique phenotype presenting several similarities with dendritic cells, as well as a unique function of antigen-presenting cells for T lymphocytes (Zhang et al., 2013). These cells are likely involved in the control of the immune response and tolerance within the liver.

Despite their ability to bind HCV particles, it has been shown that the expression of these 2 molecules do not allow HCV entry and infection (Lai et al., 2006) highlighting

the need of other cellular factors to ensure a complete entry process. However, the role of these molecules might be essential for the capture and delivery of viral particles within hepatocytes (Bartsoch et al., 2006).

Another CLR, the Asialoglycoprotein receptor (ASGP-R), has been proposed to act as a receptor during HCV entry (Saunier et al., 2003). This molecule that is mainly expressed on hepatocytes surface has been shown to bind a soluble form of E2 produced from a baculovirus expression system. This binding was specific as it could be abrogate with ASPG-R specific ligands. However, it is known that baculovirus expression derived-E2 glycoprotein displays particular glycosylation patterns that may bias the relevance of the observed binding. Thus, this binding needs to be confirmed with the use of more relevant experimental models.

ii.   *Glycosaminoglycans*

Glycosaminoglycans (GAGs) are large polysaccharides composed of repeated disaccharide units. These molecules are expressed on the surface of most of the mammalian cells and serve as cellular attachment molecules for a large number of viruses (Lin et al., 2013). Interestingly, several *Flaviviridae* such as Dengue or CFSV use a particular GAG, the heparin sulfate proteoglycan (HSPG) as an initial attachment factor before binding to more specific receptors (Hulst et al., 2001; Lin et al., 2013). Consistently, it has been shown that HCV sE2 attachment to cell surface was abrogated through binding to heparin, a heparin sulfate homolog, as well as by the heparinase, an enzyme able to degrade heparan sulfate at the cell surface (Barth et al., 2003; Koutsoudakis et al., 2006; Jiang et al., 2012). Moreover, both intracellular and secreted HCVcc viral particles were also able to bind heparin (Koutsoudakis et al., 2006). Kinetics studies with the HCVcc system have demonstrated that HSPG indeed plays a role in cell entry, but only as an initial attachment factor and not as specific HCV cellular receptor (Koutsoudakis et al., 2006).

However, the molecular determinants of this interaction remain difficult to determine. No GAG binding motifs have been identified so far on HCV E2 but the deletion of the HVR1 E2 and antibodies targeting this domain impaired sE2 binding

to heparin (Barth et al., 2006), indicating that HVR1 may play a role in this attachment. This assumption has also been confirmed by the high affinity of a HVR1-synthetic peptide for heparin. However, surprisingly, HCVpp binding is not inhibited by heparin (Callens et al., 2005), suggesting that HSPG attachment of viral particles is independent of functional E1E2 envelope glycoproteins complexes.

ApoE is known to be harbored by lipo-viro particles but is also considered to be a GAGs ligand. Consistently, suppression of the endogenous expression of ApoE in Huh7.5 hepatoma cell lines and complementation with ApoE mutants deficient for HSPG binding resulted in the production of poorly infectious viral particles with a reduced attachment phenotype (Jiang et al., 2012). Moreover, a synthetic peptide derived from the ApoE receptor binding domain to HSPG was also able to inhibit HCVcc infection. In conclusion, GAGs appears to be important initial attachment factors for HCV particles during entry, through the mediation of ApoE.

iii.   The low-density lipoprotein receptor (LDL-r)

The low-density lipoprotein receptor is expressed in a large variety of tissues (Hussain et al., 1999). However, its major function, which consists to capture and clear from the blood circulation the low density lipoprotein, is restricted to the liver (Hussain et al., 1999; Rigotti et al., 2003). More precisely, the LDL-r internalizes the LDLs through a clathrin dependent endocytosis mechanism before delivery into the early endosome. Although the LDL-r is recycled onto the cell surface, the LDLs are degraded into the lysosomes. The LDL-r is composed of three major extracellular domains (Figure 38) (Innerarity, 2002). The N terminal domain is a repeat of seven cystein-rich ligand binding domains that are involved in the binding of LDL. The central domain is composed of three epidermal growth factor (EGF) precursor–like repeats linked to a β-propeller structure (Figure 38). The third extracellular domain is represented by a heavily glycosylated region. The cystein-rich ligand binding domains interact with two particular ligands. The first one is the apolipoprotein B-100 that is the main component of the LDL (Hussain et al., 1999; Innerarity et al., 2002). In addition, LDL-r is also able to exhibit a high affinity for other types of

lipoprotein such as the very low density lipoprotein (VLDL) through interaction with the apolipoprotein E (Hussain et al., 1999; Innerarity et al., 2002).

The LDL-r has rapidly been suspected to be an HCV entry receptor and a potential candidate responsible for virus internalization. Indeed, the heterogeneous buoyant density of the HCV particles and their association with lipoproteins (Thomssen et al., 1992; Lindenbach et al., 2005) constituted strong arguments to hypothesize that HCV from low density fraction may bind to LDL-r. Consistently, a study demonstrated, using patient derived HCV particles, that only low density viral particles could bind to LDL-r expressing cell lines whereas soluble E2 did not (Molina et al., 2007). This interaction was correlated with the level of expression of LDL-r and could be inhibited with soluble human-LDL peptide.
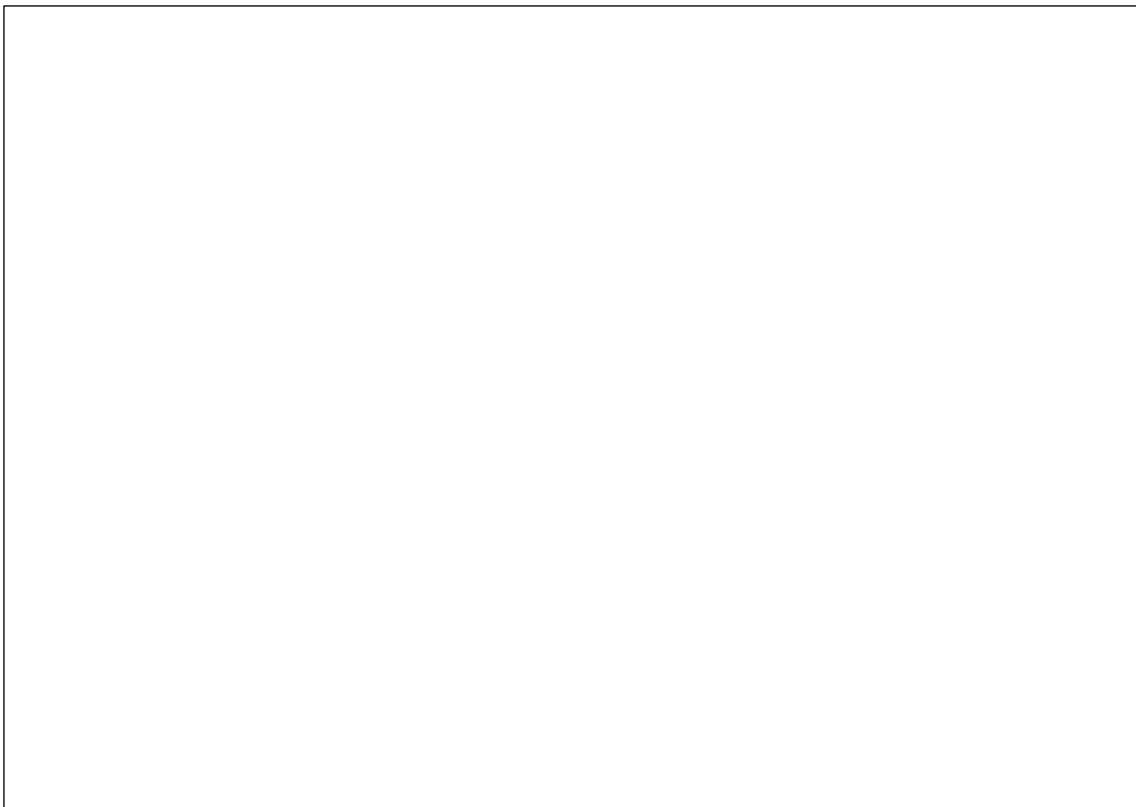
Figure 38. Structure of the LDL receptor. The N terminal domain consists of several repeats of cytidine-rich ligand binding domains that are involved in the attachment of lipoprotein. EGFR precursor-like repeats, a β-propeller structure and a glycosylated domain links the LDL-binding domain to the transmembrane (Adapted from Rebeck et al., 2006).

As no direct interaction was observed between sE2 and LDL-r (Wushmann et al., 2000; Molina et al., 2007), the HCVpp system appeared as a non-competent system to study the molecular determinants of the interaction between the viral particles and the LDL-r. In contrast, the HCVcc system seriously contributed to answer to these questions. Studies confirmed that LDL-r down regulation inhibits HCVcc infection and reported that the association between HCV particles and LDL-r is dependent on ApoE (Owen et al., 2009; Hishiki et al., 2010). Indeed, VLDL or anti-ApoE antibodies were able to inhibit HCVcc infection (Owen et al., 2009). Moreover, the exogenous expression of ApoE2 after down regulation of endogenous ApoE in HCV infected cells did not rescue HCVcc infectivity consistently with the fact that ApoE2 presents a low affinity for LDL-r (Hishiki et al., 2010). To conclude, it is now clear that ApoE constitutes the main mediator of HCV particles for LDL-r attachment such as for GAGs attachment. However, another study demonstrated recently that even though LDL-r represents a major HCV attachment factor, its role is not critical for viral entry. Indeed, this study showed that attachment to LDL-r may lead to non-productive internalization of viral particles (Albecka et al., 2012). Importantly, it also pointed out that the physiological function of this molecule is essential for efficient genome replication.

b) *Between the lines: The Scavenger Receptor B-I (SR-BI)*

The Scavenger Receptor B-I (SR-BI) is a cell surface receptor found on many cell types but that is highly expressed within the liver and the steroidogenic tissues. This protein contains two C and N terminal cytoplasmic domains and one extracellular domain forming a large loop involved in the physiological functions of the receptor (Figure 39A) (Dao thi et al., 2011). SR-BI is able to bind a large variety of molecules as it is involved in the lipid metabolism of different classes of lipoproteins such as the VLDL, LDL or HDL (Van Eck et al., 2008). More particularly, this receptor ensures the selective uptake of HDL cholesteryl ester (CE) via the control of the bidirectional flux of cholesterol between cellular plasma membrane and lipoprotein (Krieger et al., 1999). After binding to SR-BI, the CE is delivered from the HDL to the plasma membrane. The HDL is not degraded in contrast to LDL after internalization by the

LDL-r and the cholesterol mass of the plasma membrane is increased (Silver et al., 2001).

SR-B1 has been identified as a potential HCV receptor through binding assays with sE2 and neutralization assays using anti-SR-BI antibodies (Scarselli et al., 2002, Bartosch et al., 2003; Catanese et al., 2007). HVR1 E2 region was shown to mediate SR-BI binding and specific SR-BI residues involved in this interaction were identified (Bartosch et al., 2003; Catanese et al., 2010; Bankwitz et al., 2011; Dao thi et al., 2012). Moreover, SR-BI down-regulation was shown to decrease both HCVpp and HCVcc infection, confirming the critical role of this receptor for HCV entry (Bartosch et al., 2003; Lavillette et al., 2005; Catanese et al., 2007; Zeisel et al., 2007).

Recently, many efforts attempted to precisely define the role of SR-BI during entry. It has been observed that purified VLDL and antibodies targeting β-lipoproteins were able to inhibit SR-BI binding of patient derived HCV particles (Maillard et al., 2006). Furthermore, SR-BI was shown to act simultaneously with CD81 during HCV entry (Zeisel et al., 2007).
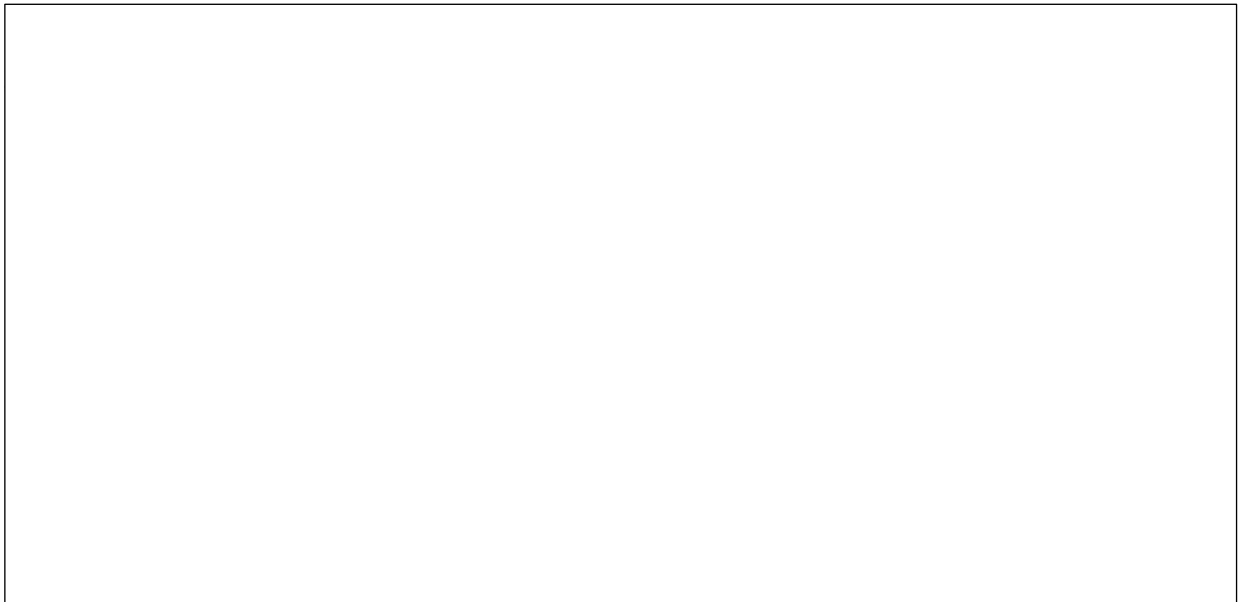
Figure 39. Structure and functions of the Scavenger Receptor B-I. **A.** Schematic representation of the Scavenger Receptor B-I (SR-BI). SR-BI is composed of a single large extracellular loop anchored to the plasma membrane by two transmembrane domains. The extracellular loop contains nine N-glycosylation sites and six cysteins (purple pearls) (Cocquerel et al., 2006). **B.** Summary of the different SR-BI functions during virus entry. For each SR-BI function, the viral and cellular mediators are indicated as well as the related viral sub-population. Each sub-population is indicated through its buoyant density (Dao thi et al., 2012). HDL, high-density lipoprotein; HCV, hepatitis C virus; VLDL, very-low-density lipoprotein; Apo, Apolipoprotein.

Despite deletion of HVRI or the use of anti-HVRI, binding of viral particles was not completely inhibited (Bartosch et al., 2003; Bankwitz et al., 2011; Dao thi et al., 2012). Thus, it has been suggested that HCV can probably bind SR-BI in an envelope glycoprotein- independent way, likely via lipoprotein components associated with viral particles.

Indeed, this role of SR-BI has been recently described and decomposed in two particular functions, the attachment function and the access function (Dao thi et al., 2012). The attachment function has been determined after observation that deletion of HVR1 did not abrogate the binding of viral particles from intermediate density (Bartosch et al., 2003; Bankwitz et al., 2011; Dao Thi et al., 2012), suggesting that SR-BI allows the attachment of these particles to hepatocytes independently of viral glycoproteins (Figure 39B). Additionally, this also suggests that viral particles from low (that constitutes the most infectious particles) and high density fractions may attach to cell surface via alternative cellular molecules, such as GAGs or LDL-r. The access function has been identified via the observation that blocking of the lipid transfer activity of SR-BI blocks entry of HCVcc particles from all types of density (Dao thi et al., 2012). Indeed, SR-BI appeared as critical for entry of viral particles from all density (Figure 39B), likely through the ability of SR-BI to bind HCV lipoprotein components. This statement is also supported by the ability of mice SR-BI to mediate HCV infection without being able to bind sE2 (Catanese et al., 2010; Dao thi et al., 2012). Thus, SR-BI seems to represent a critical factor that allows the access of HCV particles to subsequent HCV receptors. More precisely, SR-BI has been suggested to be involved in the delipidation of viral particles, contributing to enrich the cellular membrane with cholesterol and allowing E1E2 binding to SR-BI and to other HCV cell entry receptors, then leading to virus internalization (Dao thi et al., 2012).

However, as HVR1 E2 was shown to also bind SR-BI, the role of this interaction has been characterized. It has been shown that HDL is able to enhance HCVpp infection by facilitating virus entry (Bartosch et al., 2005; Voisset et al., 2005). This enhancement was dependent on HVR1 E2 although no direct interaction was

observed between HDL and HCVpp as well as no increase of HCV binding to SR-BI (Bartosch et al., 2005; Voisset et al., 2005; Dreux et al., 2006). It has been proposed that the transfer of Apolipoprotein C-1, a major component of HDL that is also present on HCVcc particles, from HDL to HCV particles enhances viral fusion (Meunier et al., 2005; Dreux et al., 2007). A single E2 mutation in the HCVcc system, abrogating infection enhancement but not infectivity, decreased infectivity of viral particles from lower intermediate density fraction (Dao thi et al., 2012), thus confirming that such enhancement is dependent on both E2-SR-BI binding and SR-BI lipid transfer function (Figure 39B). Moreover, as mice SR-BI do not support E2 binding and an enhancement function, the generation of chimeric human/mice SR-BI constructs showed that the enhancement function is mediated by the first domain of human SR-BI.

Finally, SR-BI has also been shown to exert post binding functions. Indeed, SR-BI lipid intake may induce several alterations in the cholesterol distribution of the plasma membrane (Zahid et al., 2010). These alterations may lead to the activation of particular signaling pathways that allow the internalization of HCV and likely, its escape from neutralizing antibodies. Moreover, the SR-BI post binding functions have also been shown to be critical for the initiation and dissemination of virus infection.

In conclusion, SR-BI appears to be a multifunctional and critical factor for HCV entry, notable through its complex interplay with lipoproteins and HCV envelope glycoproteins.

c) The tetraspanin CD81

The tetraspanin CD81 is a small cell surface molecule expressed on a very large panel of cell types. Tetraspanins are adaptor molecules that interact with others cell surface molecules or trigger intracellular signal through their cytoplasmic tail (Van Spriel et al., 2010 ; Monk et al., 2012). All the members of the tetraspanin family share similar functions and structural features. These molecules harbor two extracellular domains, a small and a large extracellular loop (respectively SEL and LEL) anchored to the cell

membrane through four transmembrane domains (Figure 40). The LEL domain adopts a homodimeric conformation. Each monomer is composed of five α-helices (from A to E) that are arranged as a head subdomain. The LEL contains several conserved cysteins residues that notably stabilize the head subdomains (Figure 40) (Van Spriel et al., 2010).

A major HCV cell entry receptor. The tetraspanin CD81 was the first cell surface molecule identified as a specific cell entry receptor critical for HCV entry. This receptor was initially identified by using mouse fibroblasts expressing a cDNA library derived from human cell lines that display a high E2 binding ability. Binding assays with sE2 on these cDNA expressing cell lines allowed the identification of the CD81 tetraspanin as a strong cell receptor for HCV E2 (Pileri et al., 1998). More particularly, this binding was mediated by the LEL domain, through residues located on the D helix (Drummer et al., 2002).

Both HCVpp and sE2 have been shown to bind CD81 highlighting that functional E1E2 heterodimer complexes are able to bind CD81. Use of anti-CD81 antibodies blocked HCVpp infection, as well as competition with soluble LEL (Bartosch et al, 2003; Bartosch et al., 2003; Hsu et al., 2003; Lavillette et al., 2005).
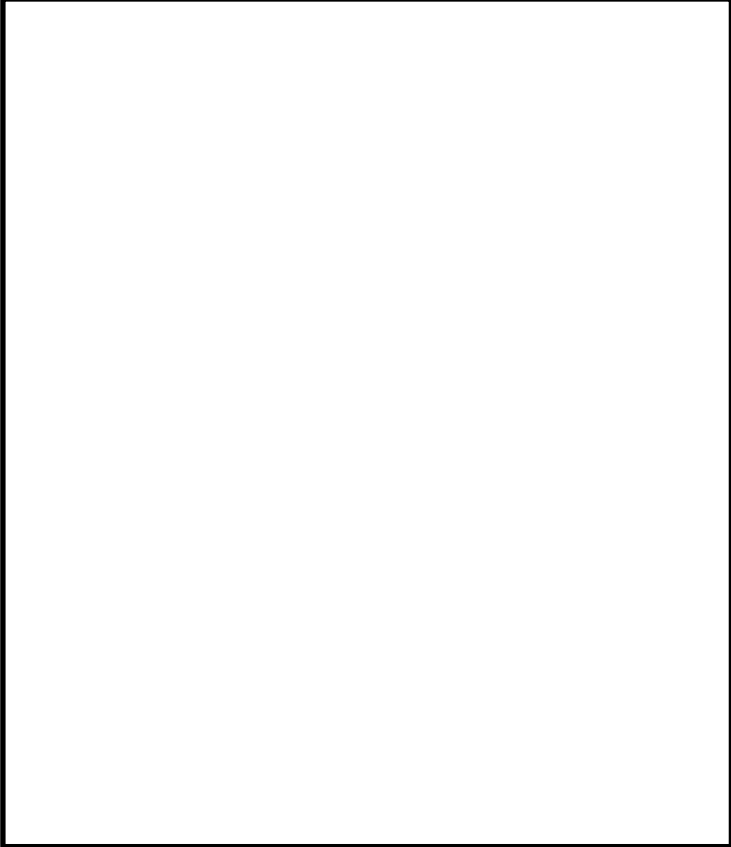
Figure 40. Structure of the tetraspanin CD81. CD81 harbors two extracellular domains (SEL and LEL) anchored to the plasma membrane through four transmembrane domains. LEL harbors a conserved core structure formed by helices a, b and e. This large extracellular loop mediates HCV E2 binding and and primes E1E2 heterodimer for fusion (Levy et al., 2005).

Moreover, exogenous expression of CD81 in HepG2 hepatoma cell lines that do not express CD81 restored HCVpp entry and infection (Bartosch et al., 2003). Interestingly, use of anti-CD81 antibodies also inhibited HCVcc infection although no interaction between HCVcc particles and CD81 could be observed (Wakita et al., 2005; Zhong et al., 2005; Kapadia et al, 2007). Consistently, entry of pre-bound HCVpps was inhibited by anti-CD81 antibodies indicating that CD81 binding may occurs during later stage of viral entry after the initial attachment of the viral particles (Flint et al., 2006; Koutsoudakis et al., 2007).

