

Role of cholesterol and N-glycosylation in apical sorting of GPI- APs in polarized epithelial FRT cells

Naga Sailaja Imjeti

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DISCIPLINE : Biologie Cellulaire

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par

Naga Sailaja IMJETI

Role of cholesterol and N-glycosylation in apical sorting of GPI-APs in polarized epithelial FRT(Fisher Rat thyroid) cells.

Directeur de thèse : Dr.Chiara Zurzolo Directrice de unit (UTRAF), Institute Pasteur

Composition du jury :

Président du jury :	Dr. Jean-Pierre Rousset	Professor (Paris sud)
Rapporteurs :	Dr. Nathalie Sauvonnet	Chargé de recherche, (BIC), InstitutePasteur
	Dr. Ama Gassama-Diagne	Chargé de recherche, Inserm U785, Hopital Paul Brousse
Examinateurs :	Dr. Delphine Delacour	Chargé de recherche, UMR 7592 CNRS, Université Paris Diderot
	Dr. Germain Trugnan	Professor, UMRS 538, University Paris 6.

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Pour obtenir le grade de

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Sujet de la thèse :

Role of cholesterol and N-glycosylation in apical sorting of GPI- APs in polarized epithelial FRT cells.

Soutenue le 1er Juillet 2011 devant le jury composé de:

Dr. Rousset Jean-Pierre	Président
Dr. Sauvonnet Nathalie	Rapporteur
Dr. Gassama-Diagne Ama	Rapporteur
Dr. Delacour Delphine	Examinateur
Dr. TRUGNAN Germain	Examinateur
Dr. Zurzolo Chiara	Directrice de thèse

The whole of science is nothing more than a refinement of everyday thinking.

Albert Einstein

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LIST OF ABBREVATIONS:

AP: Adaptor protein

DAF: Decay accelerating factor

DPP IV: Dipeptidyl peptidase

DRM: Detergent resistant membrane

ECM: Extracellular matrix

ECL: Enchanced chemiluminescence

ER: Endoplasmic Reticulum

FR: Folate receptor

FRAP: Fluorescence recovery after photo bleaching

FRT: Fisher Rat Thyroid

GFP: Green fluorescent protein

GPI: Glycosyl phosphatidyl inositol

GPI-APs: GPI-anchored proteins

GSL: Glycosphingolipid

HA: Hemaglutinin A

HMW: High molecular weight

LDL: Low density lipoprotein

MDCK: Madin-Darby Canine Kidney

PI: phosphoinositide

PLAP: Placental alkaline phosphatase

PrP: Prion protein

P75NTR: Neurotrophin receptor p75

REs: Recycling endosomes

SI: Sucrase-isomaltase

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SNAP: Soluble NSF attachment protein

SNARE: Soluble NSF attachment protein receptor

TCA: Trichloro acetic acid

Tf: Transferrine

TGN: Trans Golgi Network

TI-VAMP: Tetanus-insensitive VAMP

t-SNARE: target SNARE

VAMP: Vesicle associated membrane protein

v-SNARE: Vesicular SNARE

RESUME

L'épithelium constitue une interface entre le monde externe et le système interne de l'organisme. En fonction de l'organe considéré ou de son état de différentiation, les cellules épithéliales sont spécialisées dans des fonctions particulières comme l'absorption, la sécrétion, le transport vectorial de fluide, électrolytes et protéines. Afin de bien réguler ces différentes fonctions, les cellules épithéliales établissent et maintiennent leur polarization. La membrane plasmique des cellules épithéliales polarisées est asymmétrique et divisée en deux domaines distincts nommés : domaine apical qui est en contact direct avec l'environnement externe et le domaine basolatéral qui est en contact avec les cellules voisines, la membrane basale et le milieu interne (Delacour and Jacob, 2006) (Mostov *et al.*, 2003). Les deux domaines diffèrent dans leur fonctions ainsi que dans leur composition protéique et lipidique. Cette distribution asymmétrique de la membrane plasmique résultent selectif des lipides ainsi que d'un tri continue des protéines nouvellement synthétisées à chacune de ces deux surfaces ainsi que de leur internalization régulée (Mellman et al., 1996 and Matter et al., 2000).

De récentes études biochimiques et d'imagerie sur cellules vivantes ont montré que les protéines nouvellement synthétisées divergent dans des vésicules différentes après leur sortie du TGN (Trans Golgi Network) afin de rejoindre la membrane plasmique (Kreitzer et al., 2003; Christiansen et al., 2005 and Paladino et al., 2006; Folsch et al., 2009). Il a également été reporté que la ségrégation se produit aussi bien après le TGN au niveau des endosomes de recyclage. Depuis le TGN, les protéines peuvent être dirigées au niveau des surfaces apicales ou basolatérales soit

directement ou indirectement via les endosomes de recyclage (Ang et al., 2004; Cancino et al., 2007; Cresawn et al., 2007; Gravotta et al., 2007). La ségrégation entre les protéines apicales et basolatérales reposent sur la présence de signaux apicaux ou basolatéraux intrinsèques aux protéines reconnus par la machinerie intracellulaire. Cette reconnaissance spécifique médie alors l'incorporation de ces protéines dans des vésicules cargo (Ellis et al., 2006; Matter et al., 2000; Mellman et al., 2008 and Wandinger-ness et al., 1990).

Alors que les signaux responsables du tri basolatéral des protéines ont été clairement identifié, la situation concernant le tri apical des protéines est plus complexe (Ellis et al., 2006; Gravotta et al., 2007; Matter, 2000; Mellman and Nelson, 2008). Les motifs assurant un tri basolatéral sont, avec quelques exceptions, généralement trouvés dans le domaine cytoplasmique des protéines et sont composés de séquences amino acides discrètes dans la séquence primaire des protéines (Puthenveedu et al., 2003).

Différemment, les signaux de tri apicaux peuvent être localisés dans les domaines extra, intra cellulaire ou au sein même du domaine transmembranaire des protéines (Arreaza and Brown, 1995). Généralement, ils ne sont pas formés par des séquences d'aminoacides mais plutôt par des modifications post-traductionnelles comme la *N* ou/et *O*-glycosylation (Alfalah et al., 1999; Potter et al., 2006; Yeaman et al., 1997). L'un des signaux supposés responsable du tri apical était l'ancre GPI (glycosylphosphotidyl inositol) (Brown et al., 1989; Lisanti et al., 1989; Lisanti et al., 1988; Simons and Ikonen, 1997).

Les protéines glypiées ou à ancre GPI apicale ou basolatérale via leur ancre GPI sont associés à des domaines membranaires appelés Rafts. Rafts sont définis comme des domaines membranaires enrichis en cholestérol et sphingolipides (Simons and Ikonen, 1997) . Différent types de protéines comme les protéines glypiées, les protéines acylées et les protéines transmembranaires ségrègent dans ces domaines rafts. Ces domaines réguleraient différents méchanismes cellulaires comme le tri de protéines, l' endocytose, l'infection bactérienne et ce en permettant de compartimentaliser les protéines et les lipides dans les membranes (Cherukuri et al., 2001; Leser and Lamb, 2005; Manes et al., 2000; Nguyen and Taub, 2004). Ainsi, il a été proposé que le tri apical des protéines à ancre glypiée étaient mediée via l'association de ces protéines avec les domaines membranaires rafts. Cependant, cette hypothèse a été réfusée par le fait que bien qu'associé aux domaines rafts les protéines à ancre GPI peuvent être exprimé apicalement ou basolatéralement et ce dans differents types de cellules épithéliales polarisées (Lipardi et al., 1999; Paladino et al., 2004; Zurzolo et al., 1993). Cependant contrairement aux protéines triées à la surface basolatérale des cellules, seules les protéines à ancre GPI exprimées apicalement forment des complexes de haut poids moléculaires au niveau de l'appareil de Golgi (Paladino et al., 2004; Paladino et al., 2007). De plus, si on annhile la formation de ces oligomères alors la protéine à ancre GPI est envoyée à la surface basolatérale (Paladino et al., 2004). Les cellules MDCK expriment la plupart des protéines à ancre GPI à la surface apicale des cellules (Paladino et al., 2004; Paladino et al., 2006; Paladino et al., 2007) alors que les cellules FRT addressent principalement les protéines à ancre GPI à la surface basolatérale (Zurzolo et al., 1994).

De plus, dans les cellules MDCK, nous avons précédemment montré que l'ancre GPI ainsi que l'environment membranaire au niveau de l'appareil de Golgi (spécialement le contenu cellulaire en cholestérol) sont impliqués dans le tri apical des protéines glypiées (Lebreton et al., 2008; Paladino et al., 2008). Nous avons également montré que l'addition de cholestérol dans les cellules MDCK suffit à promouvoir l'oligomerization et donc le tri apical d'une protéine à ancre GPI GFP-PrP qui est dans les conditions contrôles exprimée basolatéralement (Paladino et al., 2008). D'après l'ensemble de ces résultats, nous avons proposé qu'un environment membranaire spécifique enrichi en cholestérol soit requis afin de favoriser l'oligomerization des protéines glypiées dans l'appareil de Golgi et ainsi assurer leur tri apical (Lebreton et al., 2008; Paladino et al., 2008). Cependant, le mécanisme de tri apical impliquant le cholestérol comme élément régulant la formation de complexes de haut poids moléculaires ainsi que les éléments moléculaires participant à ce procédé est toujours inconnu. Afin de comprendre si le rôle du cholestérol comme élément régulateur de la formation d'oligomères des protéines glypiées est conservé dans d'autres types de cellules épithéliales, nous avons étudié le rôle du cholestérol dans le tri apical des protéines glypiées dans les cellules épithéliales FRT.

Le cholestérol ne régule pas le tri apical des protéines glypiées dans les cellules FRT.

Pour cette étude, nous avons utilisé GFP-NO-GPI (GFP fusionné aux séquences de N et O- glycosylation du récepteur LDL suivi par le signal d'attachement de l'ancre de DAF) et la protéine native PLAP comme protéine apicale glypiée. Comme protéine glypiée triées basolatéralement, nous avons utilisé GFP-FR

(GFP est fusionné à la séquence d'attachement de l'ancre du récepteur folate) et GFP-PrP (GFP est fusionné à la séquence d'attachement de l'ancre de la protéine prion). En utilisant des analyses biochimiques (biotinylation, gradients de densité de sucrose ou de velocité) et des analyses en microscopie confocale, nous avons montré que contrairement aux cellules MDCK dans les cellules FRT, le cholestérol ne favorise pas l'oligomerization et donc le tri apical des protéines glypiées GFP-PrP et GFP-FR qui sont normalement monomérique/dimérique et basolatéralement exprimées.

De plus, en utilisant des expériences dites de FRAP au niveau de l'appareil de Golgi avec nos protéines modèles, nous avons révélé que l'environment membranaire au niveau de l'appareil de Golgi est différent dans les cellules MDCK et FRT. Pour cette étude de FRAP en plus de nos deux protéines glypiées modèles (GFP-NO-GPI et GFP-PrP), nous avons utilisé deux protéines non associées aux domaines membranaires rafts, transmembranaires GFP-PIT et P75-GFP respectivement triées aux surfaces basolatérale et apicale des cellules FRT. Différemment des cellules MDCK, toutes nos protéines étudiées présentent un coefficient apparent de diffusion bas (<0,015) et ceux indépendamment de leur association aux domaines membranaires rafts et de leur tri au sein des cellules. De plus, l'addition de cholestérol n'affecte pas le coefficient apparent de diffusion des protéines glypiées apicales et basolatérales dans les cellules FRT contrairement aux cellules MDCK (Lebreton et al., 2008).

Par une fractionation cellulaire et la quantification du contenu en cholestérol dans les fractions correspondant au complexe de Golgi, nous avons trouvé que le

contenu en cholestérol au sein de l'appareil de Golgi des cellules FRT est significativement enrichie en comparaison du Golgi des cellules MDCK. De plus après addition du cholestérol, nous révélons que l'appareil de Golgi des cellules FRT ne contient pas plus de cholestérol que dans les conditions contrôles. Finalement, ces résultats sont renforçés par le fait que le TGN dans les cellules FRT apparait plus clairsemé et reste inchangé après addition de cholestérol contrairement aux cellules MDCK (Stuven et al., 2003; Ying et al., 2003).

Nos résultats suggèrent que l'environment membranaire de l'appareil de Golgi des cellules FRT est déjà saturé en cholestérol ce qui induit une rigidification de ces membranes Golgiennes et le faible coefficient apparent de diffusion pour toutes nos protéines étudiées (indépendamment de leur association aux domaines membranaires rafts et du tri des protéines). Enfin, l'addition ou la déplétion du cholestérol n'affecte pas la polarité des protéines glypiées apicales et basolatérales dans les cellules FRT contrairement à nos observations précédentes dans les cellules MDCK (Paladino et al., 2008 ; Lebreton et al., 2008).
De ce fait, le cholestérol ne régule pas le tri apical des protéines glypiées dans les cellules FRT.

Analyse du rôle de la N-glycosylation dans le tri apical des protéines glypiées dans les cellules FRT:

Après avoir exclu un rôle du cholestérol dans le tri apical des protéines glypiées dans les cellules FRT, nous avons étudié le rôle putatif de la N-glycosylation comme signal de tri apical des protéines à ancre GPI dans les cellules FRT.

Il a été démontré que la N-glycosylation joué un rôle dans le tri apical de certaines protéines transmembranaires et sécrétées (Rodriguez-Boulan and Gonzalez, 1999; Potter et al., 2006). Cependant, le rôle de la N-glycosylation dans le tri apical des protéines glypiées est controversé (Benting et al., 1999a; Potter et al., 2004; Catino et al., 2008). Benting et al (Benting et al., 1999a) ont révélé que l'addition du signal d'attachement de l'ancre GPI du DAF à une hormone de croissance de rat ne résulte pas dans le tri apical de cette protéine chimérique. Cependant, l'addition de N-glycans conduit à un tri apical efficace de cette protéine.

De la même façon, le retrait des N-glycans sur la protéine glypiée dipeptidase conduit à une localisation préférentielle de cette protéine à la surface basolatérale dans les cellules MDCK et Caco2 (Pang, S. et al., 2004). Néanmoins, un mécanisme de tri apicale indépendant de la N-glycosylation à été reporté pour la protéine glypiée endolyn (Potter, B.A. et al., 2004). En accord avec cette étude, nos précédents travaux utilisant des mutants de glycosylation et des protéines chimériques ont montré que ni la *N* ni la *O*-glycosylation sont nécessaires pour le tri apical des protéines glypiées dans les cellules MDCK (Catino et al., 2008).

En prenant en compte les différences concernant le tri apical des protéines glypiées entre les cellules MDCK et FRT, nous avons décidé d'étudier le rôle de la N-glycosylation dans le tri apical des protéines à ancre GPI dans les cellules FRT. Le traitement des cellules FRT exprimant stablement PLAP ou GFP-NO-GPI avec de la tunicamycin qui inhibe la N-glycosylation induit un tri basolatéral de ces protéines. De plus, nous montrons que dans les cellules FRT la protéine PLAPΔN (protéine

mutante ou les deux sites de N-glycosylation sont mutés) est envoyé à la surface basolatérale des cellules et perd sa capacité de former des oligomères.