Thus, in strong contrast with GAGs and LDL-r that bind HCVcc particles (during the initial steps of entry) through lipoproteins, CD81 binding is dependent on functional E1E2 complexes. As HCVcc particles are associated with lipoproteins, the HCVcc model cannot allow the assessment of CD81 binding. This is consistent with the idea that CD81 binding likely occurs during later stages of entry and requires several protein conformational changes or particle rearrangements.

Role in internalization and fusion. Importantly, HCV binding to CD81 has been shown to induce the clustering of CD81 with the tight junction protein Claudin-1. This interaction is critical for HCV infection as it induces internalization of viral particles through endocytosis. It has been demonstrated that HCV binding to CD81 triggers actin-dependent relocalisation of the E2/CD81 complexes to tight junction molecules, thus allowing subsequent virus internalization (Brazolli et al., 2008; Harris et al, 2008; Harris et al., 2010; Facquar et al., 2012). More precisely, this receptor clustering is triggered, following HCV binding, by the CD81 cytoplasmic tail that activates downstream signaling pathways and the Rho GTPases (Brazolli et al., 2008). Indeed, the inhibition of Rho GTPases has been demonstrated to significantly inhibit HCV entry.

CD81 has also been shown to modulate for membrane fusion. It has been suggested that HCV binding to CD81 triggers E1E2 conformational changes and prime these proteins in order to induce virus fusion. Indeed, pre-incubation of viral particles with the soluble CD81 LEL was able to enhance fusion and viral entry (Sharma et al.,

2011). This feature places in strong contrast the HCV fusion process from the one of the flaviviruses that does not require such priming. However, interestingly, the pestiviruses envelope glycoproteins also required post attachment priming steps (Krey et al., 2005) to induce virus fusion which suggests that the HCV and pestiviruses fusion process may share some similarities.

Its partner EWI-2wint. Tetraspanins form at the cell surface important and dynamic clusters with several membrane proteins called tetraspanin-enriched areas (TEAs). These clusters are important for the maintenance of important cellular processes such as cytoskeleton organization, cellular migration or proliferation. Consequently, perturbations of clusters integrity may lead to a large panel of cellular anomalies and transformation such as cancer (Sala-Valdes et al., 2012). It has been shown that HCV binding to CD81 is independent of the TEAs but that its interaction with a TEAs protein, Ewi-2wint, inhibits HCV entry by constraining CD81 into the TEAs (Rocha-Perugini et al., 2008; Montpellier et al., 2011; Potel et al., 2013). Ewi-2wint is the cleavage product of Ewi-2, a cell surface molecule that belongs to the superfamily of the immunoglobulin. Ewi-2wint was shown to reduce the global diffusion of CD81 required for HCV infection and promote its clustering into TEAs enriched in CD81 tetraspanin (Potel et al., 2013).

In conclusion, CD81 appears to be a critical and complex entry receptor that likely divulges the potential complexity of the whole HCV entry process. Indeed, this molecule is a multi-competent protein involved in the major steps of entry such as binding, receptor clustering, internalization or fusion. The interaction with its partner Ewi-2wint may also represent an important molecular determinant contributing to the restricted tropism of HCV, considering that Ewi-2wint is expressed in several tissues or cell types (such as the brain or immune cells) but not in hepatocytes. Finally, as rat or murine CD81 do not support sE2 binding, this receptor also highly contributes to the narrow host species tropism of HCV.

d)   The tight junction proteins Claudin-1 and Occludin

Claudin-1 and Occludin are cell membrane proteins highly expressed within the liver that are both critical for HCV entry. Non permissive cell lines expressing ectopically these proteins with CD81 and SR-BI were able to support virus entry (Evans et al., 2007; Ploss et al., 2009), demonstrating that Claudin-1 and Occludin represented the last two cell entry factors to identify in order to recapitulate virus entry. However, in contrast to CD81 and SR-BI, these molecules are expressed into the hepatocyte apical membranes, as members of cellular tight junctions.

Tight junctions are important mediators of the intercellular junctions between hepatocytes, essential for the maintenance of the large cellular plate constituting the liver tissue. More precisely, these junctions connect together two neighboring cell membranes that lie close together to form a tight junction strand between two hepatocytes (Kojima et al., 2009; Gunzel et al., 2012). These junctions are mainly ensured by three major classes of proteins: the Claudin proteins, Occludin and the junction adhesion molecule (JAM). These molecules are linked to the actin cytoskeleton through cytoplasmic proteins of the family of the membrane-associated guanylate kinases (Kojima et al., 2009; Gunzel et al., 2012). Tight junctions have multi-functional roles. First, they determine the apico-basal polarity of the hepatocytes (Kojima et al., 2009; Gunzel et al., 2012). Second, they can mediate several signaling pathways within the hepatocytes that control gene expression or cell differentiation (Kojima et al., 2009; Gunzel et al., 2012; Runkle et al., 2013). Third, they represent a semi permeable barrier that modulates flow of molecules between paracellular spaces and block pathogens to avoid invasion of deeper tissues (Kojima et al., 2009; Gunzel et al., 2012; Runkle et al., 2013). Interestingly, these molecules can also be hijacked by pathogens to attach and invade hepatocytes as it has been reported for Adenoviruses (Coyne et al., 2006).

Consistently, both Claudin-1 and Occludin have been identified to be critical for HCV infection through screening of a cDNA library derived from highly permissive Huh7.5. Indeed, expression of the cDNA library into non permissive HEK293t cells

expressing CD81 and SR-BI allowed the identification of Claudin-1 and Occludin as critical HCV entry factor (Evans et al., 2007; Ploss et al., 2009).

i.   Claudin-1

Claudin-1 is a 211 amino acids protein composed of two extracellular domains, EL1 and EL2 (for extracellular loop 1 and 2), anchored to the cell membrane through four transmembrane domains (Figure 41A) (Gunzel et al., 2012). Down-regulation of Claudin-1 into Huh7.5 was reported to drastically inhibit HCV infection (Evans et al., 2007). Expression of Claudin-1 into non permissive cell lines as HEK293T rendered these cells permissive for HCV infection as well as the expression of other members of the Claudin family such as Claudin-6 or -9, but not Claudin-7 (Evans et al., 2007). This unability of Claudin-7 to mediate HCV infection was taken as an opportunity to generate chimeric form of Claudin-1. These mutants allowed the identification of a highly conserved motif into EL1 as critical for Claudin-1 mediated HCV infection (Evans et al., 2007).

Consistently, antibodies targeting EL1-inserted flag tags were able to neutralize HCV entry at a post binding step during viral entry, suggesting that Claudin-1 likely plays its role subsequently to CD81 and SR-BI binding (Evans et al., 2007).

Interestingly, no direct interaction between HCV particles and Claudin-1 has been demonstrated so far. A study notably showed that anti-claudin-1 antibodies were able to abrogate sE2 binding to Huh7.5 although no interaction between sE2 and Claudin-1 could be observed (Evans et al., 2007; Krieger et al., 2010).

Thus, claudin-1 has rapidly been thought to play a different role than that of a simple attachment molecule. It was demonstrated that the localization of Claudin-1 into the tight junction was critical for HCV infection (Yang et al., 2008; Liu et al., 2009), thus suggesting that viral particles migration into these junctions may be important for virus entry. Indeed, it has been shown that CD81/Claudin-1 complexes co-localize in close proximity of intercellular junctions during HCV infection of Huh7.5, likely mediated through a Claudin-1 EL1-CD81 LEL molecular interface (Figure 41B) (Harris et al, 2008; Harris et al., 2010; Farquhar et al., 2012; Davis et al., 2012).
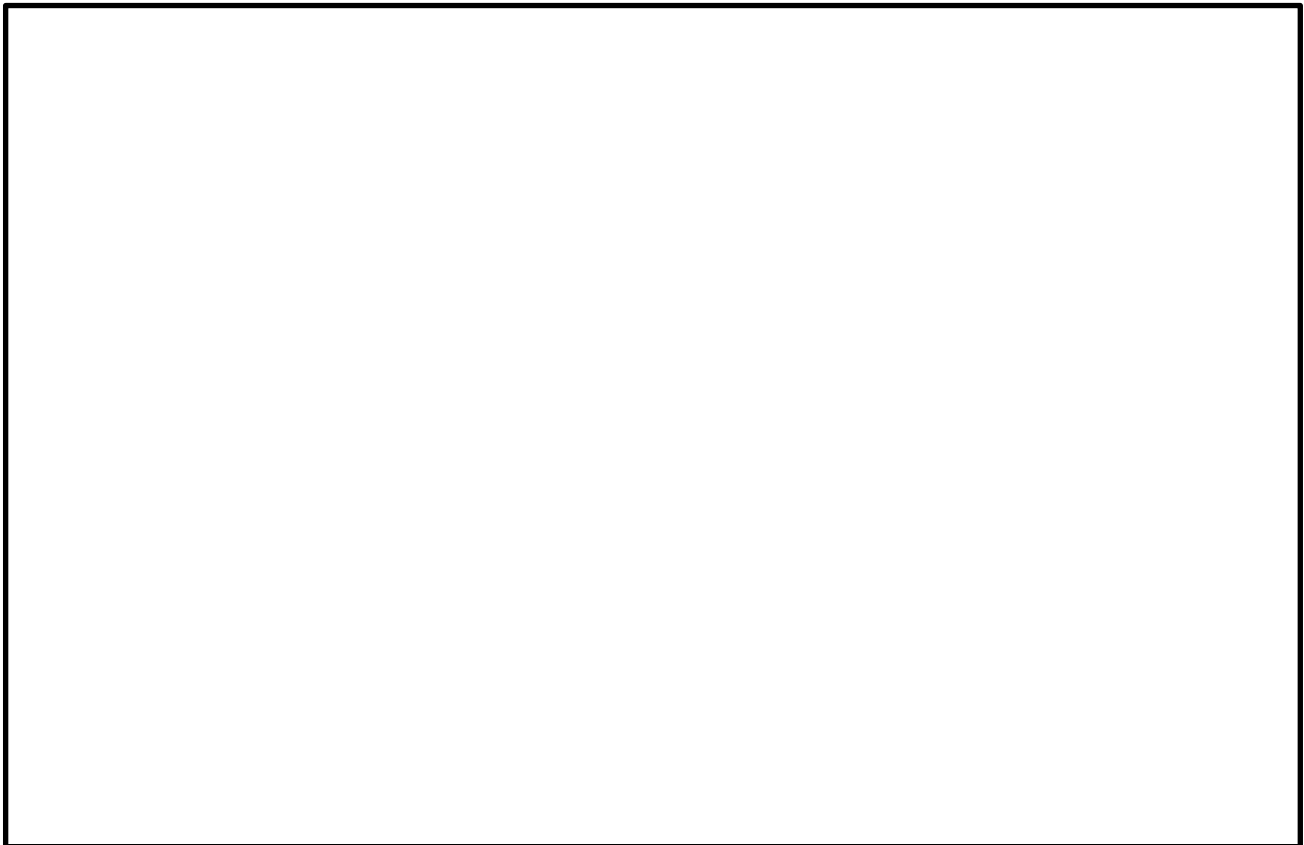
Figure 41. Structure and localization of the tight junction proteins Claudin-1 and Occludin. **A.** Claudin-1 structure. Claudin-1 is composed two extracellular loops (EL1 and EL2) anchored to the membrane through four transmembrane domains. EL1 is thought to mediate CD81 association and receptor clustering (Adapted from Lal-nag et al., 2009). **B.** Localization of Claudin-1 in intercellular junctions. After CD81 engagement by HCV, CD81 diffuse laterally toward apical membrane. Claudin-1 delocalizes from apical membrane to basolateral intercellular junctions to associate with CD81. This receptor clustering is critical for virus internalization. **C.** Occludin structure. Occludin is composed of two extracellular loops (EL1 and EL2) of similar size anchored to the plasma membrane by four extracellular loops (Adapted from Cummins et al., 2012).

Use of anti-claudin-1 antibodies able to neutralize CD81-Claudin-1 interaction was shown to neutralize HCVcc infection, demonstrating the importance of this complex for HCV entry (Krieger et al., 2010). Interestingly, these receptors complexes seem localized in basolateral intercellular junction and not in apically located tight junctions (Figure 41B), which is consistent with a viral entry into hepatocyte cell layers via the sinusoidal blood (Harris et al., 2010). The formation of CD81/Claudin-1 complexes is believed to enhance HCV association with cell receptors complexes and virus migration toward tight junctions, which is critical for virus internalization. Consistently, HCV has been shown to induce internalization of CD81-Claudin-1 complexes (Farquhar et al., 2012).

ii.   Occludin

Despite the fact that Claudin-1 is an important HCV entry, some non-permissive cell lines remained resistant to virus entry despite the ectopic expression of Claudin-1, suggesting that other entry factors are required. Based on a similar approach that allowed the identification of Claudin-1, the elusive missing entry factor was identified in 2009 to be another tight junction protein, Occludin (Ploss et al., 2009). Occludin, with 521 amino acids, is a tight junction protein larger than Claudin-1. Occludin is composed of two large extracellular loops EL1 and EL2 (the Occludin EL2 is larger than the Claudin-1 EL2) and of four transmembrane domains (Figure 41C) (Ploss et al., 2009). Once Occludin was identified, ectopic expression of SR-BI, CD81, Claudin-1 and Occludin was sufficient to render susceptible non permissive rodent cell lines such as CHO (Ploss et al, 2009), indicating that the whole collection of the critical HCV entry factors was now complete.

However, the function of Occludin remains to be precisely characterized. It has been proposed that Occludin EL2 is involved in HCVpp mediated entry as well as in E2 binding. Indeed, an interaction between E2 and Occludin has been observed through microscopic confocal analyzes and co-immunoprecipitations assays (Benedicto et al., 2008; Liu et al., 2009; Liu et al., 2010). However, the relevance of this interaction is questionable so far as it has only been observed in intracellular reticulum endoplasmic and not in a cell surface receptor binding context. A recent study also suggested a potential interaction between viral particles and Occludin, based on the observation that HCV types and sub-types present Occludin utilization differences (Sourisseau et al., 2013). Interestingly, synchronized infection assays showed that Occludin acts subsequently to CD81 and Claudin-1 during HCV entry and down regulation of Occludin decreases both HCV entry and glycoproteins-mediated cell fusion. Thus, these observations are consistent with the idea that the tight junction proteins are the last mediator of the HCV cell entry, critical for virus internalization and thus, membrane fusion.

  e) **Other** *factors involved in HCV entry.*

    i. *The Epidermal growth factor receptor (EGFR) and Ephrin A2*
      (EphA2)

EGFR and EphA2 are two members of the receptor tyrosine kinase (RTK) superfamily. These molecules harbor major physiological functions such as the control of the cell proliferation or cell differentiation. In consequence, the RTK may also be involved in several malignant diseases such as cancers (Yano et al., 2003).

In 2011, a study identified both EGFR and EphA2 to be important for HCV entry. Down regulation of these RTKs or use of specific protein kinase inhibitors impaired HCV entry both *in vitro* and *in vivo*, in chimeric humanized liver mice (Lupberger et al., 2011). Interestingly, use of RTK specific ligands such as the Epithelial Growth Factor (EGF) enhanced HCV infection through receptor dimerization and activation of intracellular signaling pathways. Moreover, EGFR appeared as critical for CD81-Claudin-1 receptors clustering as well as viral fusion (Lupberger et al., 2011). However, the mechanisms by which EGFR promotes virus entry and receptors clustering remained to be elucidated.

HCV-CD81 engagement has been shown to induce the activation of the RhoGTPases family members, MAPK or PI3K/AKT signaling pathways (Brazzoli et al., 2008; Liu et al., 2012), also known to be dependent on the phosphorylation of EGFR. Consistently, it has been observed that HCV CD81 binding activates EGFR by promoting its phosphorylation (Diao et al., 2012). More recently, HRas, a membrane-bound GTPase activated downstream of EGFR signaling, has been demonstrated to be a key signaling factor of EGRF-mediated HCV entry. It was shown that HRas associates with CD81-Claudin-1 complexes, linking the EGFR induced signaling pathway with the HCV cell entry receptor complexes (Zona et al, 2013). Importantly, this HRas mediated signaling pathway appeared as critical for CD81 lateral diffusion within plasma membrane and its stable clustering with Claudin-1 (Zona et al., 2013), thus allowing efficient virus internalization.

Interestingly, EGFR has also been suggested to be involved in cellular anti-viral response, as the inhibition of EGFR induced signaling was shown to enhance IFN-α mediated antiviral activity (Lupberger et al., 2013).

ii.   The Niemann-Pick C1-like 1 cholesterol absorption receptor (NPC1L1)

NPC1L1 is a large cell surface protein composed of 13 transmembrane domains (Figure 42) mainly expressed on the apical surface of intestinal enterocytes and human hepatocytes. NPC1L1 acts as a cholesterol-sensing receptor and is responsible for cellular cholesterol absorption and cholesterol homeostasis. The involvement of this protein in the cholesterol uptake, its preferential expression on hepatocytes and the previous reports demonstrated that this protein is a critical entry receptor for filoviruses (Carette et al., 2011) highlighting a potential role for NPC1L1 in HCV entry. Indeed, very recently, a study demonstrated that NPC1L1 is an active mediator of the HCV cell entry process.

Authors observed that HCVcc infected Huh7 presented a down regulation of NPC1L1 expression and that down regulation of this receptor significantly impaired HCVcc infection (Sainz et al., 2012). Down regulation of NPC1L1 had no effect on RNA replication, virus assembly or particle budding suggesting that this protein may play its role during virus entry. Indeed, use of anti- NPC1L1 specific antibodies significantly inhibited HCV infection and entry. Consistently, using a fluorescent based HCVcc fusion assay, NPC1L1 has been shown to be critical for post binding step events and for fusion (Sainz et al., 2012).
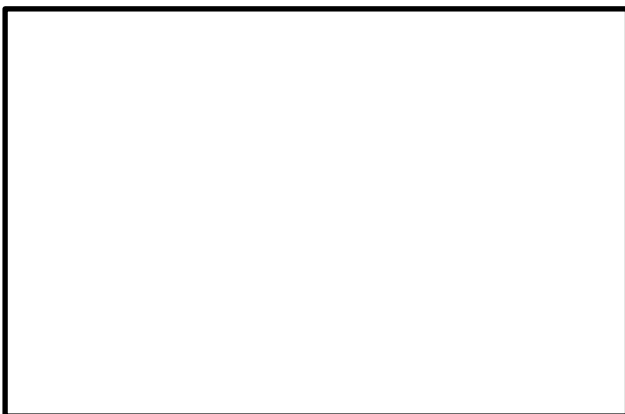

Figure 42. Structure of NPC1L1. NPC1L1 is composed of 13 transmembrane domains and seven extracellular domains. This receptor is anchored to the hepatocyte apical membrane and is exposed to the bile canaliculus , a thin tube that collects bile secreted by hepatocytes (Adapted from Weinglass et al, 2008).

The use of ezetimibe, a direct inhibitor of NPC1L1 internalization already used as a cholesterol-lowering medication, was able to affect infection and propagation of HCVcc particles harboring structural proteins from different genotypes. This result suggests that the biological function of NPC1L1 is the mediator of the NPC1L1-mediated cell entry (Sainz et al., 2012). Indeed, authors demonstrated the existence of a correlation between the cholesterol content of viral particles and their dependence on NPC1L1 during entry. The efficiency of ezetimibe was also assessed *in vivo*. A 2 weeks pretreatment of ezetimibe was able to delay the establishment of genotype 1b infection in humanized liver mice (Sainz et al., 2012).

Up to now, NPC1L1 is considered to be an important cell entry factor for HCV through its cholesterol-sensing activity. However, how NPC1L1 precisely mediates virus entry remains unknown. A possible assumption would be that NPC1L1 lipid transfer activity would play a role in viral particles delipidation or modification of lipid associated-viral particles conjointly with SR-BI.

iii.   The Transferrin receptor 1 (TfR1)

Transferrin receptor (TfR) 1 and 2 are transmembrane proteins expressed in the liver, involved in the iron uptake from blood to intracellular compartment. Iron circulates into the blood in complex with a carrier protein transferrin (Tf), which binds to the TfR and penetrates into the cells through receptor-mediated endocytosis. TfR are homodimeric proteins that harbor two protein subunits of 95 kDa (760 amino acids). Each subunit is composed of an extra- and intra-cellular domains anchored to the membrane via a 20 amino acids transmembrane domain (Macedo et al., 2008).

Interestingly, HCV infection in patient is often characterized by an iron overload. As TfRs control iron homeostasis, it has been suggested that HCV infection impaired TfR expression within hepatocytes. Indeed, a very recent study found that HCV alters the expression of TfR1 within infected cells, thus deregulating iron homeostasis (Martin et al., 2013). Moreover, authors showed that TfR1 down regulation and antibodies-mediated neutralization against TfR1 inhibited both HCVcc and HCVpp infection, suggesting that TfR1 is important for glycoproteins-dependent viral entry

(Martin et al., 2013). Kinetics experiments demonstrated that TfR1 likely acts in a post binding step after CD81 binding as the neutralizing activity of anti-TfR1 antibodies could only be observed after anti-CD81 antibodies mediated neutralization (Martin et al., 2013). Interestingly, the silencing of the TfR-1 trafficking protein, required for TfR1 internalization, was shown to inhibit HCV infection, likely suggesting a role for TfR1 in virus internalization. The precise mechanism by which TfR1 mediates this process remains to identify.

f)   HCV receptors: major determinants of HCV host tropism

As human and chimpanzees liver cells appear to be the major HCV reservoir both *in vitro* and *in vivo*, important efforts have been done to understand the role of HCV receptors in the restricted tropism of HCV. Indeed, this understanding is critical for the generation of relevant transgenic animal models able to support HCV entry.

First, CD81 has been identified, through its large extracellular loop, to be an important determinant of the narrow tropism of HCV. Indeed, only the human, and not the rodent CD81, is able to bind sE2. Consistently, soluble LEL from rodent have not been shown to inhibit HCV infection (Flint et al., 2006). However, interestingly, expression of rodent CD81 in HepG2 cells (Hepatoma cell lines that do not express CD81 and are thus non permissive for HCV infection) conferred susceptibility to HCVpp and HCVcc infection to various level, suggesting that the narrow tropism mediated by CD81 may be dependent on the cell type in which it is expressed.