L'ensemble de ces résultats indique que dans les cellules FRT, la modification post-traductionelle N-glycosylation est essentielle pour le tri apical des protéines glypiées indépendamment du contenu en cholestérol de l'environement membranaire au sein de l'appareil de Golgi. Ces resultats révèlent un rôle inattendu de la N-glycosylation dans l'oligomerization des protéines glypiées et indique que différents mécanismes peuvent conduire au tri apical des protéines glypieés dans les différents épithéliums.

Les rafts ne sont pas impliqués dans le tri apical des protéine à ancre GPI dans les cellules FRT :

Les domaines membranaires rafts enrichis en cholestérol et sphingolipides constitueraient des plateformes pour le tri apical. Cependant, le role des rafts dans le tri apical des protéines glypiées est controversé. Nos résultats démontrent que les rafts ne sont pas impliqués dans le tri apical des protéines glypiées dans les cellules FRT. Dans ces cellules, la déplétion de cholestérol augmente le coefficient apparent de diffusion des protéines apicales associées ou non aux rafts (GFP-NO-GPI et P75-GFP) alors que ce même coefficient restent faible pour les protéines basolatérales. Comme les protéines glypiées apicales et basolatérales sont associées aux DRM, leurs réponses différentielles peut reflecter la ségrégation de ces protéines dans des domaines différents de l'appareil de Golgi. Cependant, le fait que toutes les protéines apicales indépendamment de leur association aux domaines rafts sont affectées, suggère que l'effet induit par la déplétion de cholestérol ne résulte pas d'une

perturbation des domaines lipidiques rafts. En adéquation avec cette hypothèse, la déplétion en cholestérol ne conduit pas à un tri basolatéral de ces protéines glypiées contrairement à nos précédentes observations dans les cellules MDCK (paladino et al., 2004 ; Paladino et al., 2008 ; Lebreton et al., 2008). Dans l'ensemble, ces résultats supportent que dans les cellules FRT ni la ségrégation entre les protéines glypiées apicales et basolatérales dans les membranes Golgienne ni le tri apical de ces protéines ne dépend de l'association de ces protéines avec les domaines membranaires enrichis en cholestérol.

Cette hypothèse est également supportée par le fait que l'inhibition de la synthèse des sphingolipides par FB1 ne perturbe pas l'association de PLAP avec les domaines rafts alors que cela provoque un tri basolatéral de cette protéine. Au cours de notre étude, nous avons confirmé et étendu ces données à GFP-NO-GPI indiquant donc que le tri basolatéral de ces protéines glypiées apicales dans les cellules FRT est indépendant de la perturbation des domaines raft. Afin de confirmer, notre hypothèse, nous avons testé si le traitement des cellules FRT avec FB1 affectait également le tri apical des protéines transmembranaire non associées aux rafts telles que p75-GFP et DPPIV une protéine endogène. Dans les cellules FRT, ces deux protéines P75-GFP et DPPIV sont addresses à la surface basolatérale des cellules suite à l'inhibition de la synthèse des sphingolipides et ce par un méchanisme encore inconnu qui ne corrèle pas avec l'association des domaines rafts. Les résultats montrent également que les protéines glypiées peuvent être triées apicalement ou basolatéralement tout en maintenant leur association aux rafts réaffirmant que cette association aux rafts n'est pas suffisante pour assurer un tri

apical des protéines glypiées et renforçant la notion que l'oligomerization est l'élément essentiel régulant le tri apical des protéines glypiées.

Cette étude soulève de nouvelles interrogations concernant la ségrégation des protéines glypiées apicales et des proteines transmembranaire apicales (raft et non-raft). Ces deux types de protéines voyagent ensemble dans les mêmes cargos où elles se segrègent au niveau du complexe Golgien ou dans les endosomes. De plus, y-a-t-il une protéine adaptrice spécifique dans les cellules FRT ?

Nos résultats indiquent qu'il existe au moins deux mécanismes qui déterminent l'oligomerization au niveau de l'appareil de Golgi et qui conduit au tri apical des protéines glypiées dans les cellules épitheliales polarisées. L'un de ces mécanismes dépend du cholestérol et l'autre dépend de la N-glycosylation et est insensible à l'addition ou à la déplétion en cholestérol.

INTRODUCTION

INTRODUCTION

I. Polarity of epithelial cells

I.1. Epithelia:

Epithelia are one of the major tissue types found in the body (Figure 1) and they are composed of closely packed cells, which are associated to each other through intracellular junctions.

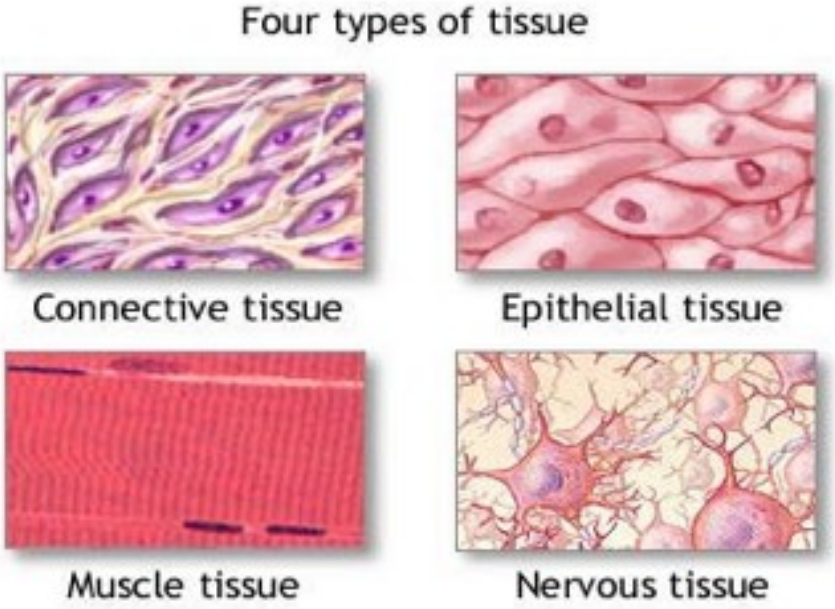


Figure 1: The four major tissue types in humans.

In the epithelial tissue, several epithelial cells are bound together to form a sheet. These sheets are held together through several types of interactions, including tight junctions, adherents, desmosomes and gap junctions. Epithelium is supported on the basal side by a basement membrane called basal lamina. Below the basal lamina a capillary bed is present, which provides epithelia with required nutrients and

disposal of waste products. There are two kinds of epithelial tissues, *protective epithelium* that covers the outer surfaces of the body and lines the internal organs and *glandular epithelium* that secretes hormones or other products.

Protective Epithelium: The epithelial tissue that covers or lines the surfaces is known as protective epithelium and it is further classified according to cell shape and by the number of cell layers. Depending on the cell shape they are classified as squamous cells (flat cells with the appearance of fried egg), cuboidal cells (cells with cuboidal or hexagonal shape, mainly involved in secretion and absorption functions), columnar cells (tall cells with column shaped, mainly involved in protection and absorption of the substances) and transitional cells (ranges from flat to tall cells that can extend or compress in response to the body movement) (Figure 2).

Depending on the number of cell layers they are classified as simple (single layer of cells), stratified (epithelium consists of multiple layers) and pseudostratified (single layer of cells with different sizes, giving the appearance of multi layered) (Figure 2).

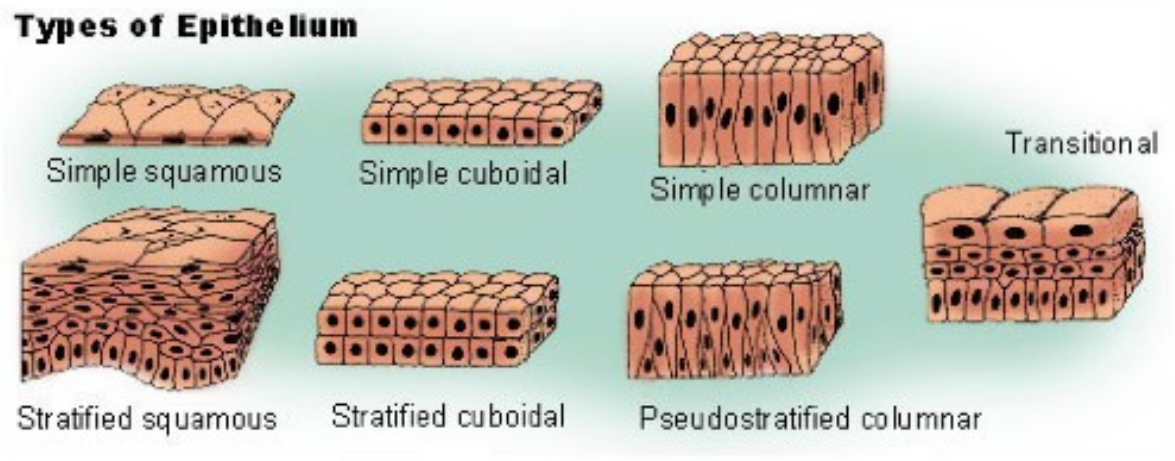


Figure 2: Different types of protective epithelium

Glandular epithelium: Glandular epithelium forms the covering of all major glands. There are two major types of glands: *endocrine glands*, which secrete hormones directly into the blood stream and *exocrine glands* that secrete their substances into

tubes or ducts, which carry these secretions to the epithelial surface. Exocrine glands are further classified according to their structure into unicellular (single cell gland) and multicellular (gland consists of a group of secretory cells and a duct) glands. The main function of glandular epithelium is the secretion. The goblet cells present in the glandular epithelium are specialized in the synthesis and secretion of several chemicals such as enzymes, hormones, milk, mucus, sweat and saliva. In addition glandular epithelium present in the intestinal linings helps in the absorption of nutrients and in the process of digestion (Figure 3).

To properly regulate all these functions epithelial cells acquire a characteristic cell polarity.

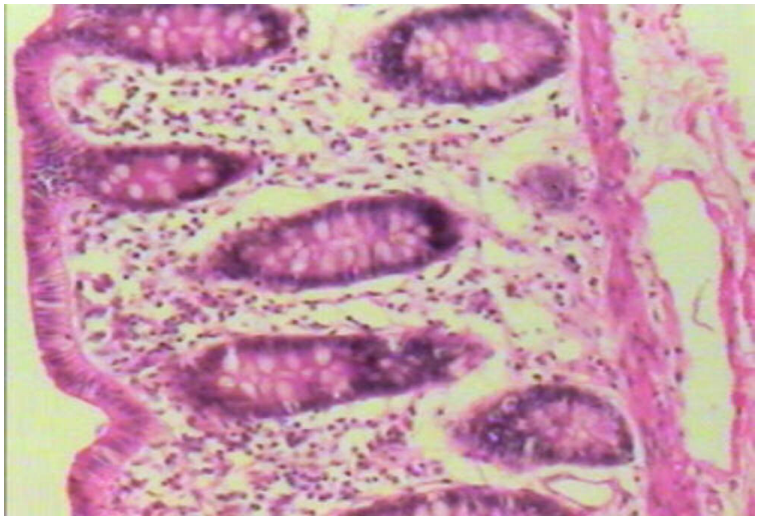


Figure 3: Glandular epithelium

I.2. Cell polarity in epithelial cells:

Epithelia represent an interface between the outside world and the internal system of the organism. Depending on the organ or the state of differentiation, epithelial cells are specialized in particular functions such as absorption, secretion and vectorial epithelial transport of fluids, electrolytes and proteins. For proper regulation of these functions and to control material flow into one precise direction epithelial cells have to maintain a polarized organization (Delacour and Jacob, 2006). The process of cell polarization is guided by different polarization events: the initial step is an influx of information from the extracellular milieu (Delacour and Jacob, 2006) and the following step is the establishment of cell-cell and cell-extracellular matrix contacts, which finally results in the formation of tight junctions (Figure 4) and in the reorganization of the cytoskeleton (Yeaman et al., 1999; Rodriguez-Boulan et al., 2005). The reorganization of the microtubule network to an apico- basolateral array also contributes to the acquisition of cell polarity (Yeaman et al., 1999). This process leads to the division of the plasma membrane in two structural, functional and biochemical domains known as apical and basolateral domains (Mostov et al., 2003).

The apical domain of epithelial cells is usually in contact with the external surface of an organism or with the body cavities, while the basolateral surface can be divided into two regions: the basal surface lies on a basement membrane and it is in close proximity to blood vessels and capillaries, whereas the lateral surface is adjacent to other cells in the same epithelium and contains specialized junctional domains that allow interactions among adjacent cells (either simple mechanical adhesion as in the case of tight junctions, adherent junctions and desmosomes or

metabolic cooperation via gap junction) (Brown and Breton, 2000). The apical and basolateral domains have distinct morphologies based on their prominent organ-specific function. The apical domain contains most of the proteins necessary for the specialized function of the organ. For example, in enterocytes the apical plasma membrane is characterized by a brush border composed of microvilli, which plays a role in the magnification of the cell surface and improve the absorption and exchange properties of the tissue. Whereas, in hepatocytes the apical pole is concentrated surrounding the bile canaliculi, where the bile secretion takes place (Aroeti et al., 1998).

In general, the apical and basolateral domains are composed of different proteins and lipids (Rodriguez-Boulan and Powell, 1992). For example, in intestinal cells the apical plasma membrane is enriched in intestinal hydrolases, whereas the basolateral domain is enriched in E-cadherin and integrins, which play a role in the formation of cell/cell or cell/ECM contacts. Lipids such as cholesterol and sphingolipids are enriched in the apical domain, whereas phosphotidylcholine is enriched in the basolateral domain (Simons and Van Meer, 1988). In epithelial cells this polarized architecture is stabilized by the tight junction complex, which prevent the diffusion of the proteins and lipids between apical and basolateral domains of the plasma membrane by determining the function of the exoplasmic leaflets of the plasma membrane of adjacent cells (Delacour and Jacob, 2006).

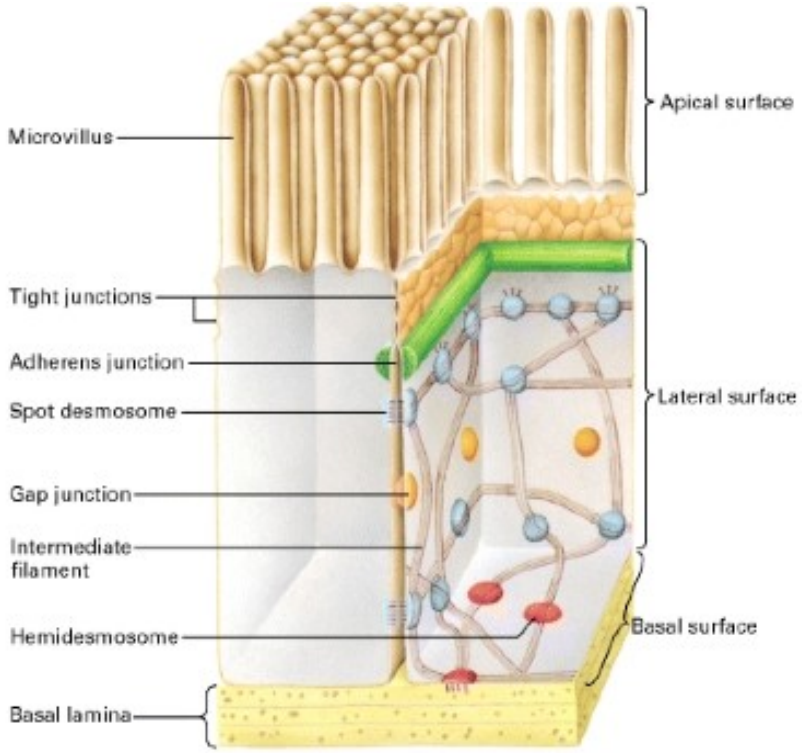


Figure 4: Epithelial cells and organization of junctional complex

Schematic diagram of epithelial cells lining the small intestine. Here the basal surface of the cells rests on the basal lamina, a fibrous network of collagen and proteoglycan that supports the epithelial cell layer. The apical surface faces the intestinal lumen. Tight junctions, lying just under the microvilli, prevent diffusion of substances between the intestinal lumen and the blood via the extracellular space between the cells. Gap junctions allow movement of small molecules and ions between the cytosol of adjacent cells. The remaining three types of junctions, adherens junctions, spot desmosomes and hemidesmosomes are critical to cell-cell and cell-matrix adhesion.