Binding of sE2 to SR-BI has been shown to be dependent on human SR-BI sequence specificities, suggesting that the interaction between E2 and human SR-BI is part of the HCV host tropism determinants. Indeed, expression of SR-B1 mutants defective for sE2 binding in SR-BI-knocked-down Huh7.5 cell line did not fully restored the HCVcc infectivity (Catanese et al., 2010). However, despite the lack of E2-SR-B1 interaction, mouse SR-BI can still mediate HCV entry through lipoprotein association. Indeed, as mentioned in Introduction-Chapter V II.1.b., mouse SR-B1 supports both an attachment and access function, which efficiently allows HCVcc

entry (Dao thi et al., 2012). Thus, human SR-BI, in contrast to CD81, does not constitute an important determinant of HCV host tropism.

Both mouse and human Claudin-1 have been shown to render HEK293T cells permissive (Evans et al., 2009), thus suggesting that Claudin-1 is not a determinant of HCV narrow host tropism. Interestingly, a study showed that mouse Claudin-1, when expressed in human hepatoblastoma cell lines HuH6 (that express low levels of endogenous Claudin-1 but are able to replicated HCV RNA), displayed moderate HCVpp and HCVcc entry although expression of human, rat and hamster Claudin-1 allowed efficient virus entry (Haid et al., 2010). Thus, similarly to CD81, entry mediation ability of mouse Claudin-1 may be dependent on the cell type in which the receptor is expressed. Nevertheless, according to these studies, Claudin-1 does not appear as a determinant of HCV host tropism.

Despite the identification of Claudin-1, some cell lines such as CHO or NIH3T3 remained non permissive to HCV infection. Identification of Occludin as the missing cellular factor able to render these cell lines permissive to infection (once expressed with CD81, SR-B1 and Claudin-1) constituted the first opportunity to perform a combined evaluation of the ability of the four cellular factors to mediate HCV host tropism. Six residues located within the Occludin second extracellular loop were shown to mediate HCV host tropism similarly to the CD81 LEL (Ploss et al., 2009; Michta et al., 2010). Moreover, identification of Occludin-1 permitted to confirm that CD81 and Occludin are the only mediators of the HCV host tropism, whereas SR-BI and Claudin-1 are not.

The identification of human CD81 and Occludin-1 as determinants of HCV host tropism permitted major advances for the development of relevant HCV animal models. Indeed, transient or stable hepatic expression of these two human genes were sufficient to render permissive for HCV infection immunocompetent inbred mice (Dorner et al., 2011), which thus represented a unique opportunity to dissect virus entry and action of viral entry inhibitors in an immunocompetent context (See Introduction – Chapter III VIII.2.b

2. Viral particle internalization and fusion

Viruses have developed an important number of mechanisms to invade a cell and release their genetic material into the host cell cytosol. Among them, the hijacking of the cell endocytic pathway is commonly used by most of the known viruses. Depending on the particle size, the receptor usage, cell tropism or mode of transmission, endocytic pathways used by viruses are extremely diverse. Endocytosis is divided into two major mechanisms: pinocytosis and phagocytosis (Figure 43). Among the viral world, pinocytosis is a largely used endocytic pathway. This cellular mechanism absorbs and internalizes particles or molecules in intracellular vesicles through internal invagination of the plasma membrane (Mercer et al., 2009).

Dynamin-dependent pinocytosis is composed of two endocytic mechanisms: the clathrin-mediated endocytosis and the caveolar mediated endocytosis (Figure 43). Clathrin mediated endocytosis is a specific internalization process that follows particle attachment to particular cell surface receptors. Particle attachment induces the inward budding of the plasma membrane and the formation of a clathrin pit that internalizes the particle-receptor complex. This pathway is used by a large number of viruses, such as HIV-1, Influenza virus, the *Bunyaviridae* and the *Flaviviridae* as HCV (Mercer et al., 20009). Indeed, it has been shown that downregulation of the clathrin heavy chain inhibited HCVpp and HCVcc infection (Blanchard et al., 2006). Use of bafilomycin A1, that affects endosomal acidic environment by preventing re-acidification, also blocked HCV infection (Meertens et al., 2006).
Moreover, during HCV infection, internalization of CD81-Claudin-1 complexes through clathrin-dependent endocytosis has been observed and CD81-Claudin-1 association has been shown to be critical for virus internalization, suggesting that HCV is internalized with cellular receptors prior fusion (Harris et al., 2008; Harris et al., 2010; Krieger et al., 2010; Farquhar et al., 2012). This observation is consistent with the fact that CD81 has been demonstrated to prime HCV fusion (Sharma et al., 2011).
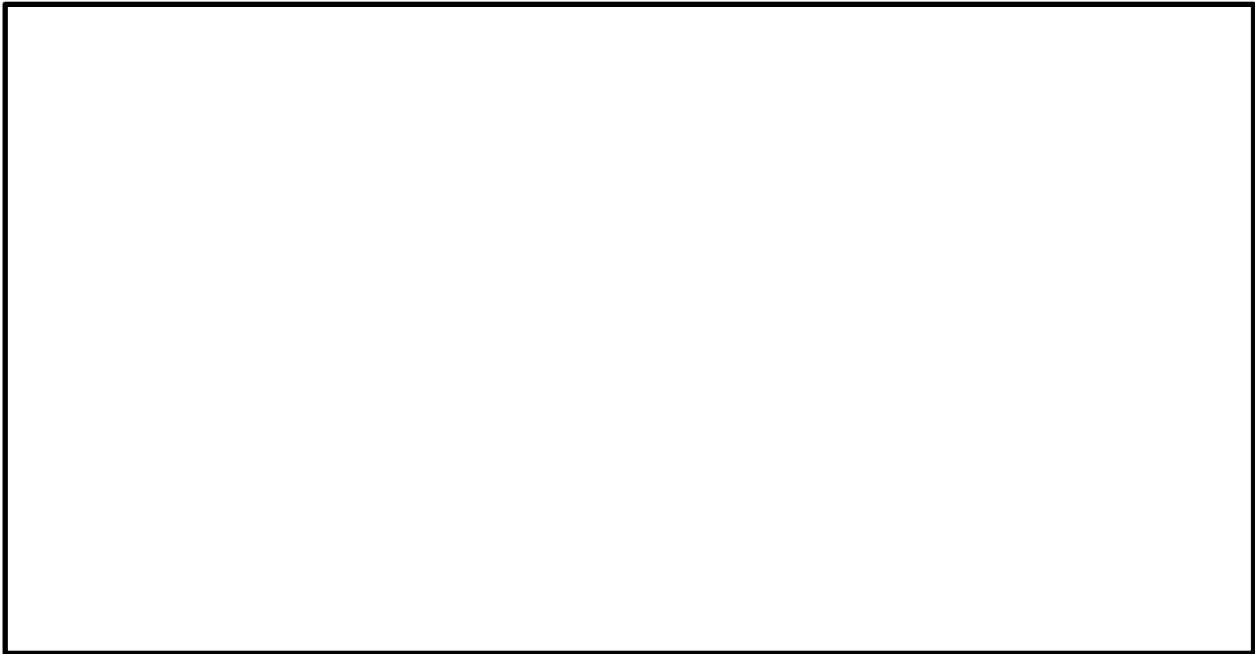
Figure 43. Viral endocytic pathways. Different endocytic pathways used by a large panel of viruses according to the particle structure, the receptor bound or the cell tropism. Endocytosis is divided into two major mechanisms: pinocytosis and phagocytosis. Pinocytosis is largely used among the viral world and is composed of several mechanisms such as clathrin-mediated endocytosis (Mercer et al., 2009).

After internalization, acidification of the endosomal compartment is thought to trigger E1E2 conformational changes that induce the fusion of the viral membrane with the endosomal membrane (Op de Beeck et al., 2004; Sharma et al., 2011). For more information, fusion process is detailed in Introduction – Chapter V I.4.c.ii.

Internalization through pinocytosis can also be dynamin-independent (Figure 43). This type of pinocytosis includes three un-specific processes of molecule internalization: the non-clathrin mediated endocytosis, the lipid-raft mediated endocytosis or the macropinocytosis (Figure 43). Macropinocytosis is characterized by the formation of large vacuoles involved in fluid uptake. This mechanism is used by a large panel of viruses such the vaccinia viruses, the adenoviruses or the picornaviruses (Mercer et al., 2009).

3. Cell-Cell transmission

It has been shown that HCV can propagate from infected cells to neighboring cells independently of the classical entry and internalization pathways used by circulating viral particles (Timpe et al., 2008; Wittevelt et al., 2009; Brimacombe et al., 2011,

Catanese et al., 2013). This cell-to-cell transfer of virus infection is thought to be an important route for efficient virus spread within liver tissue, as it constitutes an efficient strategy to escape from the host neutralizing response. SR-B1, Claudin-1 and Occludin-1 have been shown to be important for virus transmission between hepatocytes (Timpe et al., 2008; Wittevelt et al., 2009; Brimacombe et al., 2011, Catanese et al., 2013). Consistently, a very recent study demonstrated that anti-SR-B1 antibodies could bock cell to cell transmission. Moreover, authors showed that specific mutations in E1 and E2 were responsible of an increased cell-to-cell spread ability, through reduction of SR-B1 usage. However, this enhanced ability was presented as unlikely to occur *in vivo*, as mutations also increased virus sensitivity to neutralizing antibodies (Catanese et al., 2013).

In contrast with the other receptors, the role of CD81 in this process remains a matter of debates. Two studies previously reported that CD81 is dispensable for cell to cell transmission (Timpe et al., 2008; Wittevelt et al., 2009) although two others, more recent, reported that CD81 indeed, plays a role in cell to cell transfer (Brimacombe et al., 2011, Catanese et al., 2013). A very recent study notably showed that expression of CD81 on producer cells modulates HCV cell-to-cell transmission (Catanese et al., 2013).

CHAPTER VI

HCV and Peripheral Blood Mononuclear Cells infection

This last chapter will focus on the particular ability of HCV to infect other cell tissues than the liver. Indeed, HCV infection is commonly associated with a large panel of extrahepatic disorders. Among them, Peripheral blood mononuclear cells (PBMCs)-related disorders such as mixed cryoglobulinaemia (MC) or B-cells Non-Hodgkin's Lymphomas (NHLs) are of significant prevalence. Consistently, viral replication has been observed in several PBMCs, but most importantly in B lymphocytes and monocytes-derived dendritic cells. HCV variants deriving from the PBMCs compartment present particular genetic features that distinguish them from the liver-derived variants, suggesting that particular molecular determinants mediate HCV PBMCs tropism. However, these features remain poorly characterized and HCV lymphotropism, poorly understood. The ability of HCV to infect PBMCs such as B or T-lymphocytes has important clinical implications for viral persistence and immune escape, suggesting that the understanding of HCV lymphotropism may be crucial to improve patient responses to treatment. In this chapter, we will review the evidences related to PBMCs infection by HCV and the existence of a potential genetic specialization of the PBMCs-derived variants. Then, we will summarize the cellular determinants that could play a role during HCV entry in lymphocytes and describe potential object of debates that are related to HCV lymphotropism. Finally, we will discuss the clinical significance of HCV lymphotropism.

I.   Evidence of PBMCs infection

HCV infection has been widely associated with several extrahepatic disorders (see Chapter II – V). Among them, mixed cryoglobulinaemia (MC) and Non-Hodgkin's lymphoma (NHL) are of first interest. It has been notably observed that NHL of HCV patient regressed after IFN-α treatment whereas such treatment is inefficient in NHL-patient uninfected by HCV (Hermine et al., 2002), suggesting a direct link between HCV infection and the appearance of PBMCs-derived disorders. Consequently, these observations suggest that PBMCs may represent an infection site for HCV, similarly to Hepatitis B Virus (Pasquinelli et al., 1990; Brind et al., 1997) or GBV-C (Polgreen et al., 2003).

Indeed, circulating viral load in chronic patients was shown to be higher than serum viral load (Schmidt et al., 1997). Several studies have also demonstrated the presence of negative viral stranded RNA in haematopoic cells such as B cells, T cells, monocyte-derived dendritic cells, (Zignego et al., 1992; Ferri et al., 1993 ; Muller et al., 1993 ; Zignego et al., 1995; Cribier et al., 1995 ; Ferat et al., 1996; Schmidt et al., 1997 ; Morsica et al., 1999 ; Roque-Afonso et al., 1999 ; Roque-Afonso et al., 2005 ; Di Liberto et al., 2006 ; Ducoulombier et al., 2004 ; Inokuchi et al., 2009; Durand et al., 2010; Ito et al., 2010; Sarhan et al., 2012) highlighting that HCV replicates within PBMCs. Taken together, these studies also suggested that B cells and dendritic cells were more prompt to viral infection, by harboring a higher viral load than CD8+ and CD4+ T cells.

Consistently, a significant presence of HCV viral proteins in perihepatic lymph nodes *in vivo* (Pal et al., 2005) or in peripheral blood B cells (Ito et al., 2010) has also been observed. Moreover, in patients with persistent HCV infection, NHLs were shown to be more prevalent in patients with B cell infection (Inokuchi et al., 2009). Finally, a recent study also reported that as a small fraction of serum-derived viral particles were able to slightly infect T cell lines *in vitro* (Sarhan et al., 2012).

These evidences highly suggest that the PBMCs may represent a circulating reservoir for HCV, thus raising important clinical implications for patient treatments. Indeed,

it has been shown that patients who developed a strong sustainable viral response (SVR) after IFN-α treatment still harbored persistent viral RNA within lymphocytes and monocytes (Pham et al., 2004), suggesting that PBMCs infection may constitute an important factor of viral persistence and immune escape.

II.   Compartmentalization and viral determinants

As several evidences pointed out the possible infection of PBMCs by HCV, it has been rapidly hypothesized that PBMCs infection could be correlated to the presence of specific genetic features of PBMCs-derived variants, highlighting the potential specialization of these variants to the PBMCs reservoir. Indeed, several studies pointed out the presence of a non-random genetic distribution of HCV variants deriving from PBMCs and serum compartments. The RNA-dependent RNA-polymerase (RdRp) is highly error prone and is responsible for an important intra-individual HCV variants diversity (Clementi et al., 2003; Pawlotsky, 2006). The genetic profile of these variants, called quasi-species, likely constitutes a hallmark of the virus adaptation to its host. Indeed, this genetic variability may contribute *in vivo* to the appearance of specific quasi-species able to infect lymphocytes, via the selection of specific genetic properties.

Several studies reported the presence of a non-random phylogenetic distribution of HCV quasi-species deriving from PBMCs and serum compartment (Figure 44). Indeed, sequencing of HVR1 sequences deriving from patient serum- and PBMCs-derived HCV RNA demonstrated a genetic compartimentalization of this E2 domain between these two compartments (Roque-Afonso et al., 1999; Ducoulombier et al., 2004; Schramm et al., 2008). HVR1 is highly exposed to the host neutralizing response and is an important hallmark of the virus adaptation to its environment. Thus, the genetic compartmentalization of this sequence may suggest that this region harbors genetic adaptation features to lymphotropism.

Moreover, compartmentalization of HCV IRES between serum and PBMCs compartments has also been reported (Laporte et al., 2003; Roque-Afonso et al., 2005; Di liberto et al., 2006 ; Durand et al., 2010) suggesting a translational specialization of HCV variants for lymphocytes (Figure 44). Indeed, such specialization of IRES-deriving lymphotropic quasi-species was demonstrated *in vitro* in B cell lines (Durand et al., 2010). IRES-deriving lymphotropic variants were shown to have a lower translational efficiency in hepatocytes than plasma-derived IRES, suggesting a virus adaptation for polyprotein translation in PBMCs.

Altogether, genetic compartimentalization and translational specialization of PBMCs-deriving variants strongly support the idea that PBMCs likely represent a viral recipient in chronically infected patients, colonized by specific HCV variants.

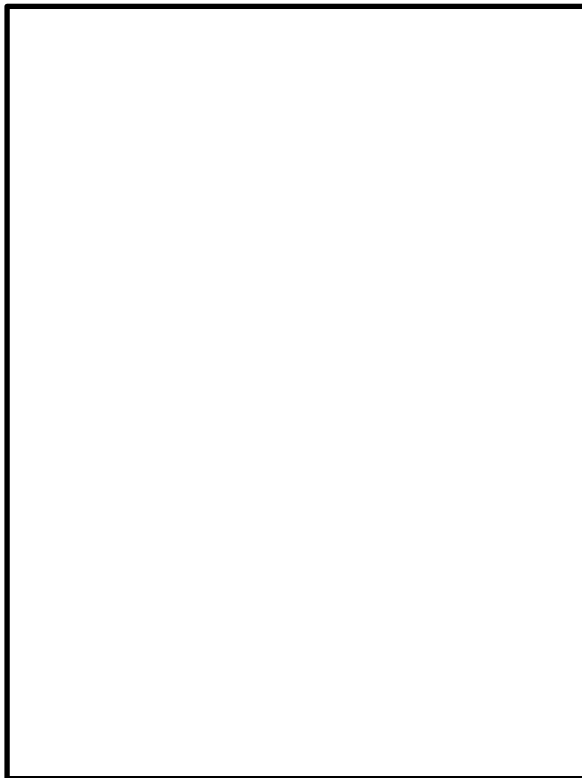

Figure 44. Phylogenetic analysis of the 5'UTR of the plasma and PBMC-derived HCV variants in a chronically infected patient. HCV RNA was amplified between nucleotides 100 to 350 in plasma and PBMC and then cloned (Roque-Afonso et al., 2005).

III. HCV infection of PBMCs:
An object of debate?

Despite these evidences, infection of PBMCs by HCV remains a matter of debate. Indeed, several studies reported the unability of the JFH-1 HCVcc virus to mediate infection of several PBMC cell types (Marukian et al., 2008). Another study also showed that the JFH-1 virus failed to establish a productive infection in B cells (Stamataki et al., 2009). In contrast, authors showed that JFH-1 binds and use B cells as vehicles. B-cell-associated viruses presented an enhanced infectivity compared with cell-free viruses, suggesting that B cells-HCV association stimulates virus spread. Moreover, this study also provides a mechanism for B cell retention in the infected liver (Stamataki et al., 2009). B-cells express the viral receptors CD81, SR-BI, and the C-type lectins DC-SIGN and L-SIGN and use of antibodies targeting SR-BI and DC-SIGN/L-SIGN has demonstrated the involvement of these three surface molecules in HCV-B cells association.

In strong contrast to these studies, another study reported than even though JFH-1 virus is not able to establish a productive infection of PBMCs, serum-derived HCV particles are (Sarhan et al., 2012). Thus, JFH-1 unability to establish a productive infection in PBMCs would not necessarily mean that HCV does not infect PBMCs. This highlights the fact that the JFH-1 virus is not a suitable virus for the study of HCV lymphotropism, as it probably does not harbor particular genetic features that may characterize lymphotropic variants.

Alternative models have been developed to both assess and study HCV lymphotropism. A previous study reported the development of a human T-cell leukemia virus type 1-infected cell line, MT-2C, that supports the replication of particular serum-derived HCV strains (Mizutani et al., 1996). This cell line allowed the isolation of a lymphotropic variant (named H1-2) that was able to infect chimpanzee PBMCs *in vivo* but not hepatocytes (Shimizu et al., 1997). Another study reported the establishment of a B cell line isolated from a HCV-infected patient non-Hodgkin's B-cell lymphoma (Sung et al., 2003). This cell line was shown to replicate

and produce a particular HCV strain, called "SB", that was able to infect hepatocytes but also PBMCs and B lymphocyte cell lines (Sung et al., 2003). However, the H1-2 and SB lymphotropic viruses remain controversial models. Indeed, no *de novo* production of viral particles from PBMCs following virus infection *in vitro* has been observed. Moreover, the genetic properties of these strains as well as their origins remain unclear.

In consequence, the correct assessment and understanding of HCV lymphotropism is today highly dependent on the development of a proper and well characterized virus cell culture system *in vitro.* As the lymphotropism appears to be determined by particular genetic specificities, *t*he development of this system necessarily requires the identification of such genetic features. The important heterogeneity of serum-derived HCV particles, dependent on lipoprotein association, viral load and patient characteristics, makes of these particles an unproper model to study molecular features of HCV lymphotropism.

IV. Significance of PBMCs infection

Clinical significances of PBMCs infection are multiple. Although some are well characterized and address how HCV deregulates PBMCs normal functions, other clinical significances remain unclear and matter of debates.

1. HCV-induced B-cell lymphoproliferative disorders

B-cell Lymphoproliferative disorders are thought to be induced and maintained by a large panel of events, such as i) chronic and continuous antigen stimulation of lymphocyte receptors by viral antigens (such as direct interaction between HCV-E2 and B-cells receptors), ii) HCV infection and replication within B-cells and iii) B-cell genetic damages (as mutations of tumor suppressor genes) caused by transient B-cell infection cells (Forghieri et al., 2012; Peveling-Oberhag et al., 2013).

Among these mechanisms, E2 interaction with B-cell tetraspanin CD81 as a critical inducer of B-cell lymphoproliferation has been intensively studied. Indeed, this

interaction has been shown to enhance expression of activation-induced cytidine deaminase (AID) and stimulate DNA breaks (Machida et al., 2005), thus leading potentially to HCV-associated B-cell lymphoproliferative disorder. Other reports showed that E2-CD81 binding triggers the activation of several mitogen-activated protein kinase (MAPK) pathways that are commonly associated with cellular lymphoproliferation disorders: the c-Jun amino-terminal kinases (JNKs) (Rosa et al., 2005), the extracellular signal-regulated kinases (ERKs) pathway and the p38 MAPK pathway (Ogasawara et al., 2003; Zhao et al., 2005; Zhao et al., 2006). Consistently, E2-CD81 interaction has also been shown to enhance the expression of anti-apoptosis Bcl2 family proteins and thus, protect B cells from Fas-mediated apoptosis (Chen et al., 2011).

In addition to E2-CD8 binding induced-lymphoproliferative disorders, direct HCV infection core has also been shown to induce B cell proliferation, via the induction of interleukin-6 production (Feldmann et al., 2006)

2. Immune escape and persistent infection

Another important implication of PBMCs infection is the viral persistence. Indeed, it has been shown that HCV RNA can be found in lymphocytes and monocytes after efficient sustainable viral response (Pham et al., 2004). Moreover, PBMCs compartment can constitute a recipient for less fitted virus in order to escape host immune responses. Consistently, genotype 2 and 3 are known to be more sensitive to IFN response than genotype 1 viruses (Chayama et al., 2011). PBMCs can thus represent an option for these variants to persist in chronically infected patients treated by IFN. Presence of genetically specialized variants in the PBMCs compartment is consistent with this idea. Indeed, HVR1 sequences isolated from serum and PBMC-derived variants have been shown to be compartmentalized in chronic patients. As this E2 region is the hallmark of the variants adaptation of the host neutralizing response, a strong genetic compartimentalization likely means that PBMCs variants are specifically adapted to an environment that does not undergo a similar immune pressure (Roque-Afonso et al., 1999; Ducoulombier et al., 2004; Schramm et al., 2008). Thus, PBMCs likely represent an independent secondary

reservoir for HCV, containing specialized variants that could contribute to an independent viral persistence and immune escape. Deregulation of PBMCs immune functions and induction of lymphoproliferative disorders could also contribute to the persistence of these specialized variants in chronically infected patients, by promoting serum-derived variants replication in liver tissues.

3. Implications for liver re-infection

Contrasting with the idea that PBMCs may represent an independent viral reservoir, the ability of the PBMC recipient to constitute an additional source of HCV variants able to re-infect liver tissues after liver transplantation (or sustainable viral response) have been questioned. Despite the observation that immune cells rapidly colonize the liver after transplantation (Navarro et al., 2000; Garcia-Retortillo et al., 2002), it has been shown that extrahepatic contribution to liver re-infection is very low and that serum-derived strains are responsible of the liver graft re-infection (Laskus et al., 2002; Di liberto et al., 2005; Dahari et al., 2005; Gray et al., 2012). Indeed, the strong specialization for lymphocytes of HCV IRES deriving from lymphotropic variants seems to show that PBMCs-derived variants are unable to replicate within hepatocytes, and thus, to re-infect liver transplant (Durand et al., 2010). Alternatively, one study proposed that liver graft re-infection can be due to variants deriving from both plasma and PBMCs before liver transplantation (Schramm et al., 2008), suggesting that liver graft may be re-infected by dual-tropic strains present in the patient's serum during liver transplantation.

V.   Cellular determinants

How lymphotropic variants attach and invade PBMCs remains elusive. B and T cells express two HCV major receptors, the tetraspanin CD81 and SR-B1. However, B cells do not express any of the tight junction proteins, Claudin-1 and Occludin whereas T cells only express Occludin (Figure 45). Thus, HCV likely developed an alternative mechanism to infect lymphocytes that still remains to be elucidated.

Lymphocytes express particular molecules such as the LDL-r that can play a role in the primary attachment of viral particles to the cell surface. After CD81/SR-B1 binding, internalization of viral particles may likely require additional binding step that would require alternative receptor other than Claudin-1 and/or Occludin-1. Involvement of LDL-r, CD81 and SR-B1 in HCV infection of PBMCs is supported by a recent study that showed that HCV infected patients harbor lymphocytes with an increased expression of CD81 and LDL-r, as well as monocytes with an increased expression of SR-B1 and LDL-r (Roque-Cuellar et al., 2012).

It has been shown that CD5 surface molecule promotes infection of serum-derived HCV particles from chronically infected patients (Sarhan et al., 2012). Thus, CD5 molecule could represent an alternative route for HCV variants during T cell infection. However, a better characterization of the involvement of this molecule in HCV T-cell entry now requires the use of a proper cell culture system with a defined virus. Moreover, only a small subset of B-cell harbors the CD5 surface molecule and there is no evidence that this receptor promotes HCV infection in this particular B-cell subset.

	Huh7	Raji (B)	Daudi (B)	Jurkat (T)	Molt (T)
CD81	+	+	+	+	+
SR-B1	+	+	+	+	+
Claudin-1	+	-	-	-	-
Occludin	+	-	-	+	+

Figure 45. Cell surface expression of CD81, SR-BI, Claudin-1 and Occludin in B-cell lines (Raji and Daudi) and T cell lines (Molt and Jurkat). Huh7, hepatoma cell lines permissive to HCV infection.

In parallel, the CD86 molecule has also been shown to be the cellular determinant of the SB virus entry into B cells (19th HCV International Symposium, Seattle, USA). However, the elusive origins of this strain do not allow any generalization about the involvement of this molecule in HCV B-cell entry.

Finally, the expression on lymphocytes surface of EWI-2wint (See Chapter V-II 1.c), a molecule that inhibits HCV infection through the containment of CD81 into tetraspanin-enriched area (Rocha-perugini et al., 2008; Montpellier et al., 2011; Potel et al., 2013), also likely suggests that HCV lymphotropic variants developed molecular mechanisms to escape this entry inhibition and the restricted use of CD81.

Characterization of HCV entry within lymphocytes is also highly dependent on the understanding of the entry-dependent envelope glycoproteins mechanisms. Indeed, as envelope glycoproteins represent a major determinant for HCV liver tropism, these proteins likely harbor in lymphocytes-derived variants: i) particular genetic specificities that may define HCV lymphotropism and ii) particular conformational mechanisms or domains involved in the binding to alternative entry receptors.

PART

II

RESULTS

Context & Aims

During viral entry, HCV envelope glycoproteins E1E2 mediate viral particles attachment to the cell surface, notably through binding to SR-BI and CD81 (Pileri et al., 1998; Scarselli et al., 2002). Furthermore, E1 and E2 are thought to induce the fusion between the endosomal membrane and the virus membrane, allowing the release of the viral RNA into the host cytosol. Although glycoprotein-mediated entry mechanisms of most of the *Flaviviridae* are well known, HCV-E1E2-mediated entry mechanisms remain poorly characterized. Residues and domains within E1 or E2 have been intensively identified over the last decade to be critical for E1E2 heterodimerization, CD81/SR-BI binding or membrane fusion (Drummer et al., 2006, Drummer et al., 2007; Lavillette et al., 2007; Rusell et al., 2009 Albecka et al., 2011). However, the related studies often restricted their analysis to a single HCV glycoprotein and its impact on viral entry.

E1E2 are assembled and incorporated onto virus surface as a functional heterodimer (Op de Beeck et al., 2001). Thus, rather to be mediated by a single glycoprotein, HCV E1E2-mediated entry is likely a complex molecular process by involving several intra and inter-molecular dialogs between E1 and E2, which both mediate structural and conformational rearrangements of the whole heterodimer complex. This suggests that HCV E1E2 may represent a single protein entity rather than the association of two proteins with distinct functions. Consequently, an improved understanding of the E1E2-mediated viral entry likely require to analyze the interrelation between E1 and E2 during entry, rather than studying single E1 or E2 domains.

In this work, we aimed to characterize dialogs and interrelations between E1E2 during entry in order to better understand how HCV heterodimer mediates virus entry at the molecular level. The existence of several dialogs between E1 and E2 would highlight that these two proteins strongly co-evolve, suggesting both i) the presence of conserved mechanisms and dialogs that are critical for heterodimer function but also ii) a potential E1E2 functional plasticity allowing to acquire new

functions or conformations (Figure 46). Thus, characterization of E1E2 dialogs and interrelations likely provide an original approach to decipher E1E2-entry molecular mechanisms that remain unknown. Indeed, the E1E2-mediated fusion process is poorly characterized. The putative HCV fusion protein has not been identified and the E1E2-related structural rearrangements remain unclear. Thus, as fusion is a conserved mechanism, identification of E1E2 intermolecular dialogs during this process could provide important inputs in our understanding of HCV fusion.

The recent development of direct acting agents (DAAs) against HCV infection has led to serious progress in the treatment of HCV infection. However, these DAAs that target HCV proteases present some limitations. Their use as combinative agents with unspecific and side-effects inducer treatments is required to avoid the emergence of resistant variants (Reesink et al., 2006; Salloum et al., 2012), thus highlighting a need of multitherapy strategies that would target different step of the virus life cycle. As HCV entry represents the first steps of viral infection, it may constitute a promising target for patient treatment. However, the lack of information concerning potential E1E2 conserved molecular mechanisms or structural epitopes hamper the development of such therapeutic option. Thus, identification of potential conserved E1E2 dialog critical for virus entry may represent a promising option to pave the way toward the development of virus-specific entry-inhibitors.
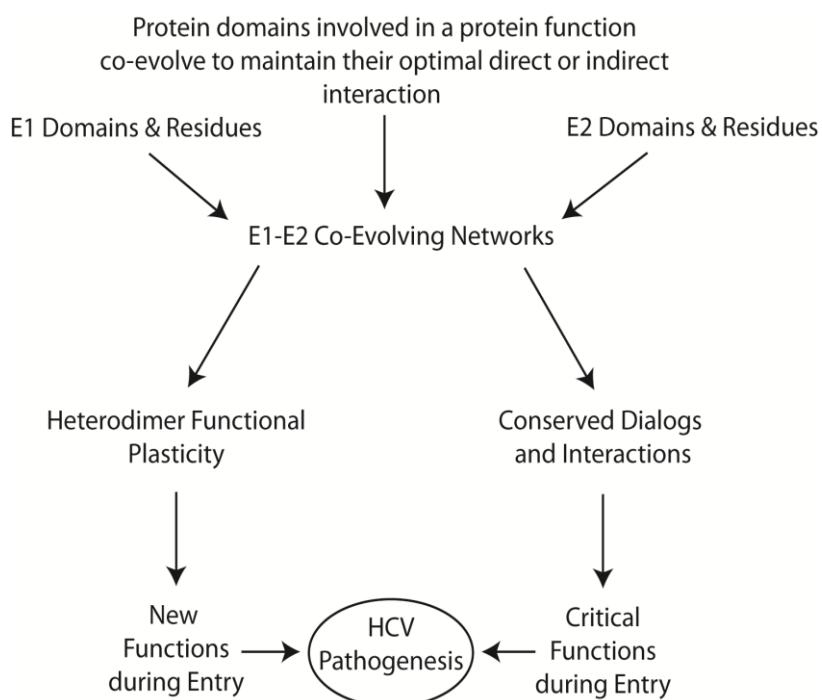

Figure 46. Schematic representation of the assumptions and aims of the thesis. E1 and E2 domains are likely involved in several co-evolution networks. These networks suggest the existence of a functional plasticity of the heterodimer, as well as the presence of conserved dialogs within E1E2 that are critical for HCV entry.

Summary of the findings

In this work, we were able to show that E1 and E2 strongly co-evolve and mediate together critical HCV functions. We highlighted several E1E2 dialogs, interrelations and structural rearrangements that govern HCV entry as well as virus fusion (Figure 47). Our results pointed out a fusogenic conformational change mediated by particular E1E2 dialogs. ████████████████████████████████████

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I. Strong specialization of HCV envelope glycoproteins is associated with the establishment of B-lymphocyte reservoirs in chronically infected patients

This first chapter showed that E1E2 harbor an important functional plasticity likely able to mediate the appearance of specific E1E2 functions that are benefic for HCV pathogenesis and viral spread. Indeed, we showed that a strong E1E2 divergence between serum-derived variants and B-cells derived variants highlights a specialization of B-cell-derived variants through preferential infection of B-cells in contrast to hepatocytes. These results demonstrated that E1E2 plasticity may have important clinical implications, by providing viral persistence in extrahepatic recipient in chronically infected patients (Figure 47).

II. A critical interaction between E1 and E2 glycoproteins determines binding and fusion properties of Hepatitis C Virus during cell entry

In this chapter, we identified a critical dialog between E1 and the domain III of E2 in genotype 1 envelope. This dialog was critical for virus entry (Figure 47), by mediating both virus binding and fusion. Our results underlined that the domain III (DIII) of E2 is potentially involved in fusogenic conformational changes, such as the domain III of flavivirus E.

III. HCV E1 and E2 co-evolving networks unveil their structural organization and suggest an unusual membrane fusion mechanism

In this chapter, we analyzed through a computational method the co-evolving networks that exist between E1 and E2. This method demonstrated that E1 and E2 strongly co-evolve and suggested a potential ability of E2 to fold over via its domain III, consistently with our previous findings. We also observed the conservation of the E1-E2 DIII dialog among genotypes, suggesting its critical importance for virus fusion (Figure 47).

CHAPTER I

Strong specialization of HCV envelope glycoproteins is associated with the establishment of B-lymphocyte reservoirs in chronically infected patients

Douam et al,

In Preparation

CHAPTER II
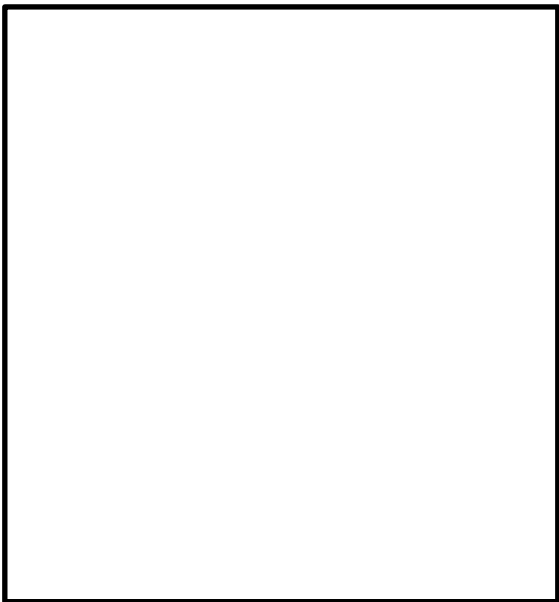
A critical interaction between E1 and E2 glycoproteins determines binding and fusion properties of Hepatitis C Virus during cell entry

Florian Douam, Viet Loan Dao Thi, Guillemette Maurin , Judith Fresquet , Dimitri Mompelat, François-Loïc Cosset and Dimitri Lavillette

Abstract

Hepatitis C virus (HCV) envelope glycoproteins E1 and E2 are important mediators for productive cell entry. However, knowledge about their structure, intra- or intermolecular dialogs and conformational changes is scarce, limiting the design of therapeutic strategies targeting E1E2. Here, we sought to investigate how certain domains of E1 and E2 have co-evolved to optimize their interactions to promote efficient HCV entry. For this purpose, we generated chimeric E1E2 heterodimers generated from two HCV 1a strains to identify and characterize cross-talks between their domains. We found an E1E2 combination that drastically impaired the infectivity of cell culture-derived HCV particles whereas the reciprocal E1E2 combination led to increased infectivity. Using HCV pseudoparticle assays, we confirmed the opposing entry phenotypes of these heterodimers. By mutagenesis analysis, we identified a particular cross talk between three amino acids of E1 and the domain III of E2. Its modulation leads to either a full restoration of the functionality of the sub-optimal heterodimer or a destabilization of the functional heterodimer. Interestingly, we found that this cross-talk modulates E1E2 binding to HCV entry receptors SR-BI and CD81. In addition, we also found for the first time that E1E2 complexes can interact with the first extracellular loop of Claudin-1 whereas soluble E2 did not. These results highlight the critical role of E1 in the modulation of HCV binding to receptors. Finally, we demonstrated that this cross-talk is involved in membrane fusion. Altogether, these results reveal a multi-functional and crucial interaction between E1 and E2 for HCV entry into cells. Our study highlights the role of E1 as a modulator of HCV binding to receptors and membrane fusion underlining its potential as an antiviral target.

Introduction

HCV cell entry is a multi-step process that is mediated by virus surface components, such as HCV E1E2 envelope glycoproteins and viral associated lipoproteins [1]. During the early steps of virus entry, the E1E2 complexes directly interact with some entry factors such as the scavenger receptor class B type 1 (SR-BI) [2] and the tetraspanin CD81 [3]. The Claudin-1 [4], Occludin [5] tight junction proteins and the epidermal growth factor receptor (EGFR) [6] are also known to be critical for HCV entry and internalization but no direct interaction with E1E2 heterodimers have been demonstrated so far. During entry, the E1 and E2 glycoproteins are thought to undergo coordinated and fine-tuned conformational changes until membrane fusion, i.e., the merging of the viral and endosomal membranes, occurs[1]. However, how E1 and E2 cooperate during HCV entry remains largely undefined. Studying the role of different domains of E1 and E2 and their temporal engagement during virus entry remains challenging and may reveal interesting information for the development of novel entry inhibitors.

The recent identification of E2 intra-molecular disulfide bonds allowed the development of an E2 structural model that presents characteristics of a typical class II fusion protein like the E glycoprotein of flaviviruses [7], which possesses both binding and fusion properties. Accordingly, E2 functions include binding to CD81 [3, 8, 9] and SR-BI [2, 10] and some domains involved in membrane fusion have been proposed [10, 11]. Conversely, the role of E1 during HCV cell entry remains poorly understood. For Flaviviruses, the E companion protein, PrM, cleaved and incorporated onto viral particles as M protein, acts mainly as a chaperone protein with no reported active role during virus entry [12]. However, no cleavage of E1 has been reported despite its size similar to PrM, which strongly suggests that E1 acts differently than PrM/M. Consistently, some segments of E1 located in both its ecto- [10, 11] and trans-membrane domains [13] were found to play a role during post-binding events. Moreover, both E1 and E2 have a chaperone activity essential for

correct folding of the heterodimer complex [14]. Altogether, the entry model of HCV seems quite original in its family.

In this study, we aimed at identifying the nature of the interrelations between E1 and E2 domains during HCV entry. Although most functional studies addressing the role of E1E2 focused on conserved residues, recent findings demonstrated importance of variable domains during entry [2,9,15,16]. Our working hypothesis was that a conserved protein function that involves different segments requires the co-evolution of variable domains in order to maintain the most optimal direct or indirect interaction (then qualified as"cross-talk" or "dialog") between these domains. We first generated a non-optimal chimeric E1E2 heterodimer in order to subsequently restore its full functionality, thereby, identify residues of E1E2 involved in cross-talks critical for efficient cell entry. The functional characterization of this interaction indicated that a specific E1E2 dialog is involved in binding to HCV receptors and membrane fusion, highlighting a multifunctional role of E1 in entry.

Methods

Cell lines and antibodies – Human Huh-7.5 (kind gift of C. Rice), BRL3A rat hepatoma (ATCC CRL-1442) and 293T kidney (ATCC CRL-1573) cells were grown in DMEM supplemented with 10% fetal bovine serum (Invitrogen). The rat anti-E2 clone 3/11 [8], the mouse anti-HCV E1 clone A4 [17] and the conformational mouse anti-HCV E2 H53 [17] or human anti-E2 AR3A [18] antibodies are kind gifts from J. McKeating (University of Birmingham, UK), H. Greenberg (Stanford University, CA, USA), J. Dubuisson (Institut Pasteur Lille, FR) and M. Law and D. Burton (Scripps Research Institute, La Jolla, USA) respectively. MLV capsid was detected by a goat anti-MLV p30 antibody (Viromed). HCVcc foci forming units were stained with a mouse anti-HCV NS5A antibody 9E10 [19] (kind gift of C. Rice,Rockefeller University, NY, USA). The rat anti-human Claudin-1 mAb OM-7D3-B3 [20] and the mouse anti-human SR-BI mAb NK8H5E3 [21] have been described previously.

HCVpp production, infection and characterization. Chimeric E1E2 heterodimers were constructed by molecular cloning between the genotype 1a envelope H77 (AF009606) and a genotype 1a envelope A40 (Maurin et al. unpublished results). HCVpp were produced and used to infect Huh-7.5 cells as previously described [16, 22]. GFP positive infected cells were quantified by FACS Canto II (BD Biosciences). Transfected 293T cells were lysed as previously described[16] and pseudoparticles were purified by ultracentrifugation at 82,000xg for 1h 45 min through 1.5 ml a 20% sucrose cushion. Cell lysates and viral pellets were subjected to western blot analysis using 3/11, A4 and an anti-MLV-CA antibody as described previously [16, 22]. Immunoprecipitation assays were performed as described previously [16] using AR3A antibody.

HCVcc production, infection and quantification - Plasmid pFK H77/JFH1/HQL displaying adaptive mutations (Y835H (in NS2), K1402Q (in NS3), and V2440L (in NS5A)) that enhance production and infectivity of HCVcc particles was used to generate recombinant genomes containing the same H77 HCV E1E2 sequences, parental or mutated, used for HCVpp production [2]. Derived HCV RNAs were electroporated in Huh-7.5 cells as described previously [2, 16, 19, 23]. Huh-7.5 cells were infected with different dilutions of cell culture supernatants harvested at 48h and 72h post electroporation or with intracellular viral particles obtained 72h post electroporation after 3 freeze/thaw cycles of Huh-7.5. Four days post-infection, foci forming units (FFUs) were visualized after NS5A immunostaining [16]. For neutralization assay, HCVcc viral particles were pre-incubated for 1h at room temperature with or without the soluble large extracellular loop (LEL) derived from CD81 (20µg/ml) before infections. For RT-qPCR analysis, viral RNAs were isolated from cell supernatants using Tri Reagent solution (Sigma-Aldrich) before reverse transcription and quantification as described previously [2].

Binding assays – Fifty microliters of concentrated virus (100x) was mixed with BRL, BRL-CD81, BRL-SR-BI or BRL-Claudin-1 cells in presence of 0.1% sodium azide for 1h at 37°C [2]. Bound viruses were detected using H53 mouse or AR3A human anti-

HCV E2 and fluorescence of APC secondary antibodies were quantified by a FACS Canto II[3,14]. For neutralization of binding, the different BRL cells were pre-incubated at 37°C with the mouse anti-human SR-BI mAb NK8H5E3 (20µg/ml), or with the rat anti-human Claudin-1 mAb OM-7D3-B3 (30µg/ml) or with control IgG antibodies for 30 minutes at 37°C, before adding concentrated pseudoparticles.

Liposome fusion assays - HCVpp/liposome lipid mixing was performed as previously described [10] using R18-labeled PC/Chol liposomes (R18 Molecular Probes; phosphatidylcholine and cholesterol Aventi). After pH decrease to 5, dequenching of R18 due to lipid mixing between HCVpp and liposomes were recorded on a FluoroMax-4 Spectrofluorometer (Horiba Jobin Yvon) with an excitation wavelength (λexc) at 560 nm and an emission wavelength (λem) at 590 nm.

Statistical Analysis. Statistical analyses were performed using a Student t test or Mann-Whitney test. Statistically significant differences relative to controls are denoted as *P <0.05 or **P <0.005.