Molecular Cell Biology, Lodish H et al., 2000, 4th edition.

The cell polarity is maintained during the lifetime of an epithelium by constant plasma membrane turnover of lipids and proteins. Hence a continuous sorting of newly synthesized molecules and recycling of membrane components are required to maintain the molecular asymmetry at the cell surface.

In addition, studies on model organisms such as yeast, worms and flies have led to the identification of core protein complexes that regulate various aspects of cell polarity. Three major polarity complexes, the PAR (CDC42-PAR3-PAR6-aPKC), Crumbs (Crb-PALS-PATI) and Scribble (Scrib-Dlg-Lgl) have been shown to be

involved in the assymetric cell division, epithelial cell polarization and cell proliferation (Glodstein et al., 2007; Macara et al, 2004). These complexes distribute assymetrically in the cells, promoting the establishment of their associated membrane domain. The PAR complex can be devided into two sub complexes: apical CDC42-PAR6-aPKC and tight junction (TJ)-localized PAR3-aPKC, which recruits the lipid phosphatase PTEN (Phosphatase and tensin homolog). In epithelial cells the PAR and Crumbs complexes localized at the apical surface co-operate in the establishment of the apical domain and in the assembly of tight junctions. More over, in MDCK cells it was shown that this complex plays a role in the biogenesis of the primary cilium (Fanf et al., 2004; Wang and Margolis, 2007; Weisz and Boulan, 2009). Conversely the Scribble complex, which is localized at the basolateral membrane, is involved in the basolateral exclusion of apical proteins (Bryant, D.M and Mostov, K.E, 2008; Tanos, B and Rodriguez-boulan, 2008). In addition, recent evidences revealed that polarity complexes are also involved in the regulation of microtubule cytoskeleton and phosphoinositol phosphate synthesis (Bryant, D.M and Mostov, K.E, 2008).

Along with the protein complexes, phosphoinositol-phosphates (PtdInsPs) have been shown to be involved in the regulation of cell polarity. In particular PtdIns(3,4,5)P_3, which is generated from PtdIns 4,5)P_2 by a family of PtdIns3-kinases (PI3K), and PtdIns(4,5)P_2 generated from PtdIns(3,4,5)P_3 by PTEN (a 3-phosphatase) seem to play an important role in the establishment of cell polarity. In polarized MDCK cells it was shown that PtdIns (3,4,5)P_3 localizes exclusively at the basolateral surface, whereas PtdIns(4,5)P_2 localizes mainly at the apical surface. The asymmetric distribution of these two phosphoinositol-phosphates is regulated by specific recruitment of PI3K (which synthesizes PtdIns(3,4,5)P_3) to the basolateral

domain and exclusion of the phosphatase PTEN from the basolateral domain and it's enrichment at the apical domain (Tanos and Rodriguez-boulan, 2008).

The PtdIns (4,5) P_2 has been shown to be involved in mainly in the endocytosis by mediating the activity of different proteins important for assembling clathrin-coated vesicles, including AP-2 and Epsin. Whereas, PtdIns(3,4,5)P_3 regulates transcytosis of basolateral membrane components. Interestingly, it was shown that the presence of PtdIns(3,4,5)P_3 is able to transform an apical membrane into a basolateral membrane (Gassama-diagne et al., 2006). In addition, growing evidences suggest that phosphoinositides can control the activity of PAR complex, there by regulating the epithelial polarity (Gassama-diagne et al., 2009).

The alteration in the expression of polarity complex genes lead to a variety of patho-physiological conditions that cause different human diseases such as Microvillus inclusion disease (Davidson's disease), an autosomal recessive disorder which is caused by the defect in microtubule mediated apical delivery of the proteins (Davidson et al., 1978). Furthermore, it was shown that in several carcinomas there is an alteration in the expression and localization of polarity complex proteins. This leads to the loss of epithelial polarity and to the acquisition of mesenchymal properties, a process involved in the progression of metastasis of the tumors (Huang et al., 2011).

To understand the so-called "diseases of protein sorting" (Brown and Breton, 2000), it is essential to understand the basic mechanisms of sorting by different cell models and study how the cell sort, deliver, insert and maintain proteins in their correct cellular location.

I.3. MDCK cells and other model systems

One of the fundamental questions is how epithelial cells establish and maintain their polarity. The understanding of these processes became experimentally approachable when it was discovered that Madin-Darby canine kidney (MDCK) cells develop a tight epithelial monolayer when they were plated on a permeable substratum (Cereijido et al., 1978) (Figure 5a).

The MDCK cells became a model cell system to study the polarized protein targeting after the finding that influenza virus assemblies from apical surface and vesicular stomatitis virus (VSV) assemblies from the basolateral surface of MDCK cells guided by the polarized distribution of their envelope glycoproteins influenza hemagglutinin (HA) and VSVG protein (VSVG) (Rodriguez-Boulan et al., 1978; 1980; 2005). These two proteins provided the first evidence of polarized biosynthetic routes and sorting at the Golgi complex and they are still widely used as apical and basolateral markers to study polarized protein targeting (Rodriguez-Boulan et al., 1978**;** 2005) (Figure 5b).

The studies of virus infected MDCK cells by influenza hemagglutinin (HA) and VSVG proteins lead to the discovery of Trans Golgi Network (TGN) (Figure 6) (Griffiths and Simons, 1986) as a major sorting compartment in the biosynthetic route (Matlin and Simons, 1984; Misek et al., 1984; Rindler et al., 1984; Rodriguez-Boulan et al., 2005). In addition recombinant live imaging studies using apical and basolateral cargo proteins tagged with green fluorescent protein (GFP) showed that these proteins accumulate in the TGN at 20°C (Polishchuk et al., 2004; Kreitzer et

al., 2003). Their exit from the Golgi complex and delivery to the cell surface is visualized using time-lapse fluorescence microscopy, while total-internal-reflection microscopy (TIRF) revealed events corresponding to the arrival of the proteins to the plasma membrane (Polishchuk et al., 2004; Kreitzer et al., 2003, Rodriguez-Boulan, 2005) (Figure 5d). Since the establishment of MDCK cells as cellular model to study protein trafficking the number of publications on epithelial cell polarity, epithelial morphogenesis and the epithelial junction complex have increased revealing the importance impact of the MDCK cellular model.