Results

Identification of a non-optimal E1E2 heterodimer for cell entry

In order to identify non-conserved domains of E1 and E2 that may act together for efficient entry, we focused on E1E2 sequences from two genotype 1a strains, H77 and A40 (Figure 1A) isolated from two different HCV-infected patients. We generated two chimeric heterodimers by swapping either E1 or E2 sequences between these two strains (E1H77/E2A40 and E1A40/E2H77; Figure 1A) and introduced them in cell culture-derived HCV (HCVcc) particles.
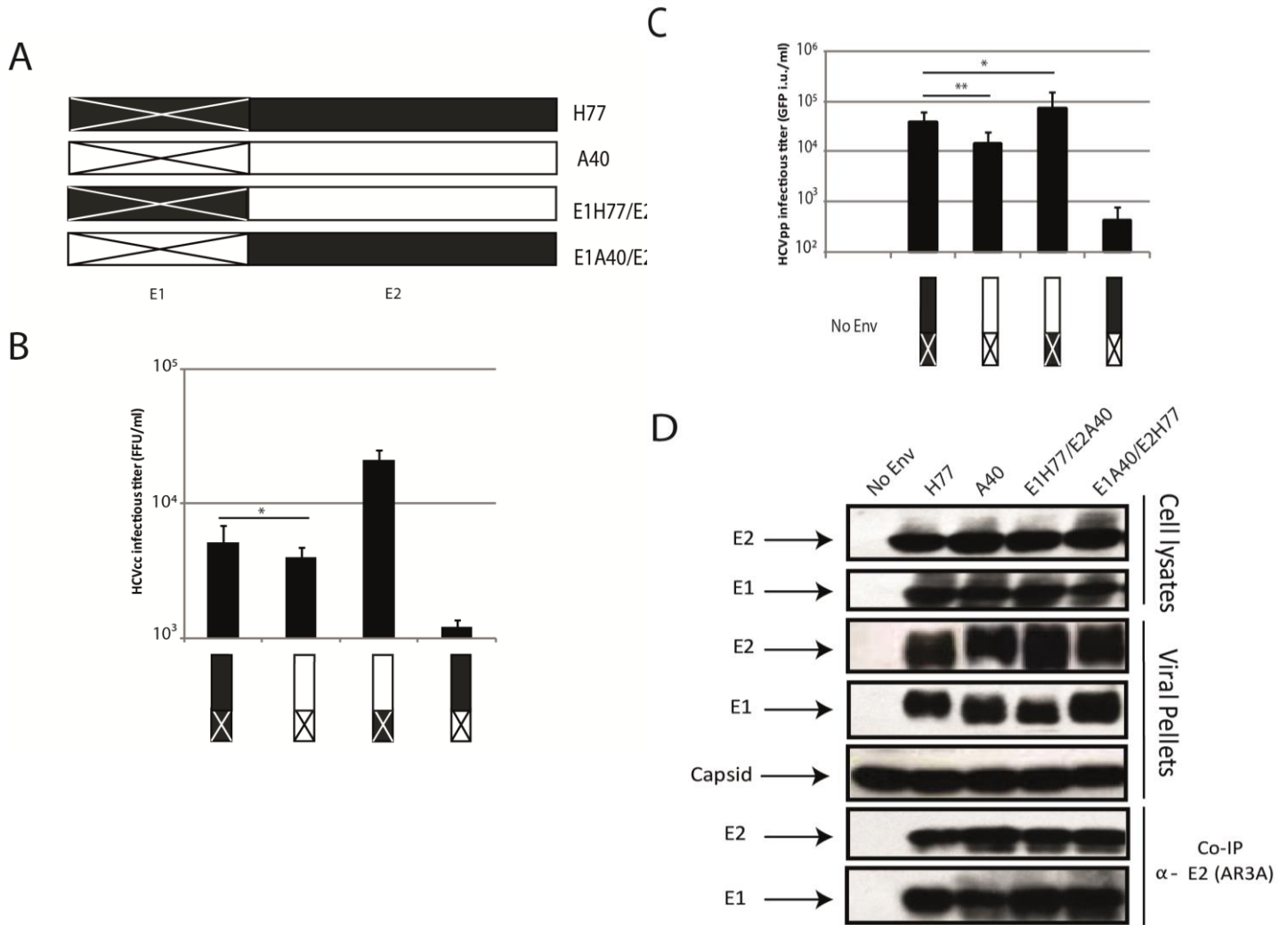
Figure 1. Identification of E1E2 conformations modulating HCV entry. A. Schematic representation of H77, A40 and of the E1H77/E2A40 and E1A40/E2H77 heterologous heterodimer. **B.** HCVcc particles (JFH-1) harboring H77, A40, E1H77/E2A40 or E1A40/E2H77 envelope glycoproteins were quantified by qRT-PCR to determine the HCV RNA GE (genome equivalent) levels in the cell culture supernatants of electroporated Huh-7.5 cells. Equivalent amount (10^5 HCV GE) of HCVcc particles were used to infect naïve Huh-7.5 cells. Infected cells were fixed and numbers of foci were counted 4 days post infection. Results are expressed as foci-forming unit per ml (ffu/mL) (mean±SD; n=4). *p < 0.05 **C.** HCV entry assays using HCVpp harboring H77, a40, E1H77/E2A40 or E1A40/E2H77 heterodimers E1E2. Huh-7.5 cells were inoculated with HCVpp harboring the indicated heterodimers E1E2 or no glycoprotein (no Env) and infectivity titers were determined 72 h after inoculation. Results represent average infectious titers, expressed as infectious unit (IU) per ml (mean±SD; n=4). *p < 0.05 ** p < 0.005. **D.** Expression in transfected 293T cells (top) and incorporation on concentrated pseudoparticles (middle) of H77, A40, E1H77/E2A40 or E1A40/E2H77 heterodimers analyzed by Western Blot using AR3A to detect E2, A4 to detect E1 and p30 to detect MLV-CA. Folding and heterodimerization of E1 and E2 glycoproteins on HCVpp were analyzed by co-immunoprecipitation with the AR3A antibody (bottom), which recognizes a conformational epitope on E2, followed by Western blot detection of pellets using anti-E1 antibody (A4).

After infection with equal quantity of physical particles measured by qRT-PCR, the titer of HCVcc harboring the E1H77/E2A40 heterodimer (then qualified as "over optimal") was higher in comparison to the one of H77 HCVcc (4 fold) whereas the titer of HCVcc harboring the mirror heterodimer E1A40/E2H77 ("sub optimal") was reduced (4 fold) (Figure 1B).

Next, to assess whether these different E1E2 combinations affect specifically HCVcc cell entry properties, we derived HCV pseudo-particles (HCVpp) and infected Huh-7.5 cells (Figure 1C). HCVpp harboring the E1A40/E2H77 heterodimer had a 20-fold reduction in entry efficiency compared to parental E1E2, whereas HCVpp harboring the inverse chimera, E1H77/E2A40, exhibited a 2-fold increased infectivity, correlating with the results obtained with the HCVcc chimeras (Figure 1B). Western blot analysis and co-immunoprecipitation assays indicated that these differences of infectivity were not due to altered expression, heterodimerization or incorporation of E1E2 complexes onto the viral particles (Figure 1D). Altogether, these results indicated that the infectivity differences between the HCVcc particles harboring the E1H77/E2A40 and E1A40/E2H77 heterodimers were mainly due to altered or improved entry.

Three amino acids in E1 are involved in a cross-talk with E2

Aiming to restore the functionality of the sub-optimal heterodimer E1A40/E2H77, we first constructed five E1A40/E2H77 point mutant heterodimers in which each residue of E1A40 was substituted one by one by those that are different in E1H77 (Figure 2A). Biochemical analysis indicated that all heterodimers were correctly expressed in cells and similarly incorporated into HCVpp (Figure S1). We found that either of three mutations (M117I, T139A or L154M) slightly improved the cell entry of the sub-optimal E1A40/E2H77 heterodimer (Figure 2B) and that the 3M mutant, combining altogether these three mutations significantly improved the functionality of the E1A40/E2H77 heterodimer (7 fold).

This suggested that determinants encompassing these three amino acids in E1 are involved in a cross-talk with E2 important for heterodimer functionality.

Figure 2. Three amino acids in E1 are important for HCV entry. A. Amino acid alignment of E1H77 and E1A40. E1A40/E2H77 mutants were constructed by substituting amino acids of E1A40 by amino acids of E1H77. **B.** Infectivity on Huh7.5 cells of HCVpp harboring H77, A40, the heterologous heterodimers, E1 mutant heterodimers or no glycoproteins (no Env) was determined as described in Figure 1B. Results represent average infectious titers, expressed as infectious unit (IU) per ml (mean±SD; n=3).

Domain III of E2 is involved in a cross-talk with E1

As E2H77 and E2A40 have many amino acid differences, we divided E2 in functional domains (Figure 3A) according to the E2 structural model proposed by *Krey et al* [7].

Thus, starting from the E1A40/E2H77 heterodimer, we constructed different chimeras by interchanging domains of E2H77 with domains of E2A40 (Figure 3B). Biochemical analysis indicated that all chimeric heterodimers were correctly expressed and incorporated similarly into viral pseudoparticles (Figure S2). Complementation of E1A40/E2H77 by the domain III of E2A40 (named DIII) restored HCVpp entry to the same level as A40 E1E2 (Figure 3B). Other domain substitutions decreased HCVpp infectivity (HVR1, Domain I, Domain II and IgVr) or did not have any significant effect (Stem Region) on heterodimer functionality. These results indicated that domain III of E2 plays an important role in the function of the E1E2 complex during cell entry.

Identified domains are important for E1E2 function during HCVcc entry

To confirm our results using HCVcc, we generated recombinant HCV genomes harboring the E1E2 chimeric heterodimers described above (Figures 2 and 3). Equivalent amounts of viral RNAs were electroporated and subsequently quantified in cell supernatants at 48hrs and 72hrs post electroporation. All viral genomes replicated in comparison to GND replication-defective HCV genome (Figure 4A). Viral particles harboring 3M and DIII heterodimers were correctly produced, showed no defect in viral release compared to control wild type and chimeric heterodimers (Figure 4A), and were all infectious compared to GND replication-defective and ΔE1E2 entry defective mutants (Figure 4B). Viral particles harboring 3M and DIII heterodimers also presented an improved infectivity in comparison to viral particles harboring sub-optimal E1A40/E2H77 heterodimers (Figure 4B). Comparison of intracellular and extracellular infectivity of HCVcc indicated that E1E2 changes did not affect budding of viral particles (Figure 4C). Based on the quantification of viral particle RNAs and on their infectivity, we then calculated the specific infectivity of viral particles harboring 3M and DIII heterodimer and confirmed that, similarly to HCVpp infectivity, HCVcc harboring the 3M or DIII heterodimers presented a restored cell entry function (Figure 4D).
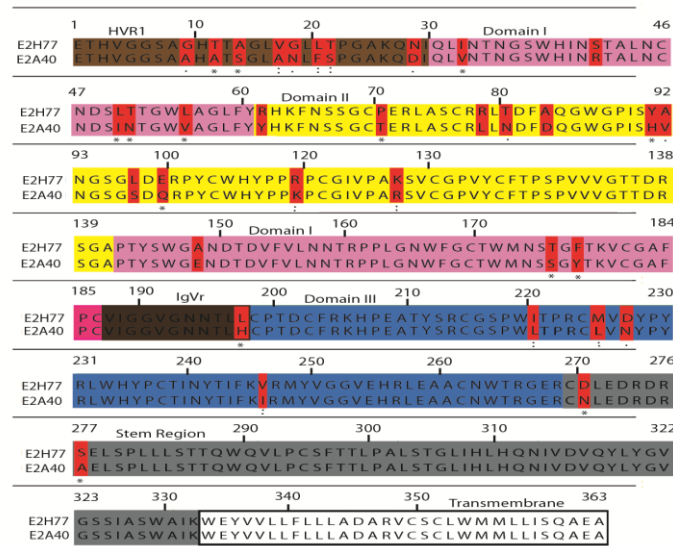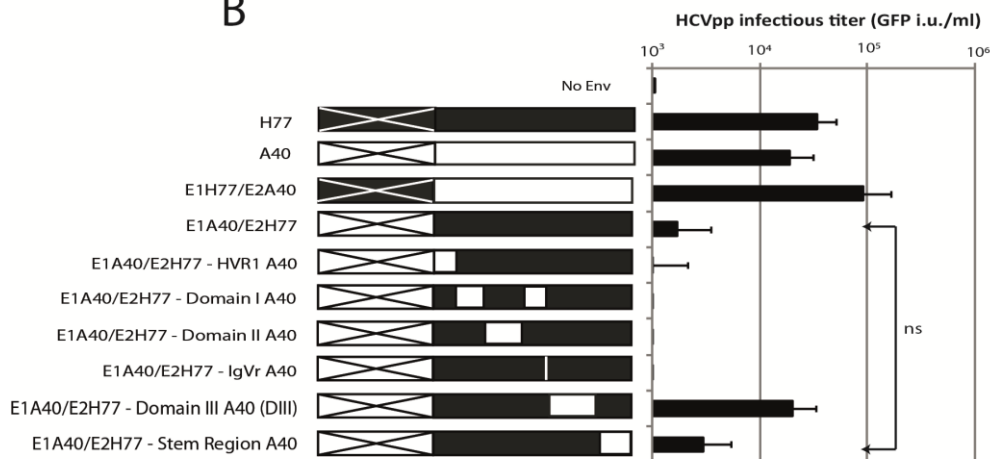
Figure 3. Domain III of HCV E2 modulates viral entry. A. Amino acid alignment of E2H77 and E2A40. Proteins were divided in functional domains according to the E2 structure model proposed by *Krey et al* [12]. Chimeric E1A40/E2H77 heterodimers were constructed by substituting domains of E2H77 by domains of E2A40. **B.** Infectivity on Huh-7.5 cells of HCVpp harboring H77, A40, the heterologous heterodimers, E2 chimeric heterodimers or no glycoproteins (no Env) was determined as described in Figure 1B. Results represent average infectious titers, expressed as infectious unit (IU) per ml (mean±SD; n=3). ns = not statistically significant.

These observations were confirmed by HCVcc infection assays using equal amount of viral particles (data not shown). Altogether, these data demonstrate that the identified amino acids in E1 and DIII in E2 are important for the functionality of E1E2 complex during HCVcc entry.
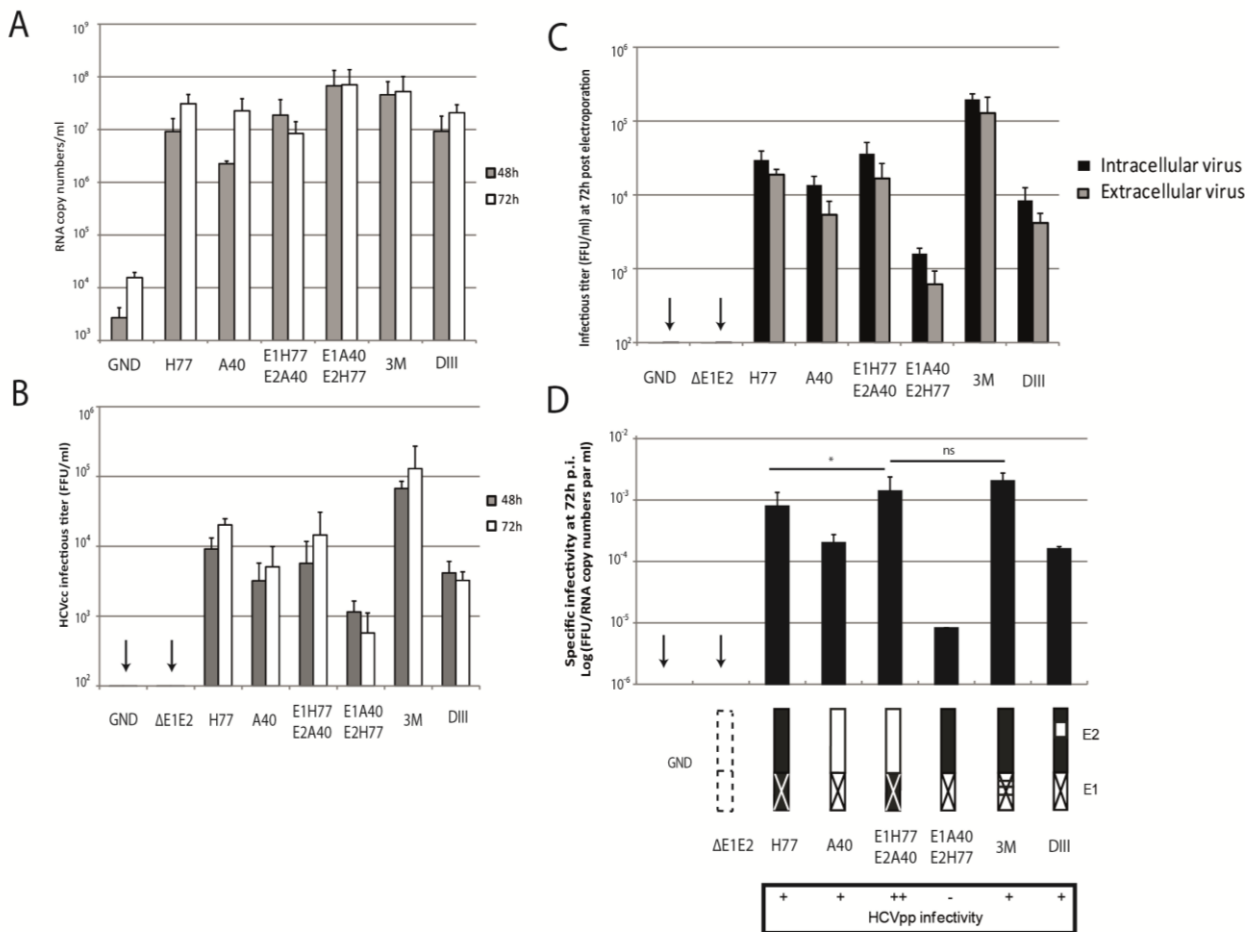
Figure 4. Identified E1 amino acids and DIII of E2 are important for HCVcc entry. HCVcc particles harboring the different E1E2 heterodimers or encoding for a replication (GND) defective genome were harvested at 48 h and 72h after electroporation of Huh7.5 cells and analyzed from filtered cell culture supernatants for HCV RNA GE (genome equivalent) levels determined by qRT-PCR (**A**) and for HCV infectivity by titration (**B**) on naive Huh7.5 cells expressed as foci-forming unit per ml (ffu/mL) (mean±SD; n=3). A genome deleted for E1 and E2 (ΔE1E2) was used as a negative control of infectivity **C.** Intracellular and extracellular infectious titer of HCVcc viral particles harboring the different E1E2 heterodimers at 72h after electroporation. Viral particles were harvested either from cell supernatant (extracellular infectivity) or after disruption of electroporated cell membranes (intracellular infectivity) and titrated on naive Huh-7.5 cells. Results are expressed as foci-forming unit per ml (ffu/mL) (mean±SD; n=3) **D.** Specific infectivity of HCVcc viral particles harboring the different E1E2 heterodimers was calculated as infectivity per HCV RNA GE at 72h post electroporation. For each envelope, the corresponding HCVpp infectivity is indicated below (mean±SD; n=4). *$p < 0.05$, ns = not statistically significant.

Identified amino acids in E1 are involved in a common cross-talk with E2

To address whether the three amino acids identified in E1 and DIII of E2 are partners of a cross-talk, we constructed H77 E1E2 mutants by substituting the three identified E1 amino acids (3M), the domain III of E2 (DIII), or both, by those of A40 E1E2

sequence. We first demonstrated that all heterodimers were expressed and incorporated onto viral particles in a similar manner (Figure S3). Mutations in E1 H77 (#1) dramatically affected HCVpp infectivity (Figure 5). In contrast, substitution of DIII of H77 by that of A40 (#2) increased entry to a level similar to that of E1H77/E2A40 (Figure 5). Substitution of 3M and DIII simultaneously in H77 heterodimer (#3) restored infectivity of HCVpp to a level similar to H77 HCVpp (Figure 5) indicating that the cross-talk helps the regulation of E1E2 function. Same phenotype modulations were observed by mutating A40 heterodimer. Insertion of the three amino acids from E1 H77 over-optimized the A40 heterodimer for entry (#4). This over-optimization was conserved when E2A40 is exchanged with E2H77 harboring the DIII of A40 (#5). Altogether, these results underlined that the three identified amino acids in E1 and DIII of E2 are likely involved in a cross-talk essential for E1E2 conformation and/or virus cell entry.
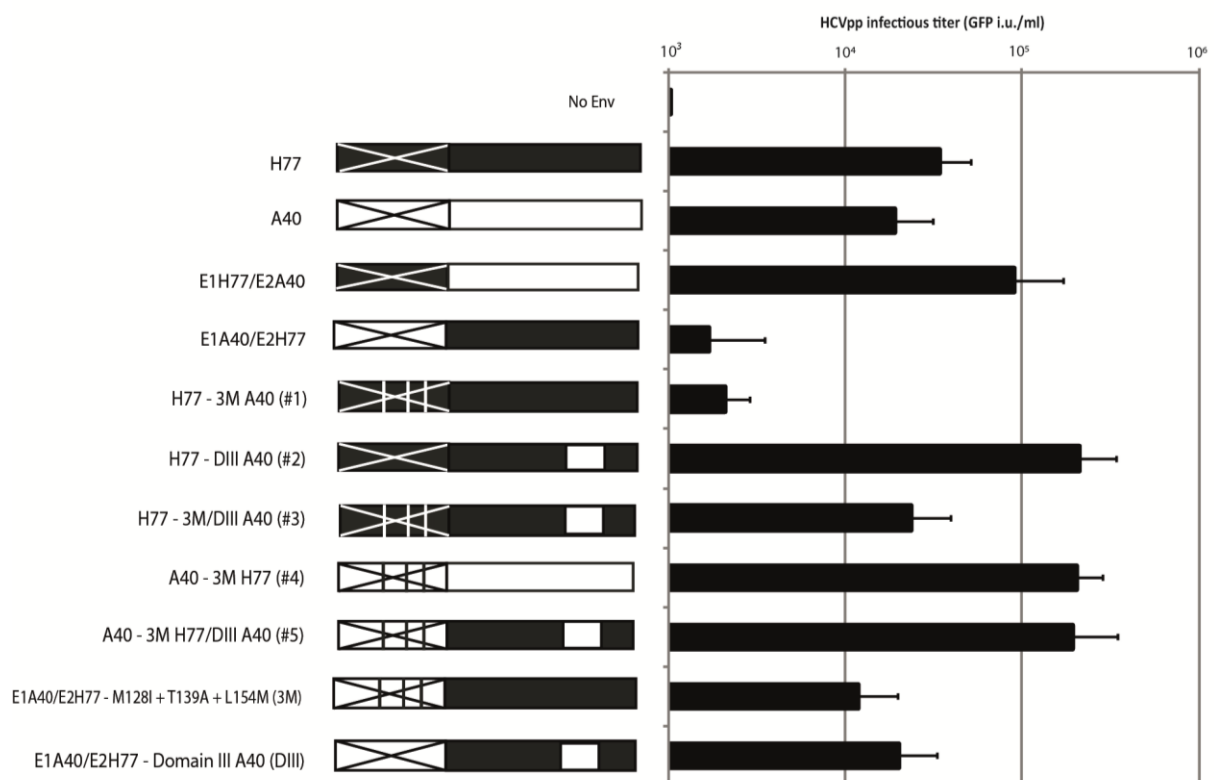

Figure 5. Identified amino acids in E1 and domain III of E2 are involved in a common cross talk. Infectivity on Huh7.5 cells of HCVpp harboring indicated E1E2 envelopes or no glycoproteins (no Env pp) was determined as described in Figure 1B. Results represent average infectious titers, expressed as infectious unit (IU) per ml (mean±SD; n=3).

Identified cross-talk participates in the modulation of binding to CD81 and SR-BI

We next analyzed at which step of the entry process could act the proposed E1E2 cross-talk. To address the possibility that it may modulate the interaction of E1E2 with HCV receptors, we performed HCVpp binding assays on BRL3A rat hepatocarcinoma cells expressing human CD81, SR-BI or Claudin-1 (Figure S4A). The loss of binding of HCVpp harboring a H77 heterodimer mutated in the CD81 binding domain on BRL-CD81 cells (Figure 6C and S4B) and the inhibition of HCVpp binding by anti-SR-BI antibody [21] on BRL-hSR-BI cells (Figure 6D) confirmed the specificity of the HCVpp binding assays. Similar amount of HCVpp were used in all assays as measured by the quantity of capsid and E2 by Western blot (Figure S4C). All binding assays were also confirmed with another anti-E2 antibody[18] (AR3A) suggesting that the differences in binding assays did not reflect affinity differences (data not shown). Interestingly, HCVpp harboring 3M and DIII mutations presented an improved binding to CD81 and SR-BI as compared to HCVpp harboring sub optimal E1A40/E2H77 heterodimer which displayed a weaker binding to these receptors (Figure 6A, B, S5A, B). These results suggested that the 3M/DIII cross-talk modulates E1E2 binding to HCV receptors. Exposure of the viral CD81 binding site was also compared between chimeras by another complementary approach [9] employing infection inhibition of HCVcc by soluble CD81 large extracellular loop (CD81-LEL). Overall, the CD81-LEL inhibition pattern of the HCVcc harboring the different chimera (Figure 6E) was similar to the pattern of HCVpp binding on BRL-CD81 (Figure 6A). Consistently, HCVcc harboring DIII mutation were less neutralized than HCVcc harboring sub optimal E1A40/E2H77. The differences with HCVcc harboring 3M mutations were however not significant (p>0.1). These results suggest that the 3M/DIII cross-talk can participate to the uncovering of the viral CD81 binding site. However, there is no strict correlation between entry and binding phenotypes (Figure 6A, B, Table 1) which indicates that other E1E2 interactions play a role within this cross-talk to modulate the binding to CD81 and/or SR-BI receptors.
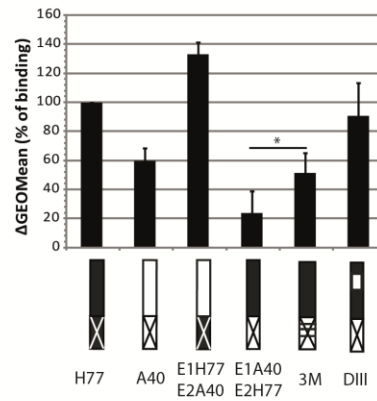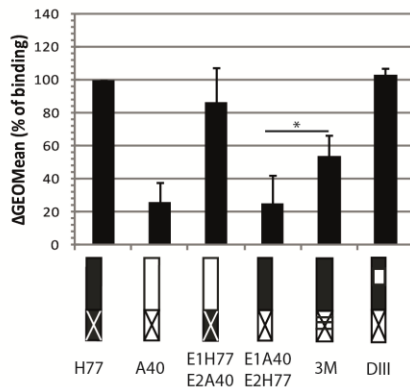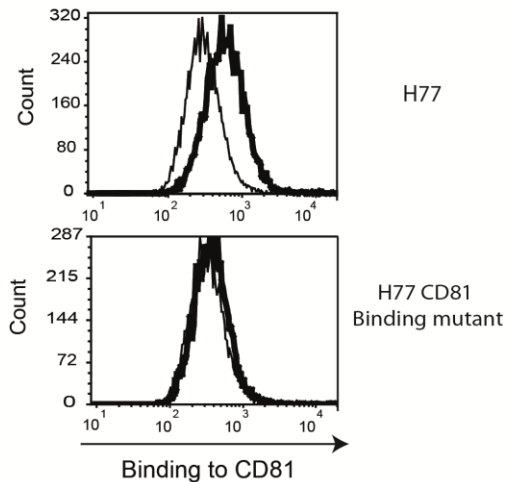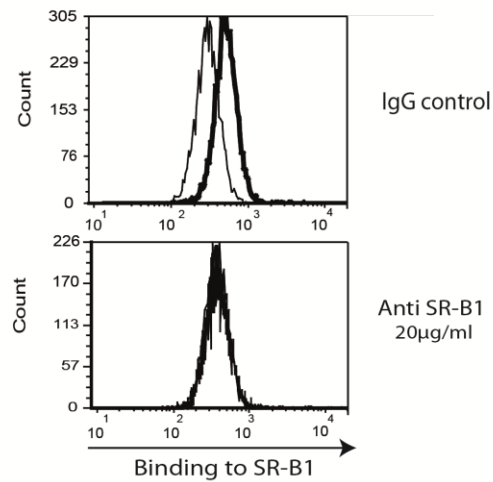
Figure 6. Cell surface binding of HCVpp is modulated by E1E2 mutations. A and B. Binding ability of HCVpp harboring the different E1E2 heterodimers to human CD81 (**A)** and to SR-BI (**B**) expressing BRL cells. BRL were incubated with pseudoparticles and E2 were stained with H53 antibody after several washes. Binding efficiencies were assessed by FACS using an APC secondary antibody. For each mutant, binding signal from native BRL were substracted to the APC GEO mean signal observed with receptor expressing BRL (ΔGEO Mean). Results are presented in percentage of binding, where ΔGEO Mean of H77 is considered as 100% of binding. (mean±SD; n=4). *p < 0.05. **C.** Mean fluorescence intensities (MFI – x axis) of APC signal after binding of pseudoparticles harboring E1E2 H77 or an E1E2 H77 CD81 binding domain mutant onto CD81 expressing-BRL (Thick lines). Thin lines represent MFI signal after binding to native BRL (n=3). **D.** Mean fluorescence intensities (MFI – x axis) of APC signal after binding of pseudoparticles harboring H77 heterodimer to native BRL (Thin lines) or SR-BI expressing BRL cell lines (Thick lines) previously incubated with control mouse IgG (20µg/ml) or with the mouse anti-human SR-BI (20 µg/ml). Results are representative of 2 independent experiments. **E.** CD81 LEL neutralization of HCVcc infections. HCVcc virus particles harboring the different heterodimers were incubated or not with a soluble peptide derived from the large extracellular loop of CD81 (20 µg/ml) before and during infection of naïve Huh-7.5. Infection inhibitions were determined for each heterodimers by quantifying the foci forming unit per ml (FFU/ml) in presence versus in absence of peptide (100% of infection). (mean±SD; n=3).
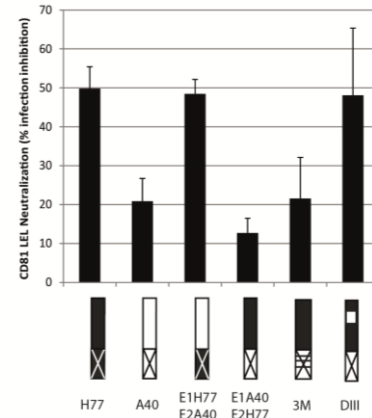
E1 allows binding of E1E2 heterodimers to Claudin-1 at the cellular surface

We then wondered whether Claudin-1 could also modulate binding of HCVpp. Interestingly, we found that HCVpp bound Claudin-1 at BRL cellular surface (Figure 7A, B C and Figure S6A) and that this binding is specifically inhibited by a monoclonal antibody, known to inhibit HCV infection [24], targeting Claudin-1 (Figure 7B). To analyze better the determinants of this binding, we generated chimeric Claudin-1 receptor harboring the first large extracellular loop (EL1) from Claudin-7 (non-functional for HCV entry [4]) and an EL1 deleted Claudin-1 mutant (Figure 7C). The Claudin-1 EL1 Claudin-7 chimera was more expressed at BRL cell surface than the wild type Claudin-1, which is similarly expressed to Claudin-1 EL1 deleted mutant (Figure S6B). Interestingly, no binding were observed on both receptor mutants (Figure 7C) suggesting that E1E2 probably bind to EL1 and that this binding play a role during HCV entry. The binding analysis of the different chimeras (Figure 7A) indicated that HCVpp harboring 3M and DIII mutations showed an improved binding to Claudin-1 (100% and 46% respectively) as compared to HCVpp harboring sub optimal heterodimer (19%) (Figure 7A, S6A, Table1). These results demonstrated that the proposed 3M and DIII cross-talk modulates Claudin-1 binding. To analyze the impact of E1 on E1E2 binding ability and regarding the technical limits to obtain a relevant folded soluble E1 protein [24], we compared binding of soluble E2A40 (sE2A40) and soluble E2H77 (sE2H77). As a control, we verified that a soluble E2H77 deleted for HVR1 (sE2H77 Δ HVR1) was able to bind CD81 but not SR-BI (Figure S6C). When equivalent amounts of either sE2 was added to cells, no binding to Claudin-1 was observed (Figure 7D). These observations highlighted the strict requirement of E1 to allow E1E2 binding to Claudin-1. In contrast to the binding of E1E2 to CD81 and SR-BI does not necessarily require E1. Our results indicated also that, consistently with the HCVpp binding phenotypes (Figure 6A), sE2H77 bound CD81 with a higher ability than sE2A40 (ΔGEO Mean = 6000 versus 1647 respectively) (Figure 7D).
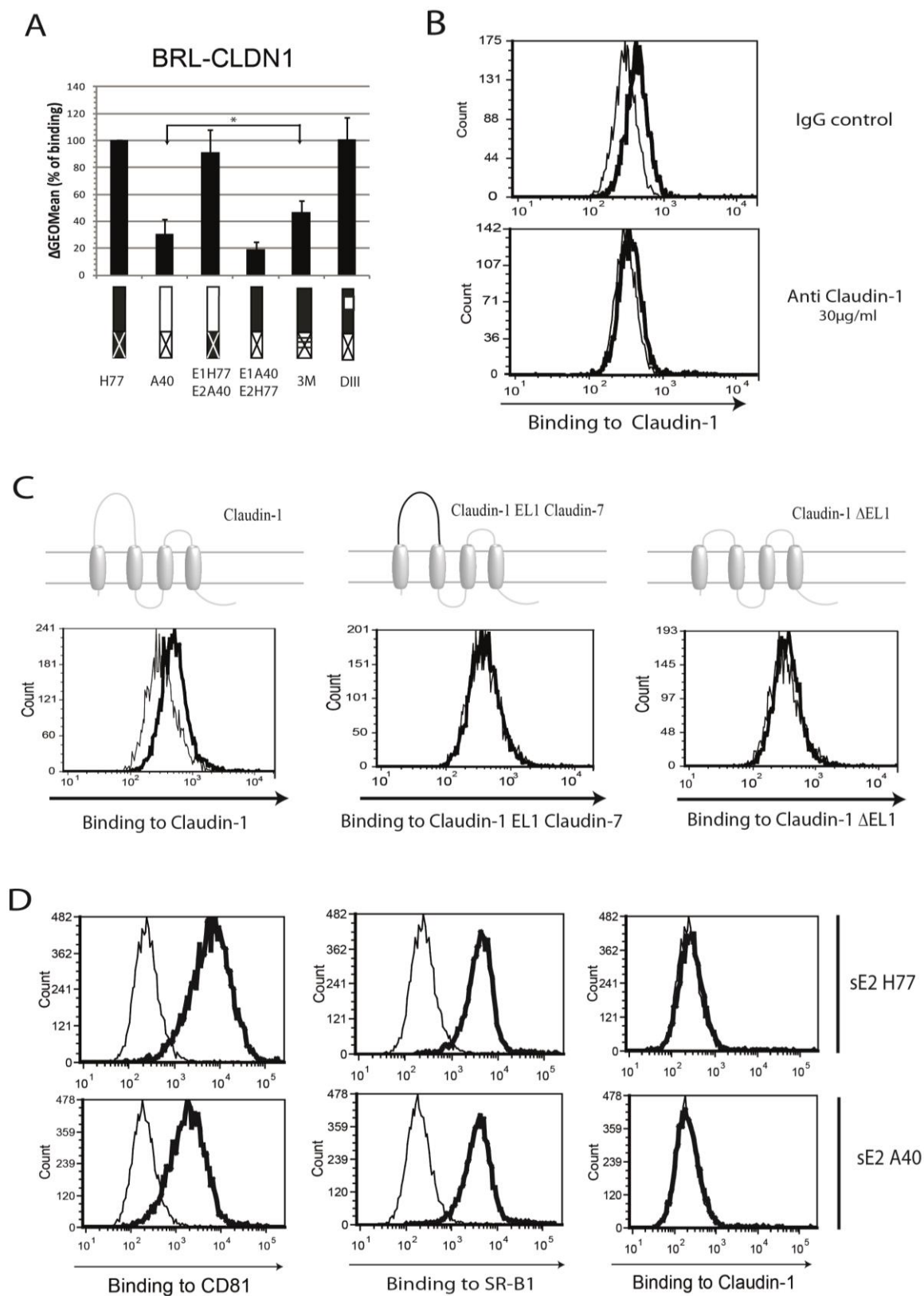
Figure 7. Heterodimers are able to bind Claudin-1 at cell surface. A. Binding ability of HCVpp harboring the different E1E2 heterodimers to Claudin-1 expressing BRL cells. Binding signal from native BRL were subtracted to the APC GEO mean signal observed with receptor expressing BRL (ΔGEO Mean) to determine heterodimer binding efficiencies as described in figure 6A. (mean±SD; n=3). *p < 0.05. **B.** Mean fluorescence intensities (MFI – *x* axis) of APC signal after binding of pseudoparticles harboring H77 heterodimer to native BRL (Thin lines) or Claudin-1 expressing BRL cell lines (Thick lines) previously incubated with a control rat IgG

(30µg/ml) or with the rat anti-human Claudin-1 mAb (30 µg/ml). Results are representative of 2 independent experiments. **C.** Schematic representations of Claudin-1 and Claudin-1 chimeric constructions, and corresponding binding assays. Mean fluorescence intensities (MFI – *x* axis) of APC signal after binding of pseudoparticles harboring H77 heterodimers to BRL expressing either Claudin-1, Claudin-1 expressing the first large extracellular loop (EL1) of Claudin-7 or Claudin-1 deleted of EL1 (Thick lines). Thin lines represent MFI signal after binding to native BRL. Results are representative of two independent experiments. **D.** Mean fluorescence intensities (MFI – *x* axis) of APC signal after binding of soluble E2 H77 (sE2 H77) or E2 A40 (sE2 A40) to CD81, SR-BI or Claudin-1 expressing-BRL (Thick lines). Thin lines represent MFI signal after binding to native BRL. Results are representative of three independent experiments.

On the contrary, sE2H77 and sE2A40 bound in a similar way SR-BI (ΔGEO Mean = 3940 versus 3659 respectively) (Figure 7D), which did not correlate with HCVpp binding results (Figure 6B; Table 1).

In conclusion, E1 seems to determine the binding ability of heterodimers to CD81, SR-BI and Claudin-1 differentially according to its sequence and its interactions with E2. E1 is mandatory for E1E2 binding to Claudin-1 and it influences binding to SR-BI and CD81. Altogether, these results suggest that E1 plays a global and active role in receptor binding modulation during entry.

The identified E1E2 cross-talk modulates conformational changes necessary for membrane fusion.

To assess the fusion abilities of our different heterodimers, we studied the ability of HCVpp to perform membrane fusion at low pH using a liposome-based fusion assay[10]. Interestingly, we found that the fusion efficiency correlated with the infectivity of the pseudoparticles harboring the set of E1E2 heterodimers (Figure 8, Table 1). Indeed, HCVpp harboring 3M and DIII heterodimers exhibited improved fusion rates (58% and 44% respectively) than HCVpp harboring E1A40/E2H77 envelope (20%). Moreover, E1H77/E2A40 heterodimer induced-fusion was over optimal (134%) in comparison to H77 and A40 fusion abilities (Figure 8). Altogether, the identified cross-talk appeared to be crucial for viral fusion, highlighting that E1 and E2 may collaborate together during this process.
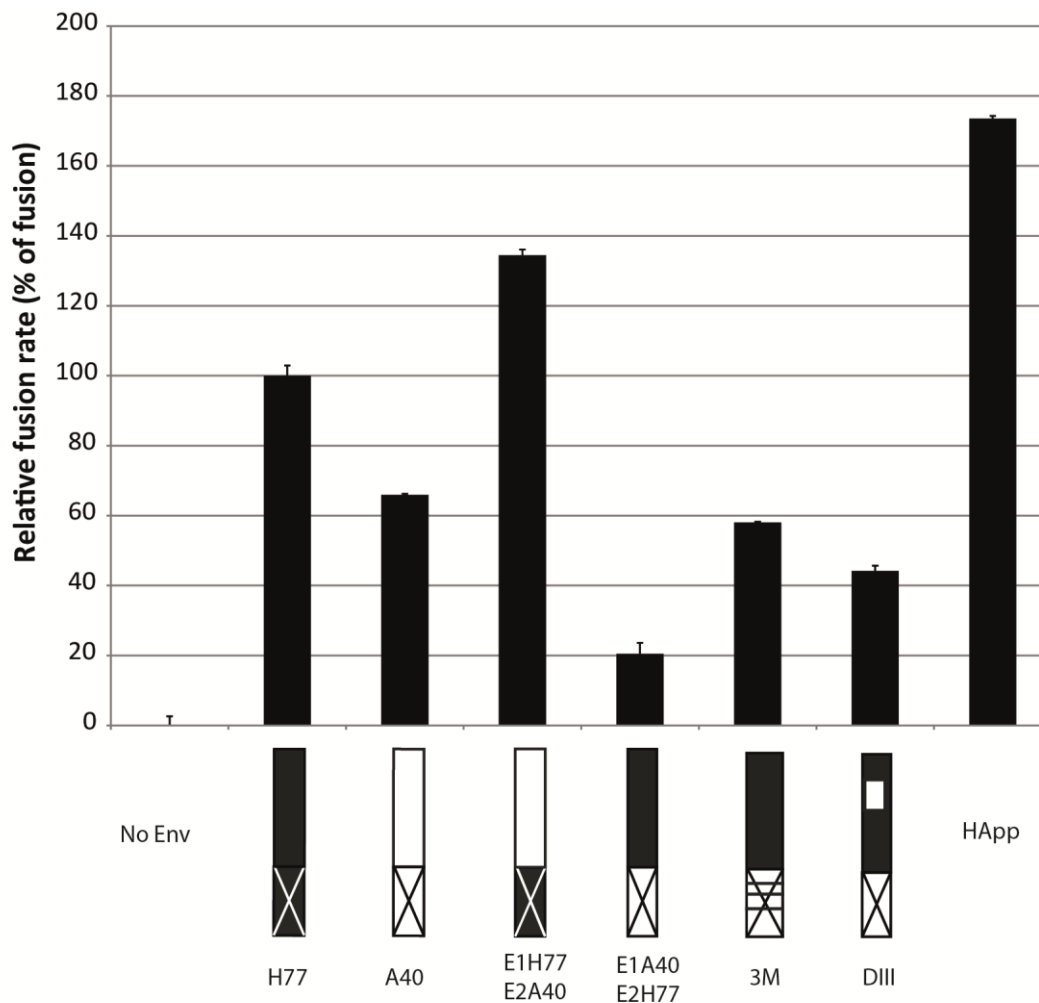
Figure 8. E1E2 membrane fusion properties are correlated to infectivity of HCV derived particles. Average membrane fusion abilities of HCVpp harboring either wild-type H77 or A40 E1E2 envelopes compared to control pseudoparticles harboring influenza (HApp), no glycoprotein (no Env) or the E1E2 mutant heterodimers. Average fusion ability of each heterodimer was determined by calculating the average dequenching signal after fusion equilibrium of HCVpp and liposome (curve plateau).Background fusion signal (HCVpp with no envelope glycoproteins) were removed from the fusion abilities of each heterodimers which were then determined according to the fusion ability of HCVpp harboring H77 envelope, normalized to 100% of fusion. Presented results are representative of 3 independent experiments.

Discussion

Even if challenging without structure data, it remains important to dissect the role of viral glycoproteins at the molecular scale and potential conformational changes during the cell entry process. Several papers have already reported cross-talks [15, 16] and important domains [9, 10, 11, 13] for entry in HCV glycoproteins. However, these reports focused only on E1 or E2. Here, to our knowledge, we performed the first study focusing on both E1 and E2 functions and behaviors in the context of their

interrelation. We identified a precise and multifunctional cross-talk, acting both during binding and fusion steps. These results contribute to a better understanding of how E1 and E2 may cooperate together during entry and suggest that E1 would not be a simple chaperone, but an active partner of E2 during entry, highlighting a very distinct entry mechanism compared to other members of the *Flaviviridae* family [12].

Identified E1E2 cross-talk modulates the binding to cellular HCV receptors.

Precise interactions between HCV E1 and E2 during receptor binding steps of the entry process are poorly characterized. We suspect that several conformational changes of the envelope glycoproteins occur and are triggered upon the interaction with the quite unique important number of identified HCV cellular receptors.In this study, we showed that i) a specific cross talk between E1 and E2 participates to the modulation of binding site accessibility to several HCV receptors, ii) that the binding phenotype is dependent on the heterodimer conformation, governed by particular E1E2 interactions and iii) that E1 plays an important role of modulation of binding, varying according to the receptor (Table 1).