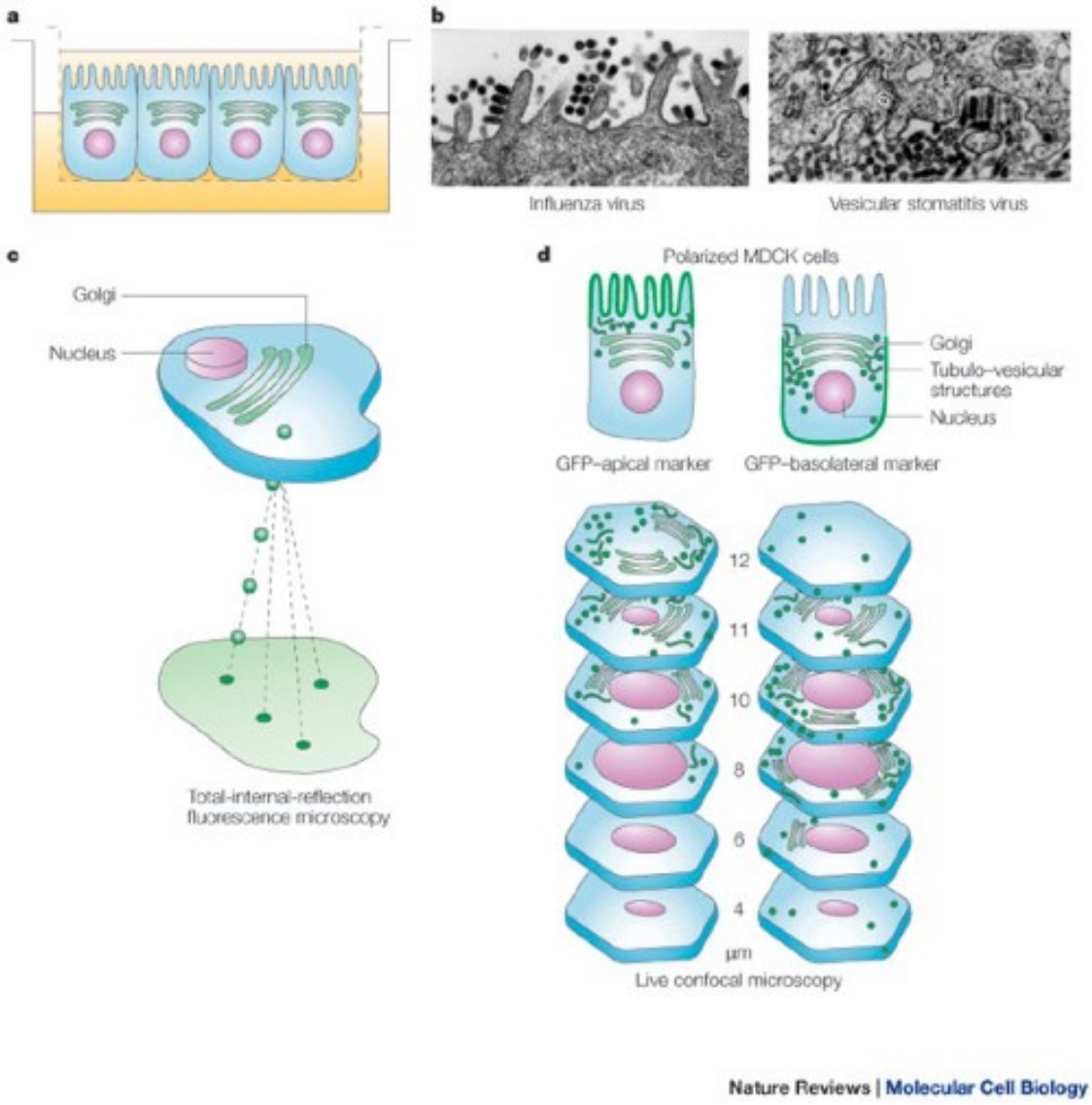


Figure 5: The Madin-Darby canine kidney (MDCK) cell model. *(from Nature Reviews, Molecular Cell Biology, March, 2005, vol 6, No 3; Rodriguez-Boulan et al., 2005).*
The exit from the Golgi complex and deliver to the cell plasma membrane of the apical and basolateral proteins was monitered by using GFP (green fluorescent protein)- tagged proteins. Their movement was followed by different microscopic techniques.

Subsequently other cell lines, like FRT (Fisher Rat Thyroid) or CaCo-2 (Colon Carcinoma) cells, were discovered to retain the ability to form polarized membrane

domains in tissue culture and provided as good experimental systems to study the mechanisms of protein sorting (Nelson and Rodriguez-Boulan, 2004). These other cell systems have allowed to confirm and extend the findings in MDCK cells to other epithelial cells allowing to demonstrate the similarities and differences in the mechanisms and molecular components of the apical and basolateral pathways (Rodriguez-Boulan *et al.*, 2005).

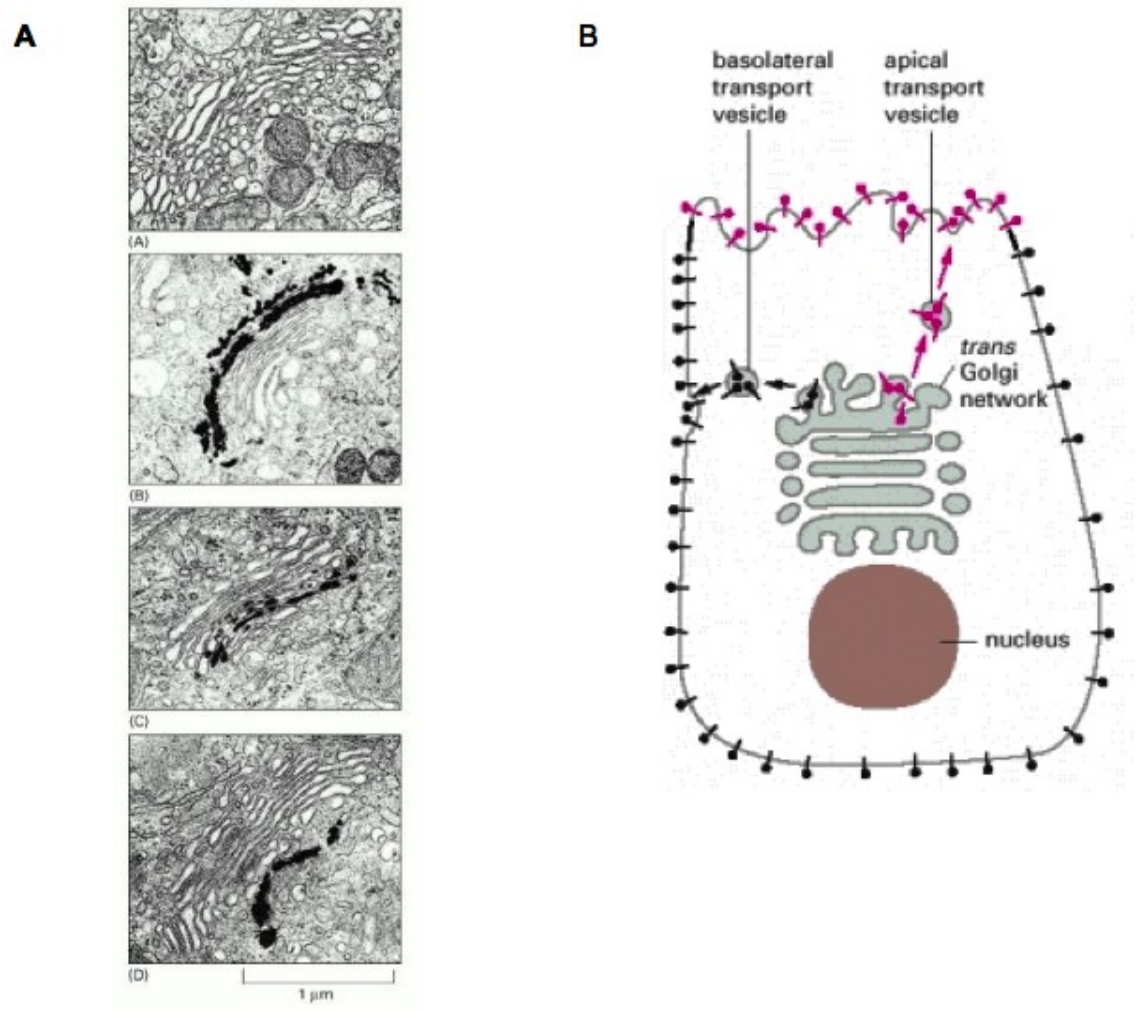


Figure 6: Gogi apparatus

A Series of electron micrographs showing the Golgi apparatus unstained (A), Cisternae of Cis compartment (B, stained with osmium), Cisternae of trans (C, stained with the resident enzyme nucleotide diphosphotase), TGN (D, stained with the resident enzyme acid phosphatase). B. The first sorting mechanism studied in epithelial cells, representing the biosynthetic pathway of apical (pink pathway in the figure) and Basolateral (black pathway in the figure) proteins from TGN to the plasma membrane.