The location of the CD81 and SR-BI binding domains in E2 are well characterized [3, 7, 8, 9, 10]. Thus, it is likely that interactions with E1 do not create new heterodimeric conformational domains where both E1 and E2 would bind directly to receptors, but E1 probably modulates the accessibility of the existing different E2 binding domains by shaping the heterodimer conformation. These results are consistent with mutagenesis studies of conserved residues such as cysteins [25], which demonstrated that E1 structurally shapes E2 binding properties to CD81. However, our study used different genetically close E1 wild type sequences and thus demonstrate that E1 functionally determines E2 binding to several receptors via their specific interactions between less conserved domains.

Due to technical limits, the identification of E1E2 conformations during entry steps remains difficult to assess. In previous studies, HCVcc binding could be detected

only on SR-BI[2]. Even if E1 or E2 domains in the context of HCVcc may mask binding domains to other receptors, it is possible that lipoprotein components also inhibit the direct interaction between the viral envelopes and the other receptors. Therefore we used HCVpp, which are devoid of lipoprotein components as an alternative model in order to characterize E1E2 interactions with CD81 and/or Claudin-1. It is important to keep in mind that such systems still do not characterize E1E2 conformational changes after each successive binding to the different receptors, which remains an important technical issue to solve.

E1 is critical for binding of the E1E2 heterodimer to Claudin-1

We demonstrated for the first time that an E1E2 complex is able to interact with Claudin-1 at the cell surface. Whereas soluble E2 does not bind Claudin-1, our results suggest the crucial need of E1 interaction with E2 to allow effective binding to Claudin-1. This result supports a model where Claudin-1 is not only an entry factor needed for receptors clustering, but is an HCV entry factor that can interact directly with E1E2 complexes. Previous studies have suggested that viral envelope glycoprotein binds exclusively to SR-BI and CD81 with subsequent interaction of the virus with the CD81-Claudin-1 complex [26, 27]. Our results suggest a model where SR-BI and CD81 may transfer the HCV particle to entry competent Claudin-1 with subsequent formation of a virus-receptors complex crucial for virus internalization, as supported by the recent observation that CD81, SR-BI and Claudin-1 are part of tetraspanin-enriched microdomains[28].

Further studies are needed to address three important questions. First, it would be relevant to determine whether E1E2 binding to Claudin-1 is critical for receptor clustering and internalization. Second, it would be of interest to map the Claudin-1 EL1 domains that are involved in E1E2 binding. Their identification would be eased by analyzing the amino acids differences between Claudin-1 and Claudin-7 EL1. In addition, Claudin-1-specific antibodies have been shown to inhibit CD81-Claudin-1 co-receptor associations [26] and Claudin-1 residues involved in CD81 association

have been identified [29]. Further mapping studies are needed to determine whether these relevant residues have a functional role in E1E2-Claudin interaction. Third, the localization of Claudin-1 binding domain in E1E2 needs to be identified. E1E2-Claudin1 interaction could be mediated by E2 with E1 required for the proper conformation of E2 interacting with Claudin-1. However, as E1 appears to be indispensable for Claudin-1 binding, another possibility would be that the binding domain is constituted either by E1 alone or by a conformational domain formed by both E1 and E2.

E1E2 cross-talk is important for conformational changes leading to membrane fusion

In this study, we brought evidences that the identified cross-talk appears to be an actor of E1E2 conformational changes that are necessary for membrane fusion. E1 has already been suggested in previous studies to be involved in viral fusion [10, 11, 16, 25]. Through the identification of this specific interaction, we provide an explanation on how E1 may mediate fusion with E2. However, the precise effects of such dialogs on heterodimer conformations during membrane fusion remain enigmatic. First, domain III of class II fusion protein is known to fold back during fusion to approach the viral membrane of the endosomal membrane [12]. We can hypothesize that E1, by interacting with DIII, may regulate this fold back. The modification or absence of this interaction would impair the ability of DIII to bring the two membranes closer. Second, E1 could also be essential to un-mask a hypothetical E2 fusion peptide via induction of conformational changes. Finally, we cannot exclude the hypothesis that E1 represent the HCV fusion protein and harbor a fusion peptide, that requires unmasking induced by conformational changes in E2 as it has been recently proposed[30] for other *Flaviviridae*.

In conclusion, we characterized a specific and multifunctional dialog between E1 and E2 which modulates binding and which is crucial for fusion. These results strongly reinforce the knowledge of the roles of E1 during entry by characterizing its

involvement in heterodimer functions and conformations. As entry represents a possible target for future therapy against HCV, an improved knowledge on E1E2 dialogs and conformations remains essential for the development of inhibitors targeting viral glycoproteins. It is likely that antibodies against domains involved in E1E2 cross-talks are generated *in vivo*. It will be important to isolate and characterize them as the strong functional pressure on these domains probably limits their mutation *in vivo* thereby providing relevant targets for B cell vaccines or immunotherapies with reduced risk of emergence of resistant viruses. Further works will be needed to validate the potential of the antiviral strategies targeting E1E2 cross-talk.

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Supplemental Informations

Methods

Establishment of BRL cell lines expressing CD81, SR-BI and Claudin-1and cell surface receptors staining - Retroviral vectors expressing human CD81 (GenBankTM accession number: NM_004356), Claudin-1 (NM_021101), and SR-BI (Z22555) were described previously [31]. Retroviral vectors containing these cDNAs were produced from 293T cells as VSV-G pseudotyped particles as described previously [32, 33]. Stable expression of either receptor in BRL cells was obtained as described previously[9]. BRL cell lines and BRL expressing CD81, SRB1 or Claudin-1 were washed and stained for 1h at 4°C. Human CD81 were detected with JS81 mAb (BD Biosciences), human SRB1 with CLA-1 mAb (BD Pharmingen) and human Claudin-1 with the MAB4618 (R&D Syst.). Cells were then washed and incubated with a secondary anti-mouse APC antibody for 1h at 4°C. Cell surface expression levels were then quantified by flow cytometry (FACS CANTO II – BD Biosciences).

Establishment of BRL cell lines expressing Claudin-1chimera and cell surface receptors staining – Deletion was introduced into the Claudin-1 encoding cDNA (NM_021101) by mutagenesis and PCR to allow the expression at the cell surface of a Claudin-1 molecule deleted for its first large extracellular loop (EL1). In parallel, the Claudin-1/7 chimera was constructed by exchanging the cDNA region encoding for the Claudin-1 EL1 with the one of Claudin-7. Construct details are available upon request. Retroviral vectors containing the corresponding cDNA were produced and used to transduced BRL cell lines as described above. Cell surface staining of Claudin-1 and Claudin-1 chimeras were performed as described above, at the difference that the human anti Claudin-1 2H10D10 mAb (Invitrogen) used targets the second extracellular loop of Claudin-1.

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Figures
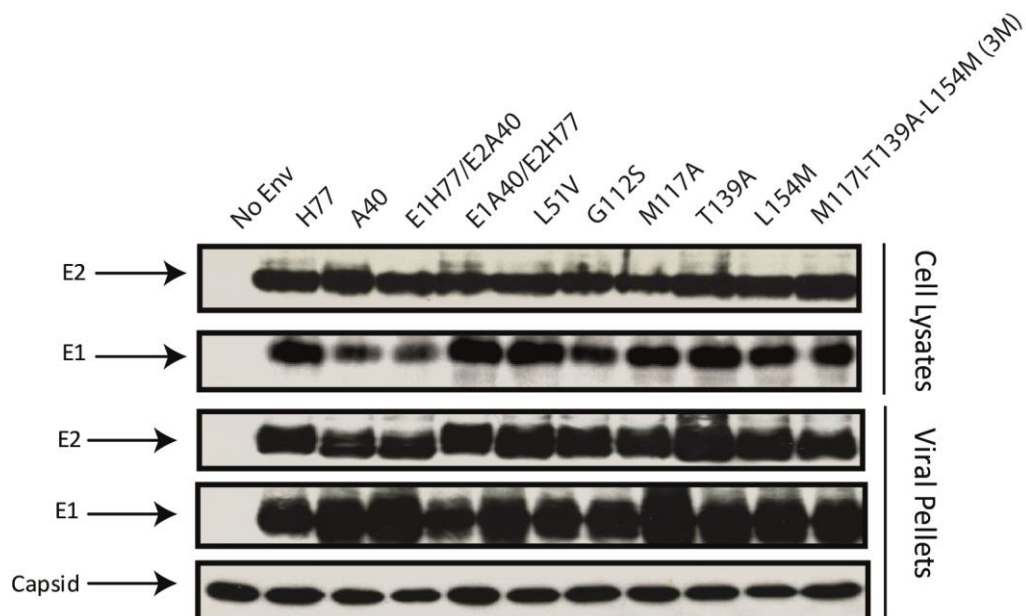
Figure S1. Expression and incorporation of H77, A40, E1H77/E2A40, E1A40/E2H77 and of the E1 mutant heterodimers onto HCVpp. Western blot were performed as described in Figure 1D.
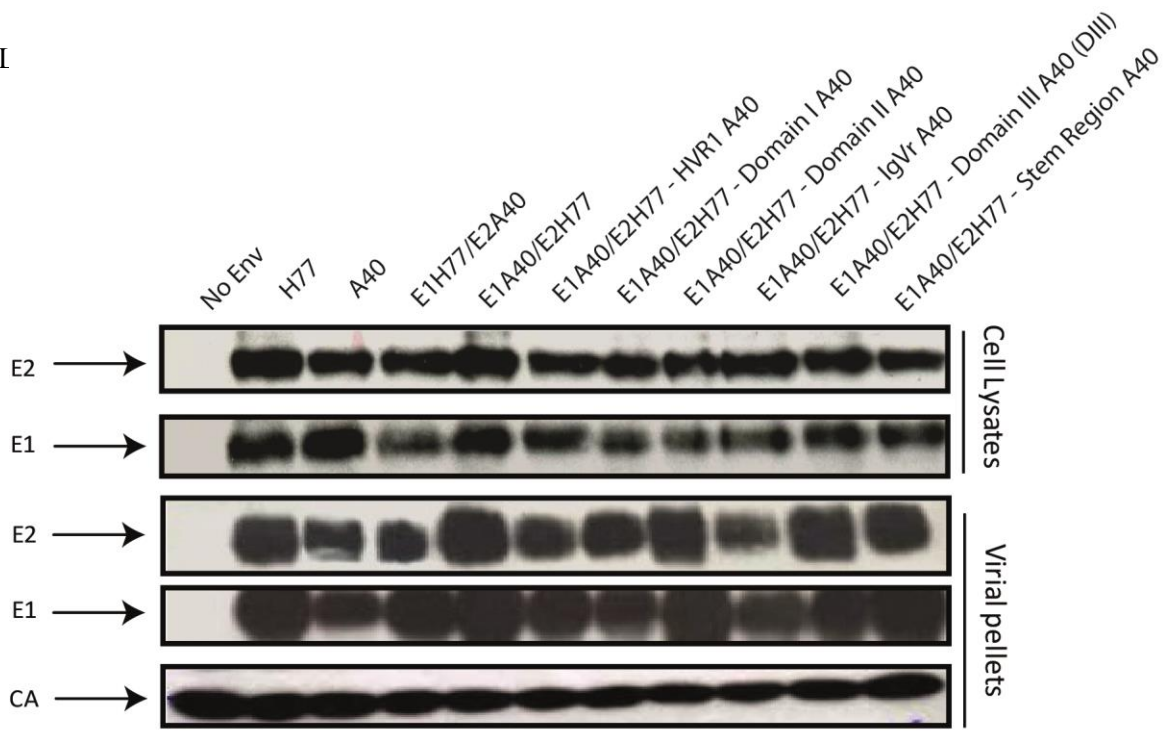
Figure S2. Expression and incorporation of H77, A40, E1H77/E2A40, E1A40/E2H77 and of the E2 chimeric heterodimers onto HCVpp. Western blot were performed as described in Figure 1D.
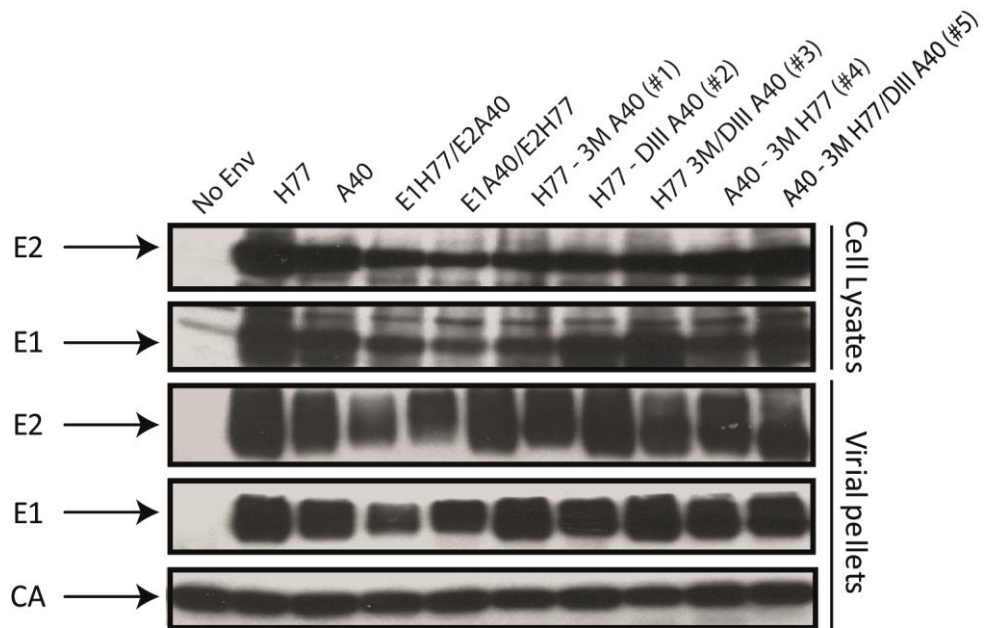
Figure S3. Expression and incorporation of H77, A40, E1H77/E2A40, E1A40/E2H77 and of the indicated mutant heterodimers onto HCVpp. Western blot were performed as described in Figure 1D.
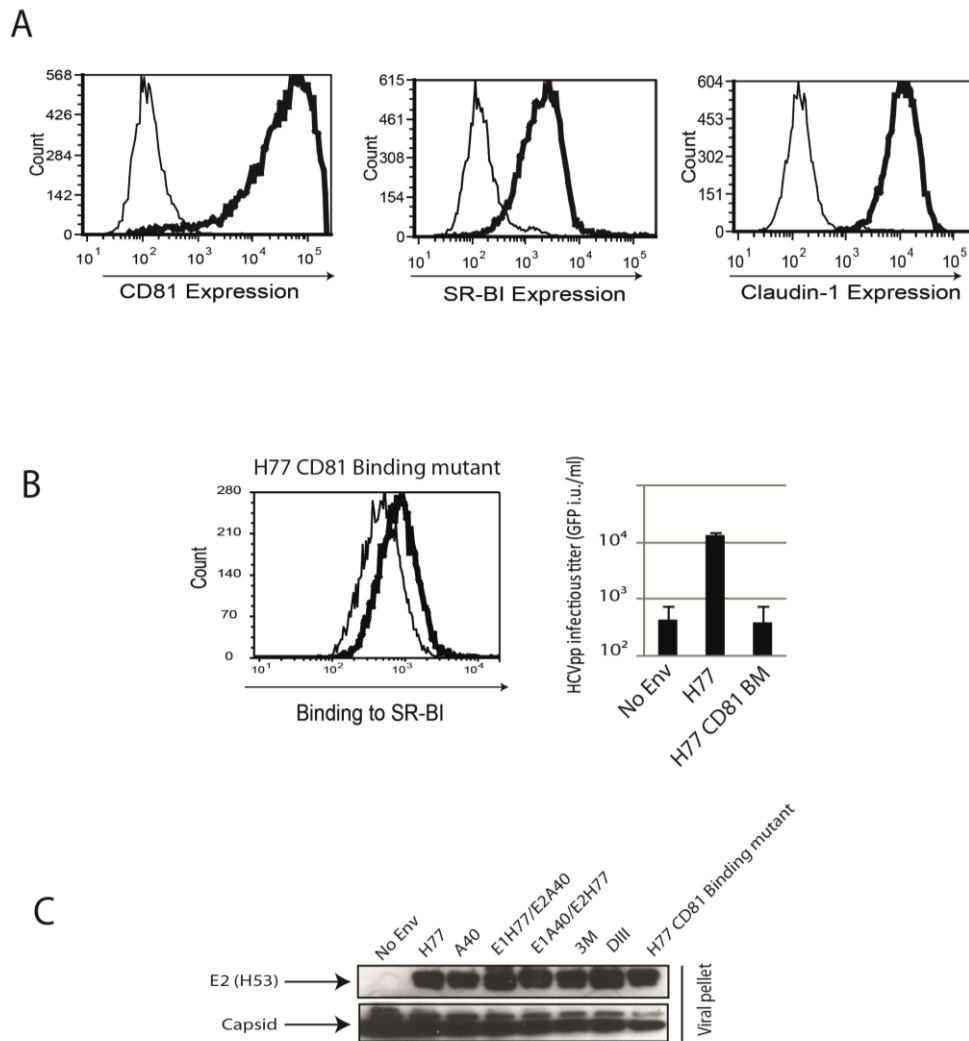
Figure S4. A. Cell surface staining of BRL expressing CD81, SRB1 or Claudin-1. Expression levels of CD81, SRB1 or Claudin-1were quantified by flow cytometry on BRL cell lines (Thin lines) and receptor transduced BRL (Thick lines). **B.** Mean fluorescence intensities (MFI − *x* axis) of APC signal after binding of pseudoparticles harboring E1E2 H77 or an E1E2 H77 CD81 binding domain mutant onto SR-B1expressing-BRL (Thick lines). Thin lines represent MFI signal after binding to native BRL. Infectivity on Huh7.5 cells of HCVpp harboring corresponding E1E2 envelopes or no glycoproteins (No Env) was also determined as described in Figure 1B. Results represent average infectious titers, expressed as infectious unit (IU) per ml (mean±SD; n=3). **C.** Western blot of the different heterodimers incorporated onto pseudoparticles. E2 were stained with anti-HCV E2 H53 conformational antibody and MLV-CA was stained by a goat anti- MLV-CA antibody anti-p30.
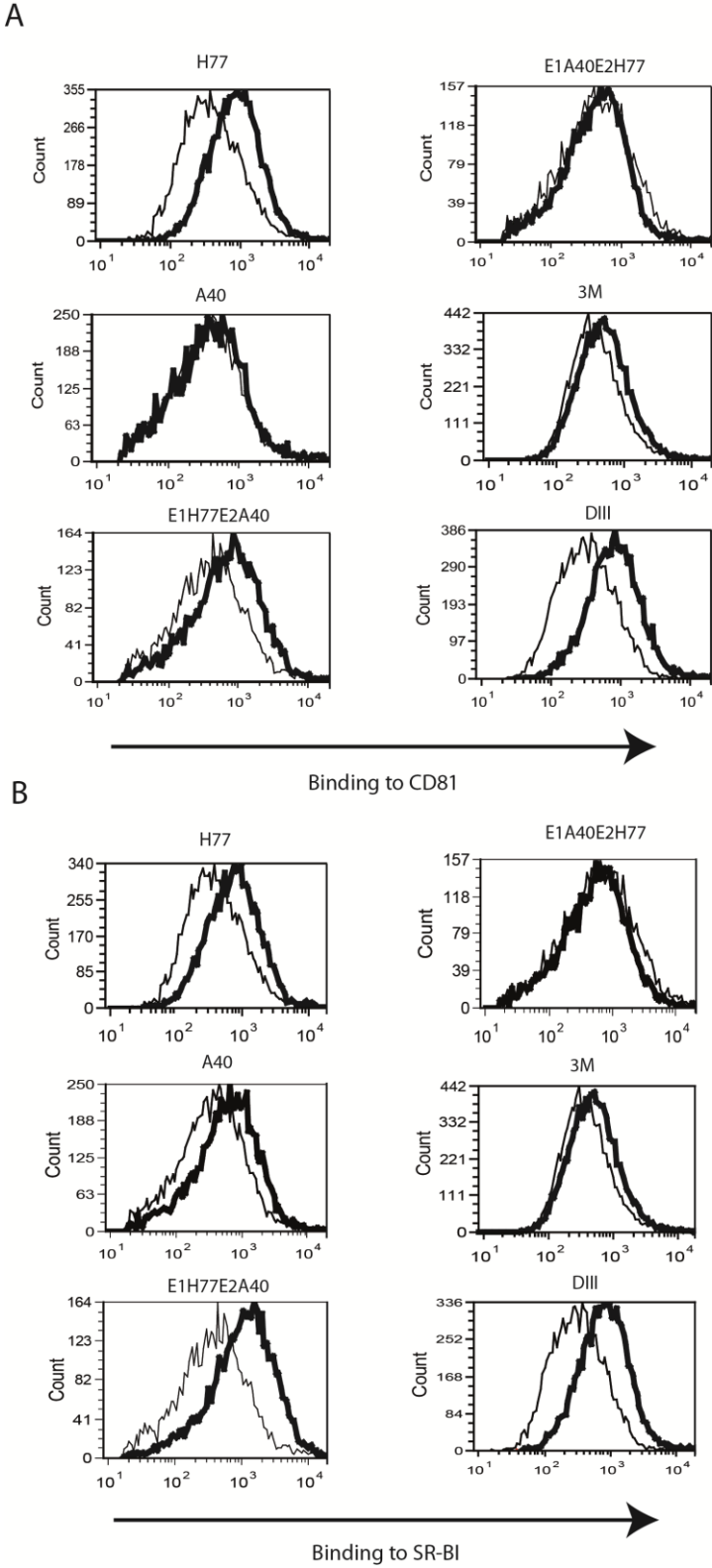
Figure S5. Mean fluorescence intensities (MFI – *x* axis) of APC signal after binding of pseudoparticles harboring the different heterodimers to **A.** CD81 or **B.** SR-B1 expressing-BRL (Thick lines). Thin lines represent MFI signal after binding to native BRL. Results are representative of 3 independent experiments.
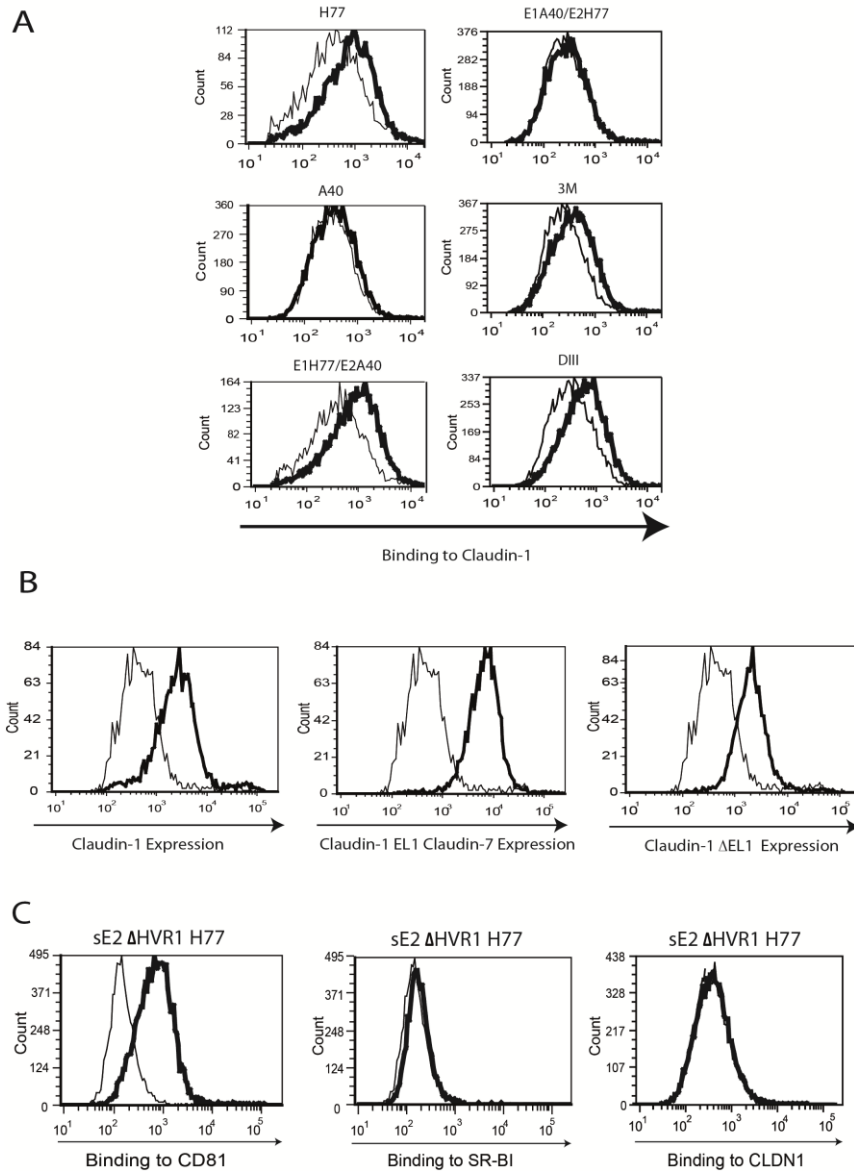
Figure S6. A. Mean fluorescence intensities (MFI − *x* axis) of APC signal after binding of pseudoparticles harboring the different heterodimers to Claudin-1 expressing-BRL (Thick lines). Thin lines represent MFI signal after binding to native BRL. Results are representative of 3 independent experiments. **B.** Cell surface staining of BRL expressing Claudin-1, Claudin-1 harboring EL1 from Claudin-7 or deleted from EL1 (ΔEL1). Expression levels were determined by flow cytometry on both BRL cell lines (Thin lines) and receptor transduced BRL (Thick lines). **C.** Mean fluorescence intensities (MFI − *x* axis) of APC signal after binding of soluble E2 ΔHVR1 H77 (sE2 ΔHVR1 H77) to CD81, SR-BI and Claudin-1 (CLDN1) expressing-BRL (Thick lines). Thin lines represent MFI signal after binding to native BRL.

CHAPTER III

Hepatitis C Virus E1 and E2 envelope glycoproteins co-evolving networks unveil their structural organization and suggest an unusual membrane fusion mechanism

Douam et al.,

In Preparation

CHAPTER IV

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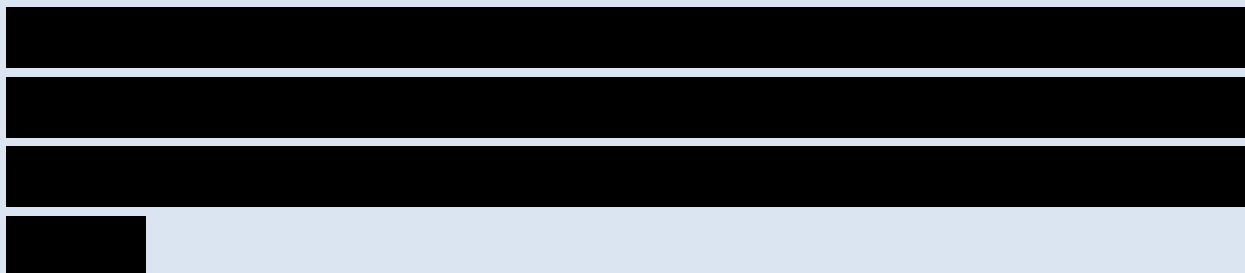
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Project On Going

PART

III

CONCLUSIONS

&

PERSPECTIVES

I.   Heterodimer as the unique HCV envelope functional entity

1. Co-evolution approach as a way to access to heterodimer function

During the last decades, roles of HCV E1 and E2 envelope glycoproteins have been essentially studied through protein analyses in a single protein context. Site directed mutagenesis allowed to identify residues and domains in these two proteins critical for heterodimerization, binding or virus fusion (Yi et al., 1997; Flint et al., 1999; Patel et al., 2000; Owisianka et al., 2001; Clayton et al., 2002; Cocquerel et al., 2002; Roccasecca et al., 2003; Ciczora et al., 2005; Owisianka et al., 2006; Drummer et al., 2006; Ciczora et al., 2007 ; Lavillette et al., 2007; Rothwangl et al., 2008; Witteveldt et al., 2009; Russel et al., 2009; Maurin et al., 2011; Drummer et al., 2012). However, this strategy never allowed to understand precisely how the HCV heterodimer functionally behaves and ensures virus entry. Moreover, the role of E1 in this complex has long been overestimated and is still poorly known in comparison to the one of E2 glycoprotein whose binding functions are well characterized. In contrast to the E/M complexes of flaviviruses where M does not play a role during entry, previous studies already suggested that E1 might likely harbor a more important role during entry than M (Drummer et al., 2007; Lavillette et al., 2007; Perez-Berna et al., 2008; Russel et al., 2009; Maurin et al., 2011). Thus, the HCV heterodimer may harbor a more complex mechanism, making it difficult to access.

E1E2 interrelations during entry have never been assessed. It is known that soluble E2 binds CD81 in a more efficient way than E1E2 complex incorporated onto surface of viral particles but soluble E2 is found in a larger extend in cell supernatants than E1E2 that are correctly incorporated onto a limited number of pseudoparticles (Bartosch et al., 2003). The proper influence of E1 on E1E2 heterodimer during entry and how E1 and E2 cooperate during this process remain to be elucidated. This missing point could be an important key to understand heterodimer function during entry, and open the way to new therapeutic strategies targeting viral entry.

In this thesis, we aimed to consider the HCV E1E2 heterodimer as a single protein entity where the role of E1 is not dissociable of the role of E2, and reversely. We hypothesized that the role of each protein is a determinant or a regulator of the functions of the other during virus entry, highlighting why their roles and functions during entry are so difficult to understand. To this purpose, we focused on the co-evolution of non-conserved residues within E1 and E2, in contrast to most of the previous studies that were based on conserved residues mutagenesis. We considered that a conserved protein function that involved different variable domains requires the co-evolution of these domains in order to maintain the most optimal direct or indirect interaction (or dialog) between these domains. Such approach was already employed in a previous study from our team (Maurin et al., 2011), where the dialog between two domains of E1, between the N terminal region and the transmembrane, has been shown to be critical for virus entry. In this work, we extended this principle to a larger context to study the entire E1E2 heterodimer.

2. A specific cross talk between E1 and E2 domain DIII in genotype 1 envelope is critical for virus entry

In one of our study, we generated several E1E2 chimera based on the envelope sequence of two genotype 1a strains, H77 and A40. Indeed, we voluntarily used two closely related envelopes in order to identify precisely non conserved residues that could be involved in an important dialog during entry. Use of two genetically more distant envelopes could have allowed the identification of more dialogs, but in a less accurate extend. By generating heterogeneous chimera between E1 and E2 from H77 and A40, we observed that the two combinations had distinct phenotypes (See Results – Chapter II). One was over-optimized for entry (E1H77E2A40) whereas the other was sub-optimized (E1A40E2H77). This observation thus constituted an important opportunity to identify potential E1 and E2 cross talk that could be present between E1 and E2 and could modulate E1E2 functionality and conformation. Indeed, we observed through different functional assays that a particular dialog between E1 and the domain III of E2 orchestrated the E1E2 functionality (Figure 48). We showed that this dialog was able to modulate E1E2 binding to several HCV

cellular receptors such as CD81 or SR-BI (See Results – Chapter II). Importantly, this result demonstrates that E1E2 conformation is able to modulate the receptor binding site accessibility to receptors, which suggest that not only E2, but the entire heterodimer determines and regulates the receptors binding phenotype during virus entry though particular dialog between its two components, E1 and E2.
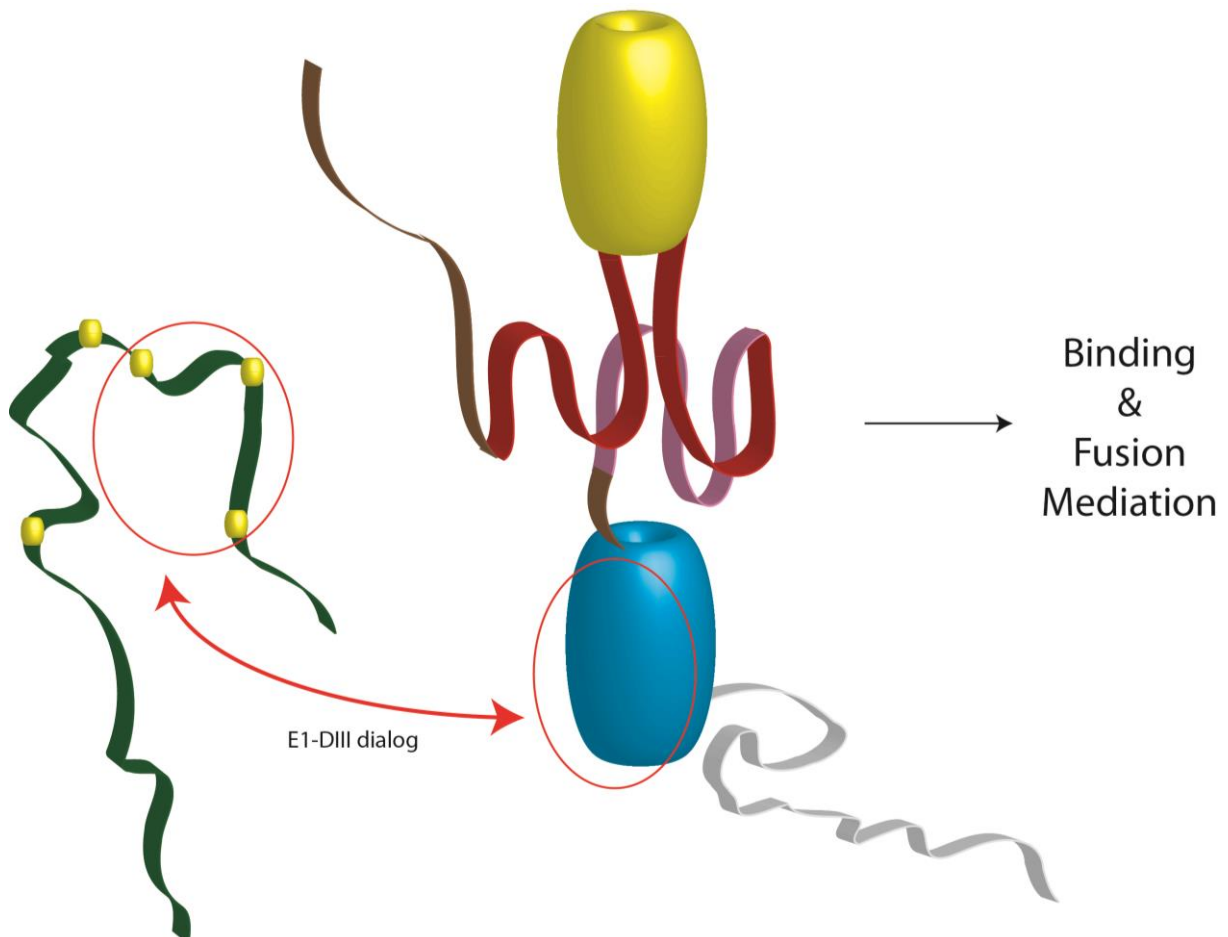
Figure 48. Tridimensional representation of the E1 – E2 DIII dialog. Yellow pearls indicate the E1 residues differences between H77 and A40. The three E1 residues involved in the E1-E2 DIII dialog and the domain III are highlighted by a red circle.

However, characterization of E1E2 dynamic conformational changes remains challenging. HCV entry has been shown to involve an important number of cellular receptors, implying viral particle successive binding leading to virus internalization (Ploss et al., 2012; Zeisel et al., 2013; Lindenbach et al., 2013). Today, characterization of HCVpp or HCVcc binding phenotypes does not take into account this dynamic and assesses the native ability of heterodimer to bind cellular receptor without

considering possible pre-conformational changes due to pre-binding steps. However until this technical issue is resolved, the HCVpp system still remains the best tool to assess the ability of E1E2 heterodimer to bind HCV receptors. Indeed, HCVcc association with lipoproteins only restricts the related binding assays to the assessment of the ability of lipoproteins to attach cell surface.

Importantly, the cross talk between E1 and the domain III of E2 was also able to modulate virus fusion (Figure 48), as observed with virus-liposome assays (See Results – Chapter II). This is an important observation because even though E1 has already been shown previously to be involved in virus fusion (Garry et al., 2003; Drummer et al., 2006; Lavillette et al., 2007; Maurin et al., 2011), no mechanism was ever been proposed to explain how E1 and E2 could mediate virus fusion together. Thus, this result highlights that E1E2 critical fusogenic conformational changes are mediated by a particular dialog between E1 and E2. This observation strongly reinforces the idea that E1E2 is a unique protein entity and places the HCV envelope heterodimer in strong contrast with Dengue E, which mediates entirely both binding and fusion (Modis et al., 2004; Kielian, 2006; Modis, 2013). Interestingly, it has been previously shown that CD81 binding (via LEL) is able to prime E1E2 complex for virus fusion (Sharma et al., 2011). As the E1-DIII dialog modulates both CD81 binding and virus fusion (Figure 48), it could be interesting to see whether these two phenotypes are linked. This could also reinforce the idea that the E1-DIII crosstalk is a critical and multifunctional dialog between E1 and E2 that shapes the function of the HCV heterodimer. One particular way to assess this question would be to design virus-liposomes assays where viral particles harboring different chimera would be pre-incubated with the soluble CD81 LEL.

3. **Multifunctional role of E1 in heterodimer function**
   a) *Binding to CD81 and SR-BI*

The E1 – DIII dialog importantly highlights the role of E1 in a heterodimeric context. Indeed, most of the previous studies focusing on E1 were based on site-directed mutagenesis of conserved residues that rendered difficult to assess the real impact of

the chosen mutations on virus entry mechanism (Lavillette et al., 2007; Russell et al., 2009; Wahid et al., 2013). Here, we used and studied different wild type E1 envelopes in combination with wild type E2 envelopes to assess the role of E1 during entry. Importantly, the binding phenotypes of the different chimera we designed underline a major role for E1 in the binding modulation of E1E2 (See Results – Chapter II). Binding of E1E2 to CD81 was shown to be dependent on both E1 and E2 sequences whereas binding to SR-B1 was mainly dependent on the E1 sequence expressed with E2. Thus, it appears that an E1 protein, once in combination with a particular E2 protein, may drastically impact the phenotype ability of the E1E2 heterodimer, depending on the E1 sequence and the receptor bound. These results suggest that E1 may modulate the accessibility of the existing different E2 receptor binding domains by shaping the heterodimer conformation.

b) Claudin-1 Binding

Strikingly, we also observed that the E1E2 heterodimer was able to bind Claudin-1 expressed at a cell surface of HCV non permissive cell lines. It has been previously shown that sE2 is not able to bind Claudin-1. As sE2 is able to bind CD81 and SR-BI, this led to the idea that HCV particles are not able to bind Claudin-1. In contrast, our work showed that HCV particles are able to bind Claudin-1 through E1E2 heterodimer (See Results – Chapter II) although sE2 is not (Evans et al., 2007). This strongly reinforces the idea that E1 plays an important role in the E1E2 binding to cellular receptors. Moreover and most importantly, this result also shows that the presence of E1 is critical to initiate the binding of E1E2 to Claudin-1.

Up to now, the role of Claudin-1 during virus entry was reported to be independent of viral particle binding (Evans et al., 2007). Indeed, it has been shown that Claudin-1 rather acts through association with CD81 and is critical for viral particle internalization (Harris et al., 2008; Kreiger et al., 2010; Harris et al., 2010; Farquhar et al., 2012). Moreover, this receptor clustering is mediated by the residues located within the first extracellular loop of Claudin-1 (EL1) (Evans et al., 2007). In our work, we also identified that the Claudin-1 EL1 is an important mediator of the viral particle binding (See Results – Chapter II). Thus, this may suggest that viral particle

association with Claudin-1 could be an important element that strengthens the molecular association between the three members of the complex (HCV, CD81 and Claudin-1). The precise role of this binding and whether it is critical or not for virus entry remains now to be elucidated. One possibility would be that this function appeared during virus evolution and was conserved because it significantly improved viral entry.

Moreover, the E1E2 domains that mediate this binding also need to be identified. One unlikely possibility would be that E1 alone harbors a Claudin-1 binding site, or that its association with E2 induces a conformational and common Claudin-1 binding site. Another possibility would be that E1 association with E2 impacts E2 conformation, which leads to the exposure of the Claudin-1 binding site.

c) Fusion

The role of E1 during fusion has only been characterized through the identification of a potential fusion-peptide like (Drummer et al., 2006; Lavillette et al., 2007). However, the identification of a dialog between particular residues of E1 and the domain III of E2 highlights for the first time a potential molecular mechanism which may explain how E1 is involved with E2 in virus fusion. Several hypotheses are possible. First, E1 would be the HCV fusion protein and would harbor a fusion peptide that requires the action of E2 to be unmasked. This hypothesis is currently consistent with the model of pestiviruses, where E1 has been highly suggested to be the fusion protein. Inversely, E1 could mask a fusion peptide located within E2, probably in the DII as suggested by *Krey et al* (Krey et al., 2010). Third, E1 and E2 would harbor a conformational fusion peptide that would require critical E1E2 conformational changes during virus fusion to be structured. ██████████████
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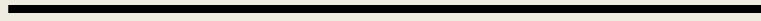
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Abstract

Hepatitis C Virus (HCV) infects more than 170 million people worldwide but no vaccine is available yet. HCV entry may represent a promising target for therapies and is mediated by two envelope glycoproteins, E1 and E2, assembled as heterodimer onto the virus surface. However, how E1 and E2 dialog, structurally rearrange and act together during these steps remain poorly defined. In this work, we aimed to clarify the interrelation of E1E2 during virus entry, thus opening ways to potential new therapeutic strategies. We first investigated whether a strong genetic divergence between E1E2 heterodimers may highlight distinct functions. We observed that B-cell derived E1E2 were specialized for B-cell infection, suggesting that new functions can emerge from the E1E2 conformational plasticity. In a second approach, we identified a conserved dialog between E1 and the domain III of E2 that was critical for virus binding and fusion. Moreover, a computational model predicted a strong co-evolution between E1 and E2 as well as potential structural rearrangements, suggesting that HCV E2 is likely a fusion protein able to fold over via its domain III through the mediation of E1. ▮▮▮▮▮ ▮▮ ▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮ Altogether, these different works highlight that E1 and E2 are involved in complex dialogs that regulate the heterodimer folding and functions, suggesting that E1E2 heterodimer is more likely a single functional protein entity than an association of two proteins with specific functions.

Résumé

Le Virus de l'Hépatite C (VHC) infecte 170 millions de personnes dans le monde mais aucun vaccin n'est encore disponible. Le processus d'entrée du VHC dans les hépatocytes représente une cible prometteuse pour le développement de stratégie thérapeutique et est finement régulé par un nombre par les deux glycoprotéines d'enveloppe du VHC, E1 et E2, assemblé sous la forme d'un hétérodimère incorporé à la surface des particules virales. Cependant, comment E1 et E2 dialoguent, modifient leurs conformations et se coordonnent mutuellement au cours de l'entrée reste encore à être définit. Dans ce travail, nous avons souhaité clarifier l'interrelation entre E1 et E2 au cours de l'entrée afin d'ouvrir la voie à de potentiels stratégies thérapeutiques. Nous avons tout d'abord examiné si une importante divergence génétique entre des hétérodimères E1E2 pouvait être liée à l'existence de fonctions particulières. Nous avons observé une spécialisation des E1E2 isolé des Lymphocytes B pour l'infection de ces mêmes cellules mais pas des hépatocytes, suggérant que de nouvelles fonctions peuvent émerger de la plasticité conformationel de E1E2. Dans un second temps, nous sommes parvenus à identifier un dialogue conservé entre E1 et le domaine III de E2 (E2 DIII), critique pour les processus d'attachement et de fusion du VHC. Nous avons aussi montré grâce à une approche bio-informatique l'existence d'une co-évolution très importante entre E1 et E2. Cette approche a également prédit de potentiel changement de conformations au sein de l'hétérodimère, suggérant que E2 est sans doute une protéine de fusion capable de se replier sur elle-même via le repliement de son domaine III et l'aide de E1. ▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮ ▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮ ▮▮▮▮▮ Ainsi, ces différents travaux soulignent l'implication de E1 et E2 au sein de dialogues fins et complexes, qui régulent à la fois les conformations et les fonctions de l'hétérodimère. Ainsi, cela suggère que l'hétérodimère E1E2 représente plutôt une unité fonctionnelle et structurale unique, plutôt que l'association de deux protéines aux fonctions distinctes.