Molecular Biology of the Cell, Alberts.B., fourth edition, 2002

II. The mechanism of polarized sorting:

The functional polarity of epithelial cells depends on the selective insertion of proteins and lipids into distinct plasma membrane domains. This polarized distribution is an important event for different categories of proteins including membrane transporters, channels, enzymes, cell adhesion molecules and junctional components and allows the cells to carry out the vectorial transport of fluid, ions and other molecules across the epithelial barrier (Brown and Breton, 2000). The mechanisms ensuring the protein transport from the TGN to the plasma membrane can be divided into following steps:

1) Protein segregation or sorting into distinct carrier vesicles.
2) Transport of carrier vesicles to the plasma membrane.
3) Docking and fusion of the carrier vesicles to the plasma membrane.

II. 1. Protein segregation or sorting:

II. 1.1 Site of segregation:

After their synthesis in the ER all the secretory proteins pass through the Golgi complex, where they are subjected to post translational modifications and undergo segregation into distinct vesicles at the level of trans-Golgi network (TGN) before being sorted to their final destinations (PM or endolysosomal compartments). Evidences such as studies on virus infected MDCK cells (Rindler et al., 1984; Rodriguez-Boulan et al., 1984) and early live imaging studies demonstrate the segregation of newly synthesized proteins at the level of the Golgi complex (TGN) into distinct tubulovesicular carriers to pursue a direct route to the plasma membrane

without apparently derouting through endosomes (Keller et al., 2001; Folsch et al., 2009).

In the past several years, this relatively simple model of segregation has been challenged by the observation that biosynthetic cargo traverses intermediate compartments en route from the TGN to the plasma membrane (Folsch et al., 2009). Orzech et al. showed that the biosynthetic route traveled by PIgRs (polymeric immunoglobulin receptors) could involve CREs (common recycling endosomes), suggesting that the CRE might also serve as a polarized sorting station on the biosynthetic pathway (Orzech et al., 2000). Evidences accumulated over a decade and most recent studies have shown that the biosynthetic route of at least some proteins includes a post-TGN transit route through REs (recycling endosomes) (Ang et al., 2004; Cancino et al., 2007; Cresawn et al., 2007; Gravotta et al., 2007). All these findings have led to the speculation that sorting of some proteins is not confined to the Golgi complex but instead may occur at multiple locations along the biosynthetic pathway (Ellis et al., 2006; Folsch et al., 2009).

According to the present understanding the segregation of proteins at the TGN and recycling endosomes is based on the recognition of specific apical and basolateral signals present on the proteins by intracellular sorting machinery, which mediates the incorporation of these proteins into specific carrier vesicles (Ellis et al., 2006; Matter et al., 2000; Mellman et al., 2008; Wandinger-ness et al., 1990).

II. 1.2 Sorting signals:

II.1.2.1 Basolateral sorting signals: The finding that basolateral proteins have a sorting signal was surprising. Indeed earlier it was thought that the transport to the

basolateral membrane occurred by a default mechanism (Simons and Wandinger-ness, 1990). Surprisingly many studies have confirmed that basolateral sorting signals exists in the cytoplasmic domain of the proteins and they play a role in the protein biosynthetic and recycling trafficking (Hunziker et al., 1991; Brewer and Roth, 1991). According to the present understanding basolateral sorting signals are relatively well defined compared to the apical sorting signals. Different types of basolateral sorting signals and their bibliographic references are shown in the table (1).

The existence of basolateral sorting signal in the cytoplasmic domain of the protein was first shown by Keith Mostov and his colleagues for the Polymeric immunoglobulin-A receptor (PIgR) (Mostov et al., 1986). The deletion of the cytoplasmic domain in PIgR induced its apical missorting. Later it was shown that a specific 14 amino acid sequence present in this domain was sufficient to redirect an apical placental alkaline phosphatase (PLAP) to the basolateral domain of the plasma membrane (Casanova et al., 1991) indicating that this amino acid sequence was playing a role specifically in the basolateral localization of the protein.

Later it was shown that the protein motifs containing Tyr, plus Leu-Leu or Leu-Val, localized in the cytoplasmic region near the transmembrane domain of the protein, were able to direct proteins to the basolateral plasma membrane domains (Rodriguez-Boulan and Powell, 1992) and the aminoacid sequence YXXΦ has also been found mediating basolateral sorting (Nelson and Yeaman, 2001).

Many findings demonstrates the role of tyrosine based motifs in the basolateral sorting such as replacement of the cysteine with tyrosine in the cytoplasmic domain of the apical Hemagglutinin (HA) protein leads to its basolateral missorting (Brewer and Roth, 1991). In addition, for the influenza virus hemagglutinin protein it was shown that the conversion of cysteine 543 to tyrosine is enough to missort the protein to the basolateral surface (Lin et al., 1997). Furthermore, it was reported that an internal deletion in the cytoplasmic tail of the P75 NTR, which placed of the cytoplasmic tyrosine residue closer to the membrane in a highly charged environment resulted in the basolateral distribution of the mutant protein.

Interestingly, it was shown that tyrosine-based motifs (Matter et al., 1992; Matter and Mellman, 1994) are also involved in the localization to coated pits and clathrin-mediated endocytosis (Chen et al., 1990; Collawn et al., 1990), and for some proteins such as low-density lipoprotein receptor (LDLR) it coincides with basolateral determinants (Hunziker *et al.*, 1991; Hunziker and Mellman, 1991). For example 10 residues (including the tyrosine) in the cytoplasmic domain of the human asialoglycoprotein receptor are sufficient for its basolateral polarity and efficient endocytosis (Geffen *et al.*, 1993). These motifs were identified in several proteins such as the low-density lipoprotein (LDL)-receptor (NPXY) (Matter et al., 1992), the transferrin receptor (YXRF) and the PIgA-R (YXXΦ) (Matter and Mellman, 1994).

The basolateral sorting activity of the signal is essential to establish the polarity of the protein, whereas the endocytosis signal permits the proper surface regulation of the protein (Muth and Caplan, 2003). The importance of a correct regulation of these processes has been demonstrated in the case of familial hypercholesterolemia,

where mutations to the LDL-receptor internalization domain prevent the uptake of low-density lipoproteins from the plasma membrane and into liver cells (Chen et al., 1990; Goldstein and Brown, 2001).

The finding that tyrosine dependent basolateral motifs are collinear with endocytic determinants suggests that the basolateral and the endocytic-sorting machineries share some common elements (Rodriguez-Boulan and Musch, 2005). Further, studies involving clathrin adaptors and most recent finding that clathrin itself involved in basolateral sorting support this notion (Deborde et al., 2008).

Another amino acid motif, which was shown to be acting as basolateral determinant, is the dileucine (LL) motif found within the cytoplasmic tail of some proteins, such as epithelial adhesion molecule E-cadherin (Miranda et al., 2001), sulfate/bicarbonate/oxalate anion exchange sat-1 (Regeer and Markovich, 2004), Fc receptors (Newton et al., 2005), MCAM-1 (melanoma cell adhesion molecule-1) (Guezguez et al., 2006) and NKCC1 (Carmosino et al., 2008). Furthermore, it has been shown that a single leucine motif also acts as basolateral sorting signal (Wehrle-Haller and Imhof, 2001).

Whereas basolateral motifs lacking any canonical consensus sequence have been also described in PIgRs (Casanova et al., 1991), transferrin (Odorizzi and Trowbridge, 1997), NCAM (neural cell adhesion molecule) (Le Gall et al., 1997) and TGF-β (transforming growth factor-β) receptor (Donoso et al., 2009).

In addition, other basolateral sorting signals like those in Kir 2.3 (inwardly rectifying potassium channels 2.3) (Le Maout et al., 2001), GAT-2 [GABA (γ-aminobutyric acid) transporter 2] (Perego et al., 1999; Brown et al., 2004) and

Syndecan-1 (Maday et al., 2008) are found at the extreme C-terminus and appear to involve juxtaposed PDZ-binding motifs.

All these findings contributed to demonstrate that basolateral sorting signals as endocytic recycling signals belonging to a family of peptide signals that interact with a family of organelle specific adaptor protein (AP) complexes.

II.1.2.1.1 Adaptors for basolateral sorting:

To ensure proper protein sorting the respective sorting signals present on the proteins need to be recognized by specific cytosolic complexes of clathrin adaptor protein (AP) family (Nakatsu and Ohno, 2003). These complexes are hetero tetrameric and comprise two large subunits (γ, α, δ, or ε and β1-β4): one medium (μ1-μ4) and one small subunit (σ1-σ2). There are four major species in this family: AP1, AP2, AP3 & AP4 (Heike Folsch, 2005). AP-1, AP-3 and AP-4 facilitates the sorting at the TGN or endosomes, whereas AP-2 acts at the plasma membrane to mediate internalization (Nakatsu and Ohno, 2003). In many cases tyrosine based basolateral sorting signals are recognized by an epithelial specific variant of AP-1, AP-1B, which is not expressed in fibroblasts and other non-polarized cells (Ohno et al., 1999). The role of AP-1B in the basolateral sorting has been shown in μ1B-deficient LLC-PK1 kidney cells. In these cells the two-basolateral proteins, low-density lipoprotein receptor (LDLR) and transferrin receptor (TfR), even though contains tyrosine based sorting signals, are missorted to the apical domain. This phenotype could be corrected by exogenous expression of μ1B (Folsch et al., 1999; Boulan, 2005). The two major functions of AP-1B are shown as interacting with basolateral cargos (Ohno et al., 1999; Fields et al., 2007) and facilitates the membrane recruitment of subunits of the mammalian exocyst, which is a vesicle tethering complex (Folsch et al., 2003)

mainly involved in fusion of basolateral carriers with the target membrane (Yeaman et al., 2004). In addition, recently, AP-4 has been shown to be involved in the basolateral sorting. Knockdown of the medium subunit of the AP-4 complex resulted in the missorting of LDL receptor in MDCK cells (Simmen et al., 2002). But until now it is not clear whether AP-1B and AP-4 interact together in the basolateral secretion.

Furthermore, where exactly these adaptors exert their functions in the basolateral pathway is highly debated especially for AP1B, which appears to function both in the TGN and recycling endosomal compartments (Heike Folsch, 2005; Boulan, 2005).

Proteins possessing a basolateral sorting signal	Reference
HA (mutant tyrosine)	Brewer and Roth 1991
P75 NGF Receptor (mutant PS)	LeBivic et al 1991
IgP 120 (lysosomal membrane glyco protein)	Hunziker et al 1991
Lysosomal acid phosphatase	Prill et al 1992
Asiao glycoprotein	Geffen and Spiess
Poly Ig Receptor (PIgR)	Casanova et al 1991
Fc Receptor	Hunziker et al 1991
LDL Receptor	Hunziker et al 1991
Transferrin Receptor	Huhn and Mellman, 1992

Proteins possessing a basolateral sorting signal	Type of signal
LDL receptor, Tfn receptor, VSVG, H/K-ATPase b subunit	Tyrosine (NPXY and Yxxϕ)
Fc II B2 receptor, E-cadherin	Di-Leucine (LL)
CD147	Leucine (L)

Table 1: Basolateral sorting signals

II.1.2.3 Apical sorting signals:

The delivery of proteins to the apical surfaces of epithelial cells depends on the coordination of multiple distinct mechanisms. In contrast to the basolateral sorting signals, apical sorting signals are of variable nature including peptide sequences and post-translational modifications (Matter, 2000; Rodriguez-Boulan and Musch, 2005; Mellman and Nelson, 2008; Gonzalez and Rodriguez-Boulan, 2009), such as lipid and sugar moieties, and they can be localized in the extracellular, transmembrane or intracellular domains of the cargo proteins (Weisz and Rodriguez-Boulan, 2009).

Among them is the lipid anchoring glycosylphosphotidylinositol (GPI), a post-translational modification that occurs in the ER. The GPI-anchor was the first apical sorting signal that was described. It was shown that addition of a GPI-anchor to the ectodomain of a basolateral protein resulted in its apical sorting (Lisanti et al., 1988; Lisanti et al., 1989; Brown et al., 1989). In addition, GPI-anchored proteins (GPI-APs)

were found to be preferentially localized at the apical plasma membrane of epithelial cells and are resistant to the detergent extraction at 4°C indicating that they are enriched in detergent resistant microdomains (DRMs) or rafts (see the introduction of GPI-anchored proteins part III) (Lisanti et al., 1989; Brown and Rose, 1992; Garcia et al., 1993; Wilson et al., 1990; Powell et al., 1991). Therefore, it was proposed that GPI-anchor mediates the apical sorting by raft association. Nevertheless, although GPI-anchor provides raft association, it is not sufficient for apical sorting. Indeed, it was shown that addition of GPI-anchoring motif to a soluble protein: rat growth hormone (rGH) is not sufficient for it's apical targeting (Benting et al., 1999). In addition it was shown that in FRT cells majority of endogenous GPI-APs are basolaterally sorted and in MDCK cells some GPI-APs, although DRM-associated, are sorted to both domains (Zurzolo et al., 1993; Lipardi et al., 1998; Paladino et al., 2002; 2004; 2006; 2007). All these findings indicate that GPI-anchor is not an apical sorting signal and association of rafts is not sufficient for apical sorting of GPI-APs (Paladino et al., 2004; 2007).

Hence, other determinants apart from GPI anchoring ensure proper apical delivery. Only good candidates for apical sorting signals are *N*-or *O*-glycans (Benting et al., 1999; Alfalah et al., 1999; Jacob et al., 2000; Yeaman et al., 1997).

Early evidences for the involvement of N-glycosyl chains in apical protein targeting came from the studies using *N*-glycosylation inhibitors. Treatment of MDCK cells with tunicamycin, a GlcNAc-analogue that inhibits the first step of *N*-glycosylation, led to the missorting of apical gp80 to both the membrane domains of MDCK cells (Urban et al., 1987). Later, by using glycosylation deficient cell lines,

glycans were showed to be involved in apical protein sorting. The impairment of *N*-glycosylation in ricin-resistant MDCK cell line induced mistargeting of apical glycoprotein gp114 (Le Bivic et al., 1993). Furthermore, recombinant addition of *N*-glycosylation chains to rat growth harmone (rGH) led to its apical localization (Scheiffele et al., 1995). Conversely, deletion of one *N*-glycosylation chain in erythropoietin resulted in impaired apical secretion in MDCK cells (Kitagawa et al., 1994). *N*-glycans have been shown to play a role in the apical delivery of endolyn (Ihrke et al., 2001), the glycine transporters (Martinez-Maza et al., 2001) and dipeptidase (Pang et al., 2004).

Nevertheless, *N*-glycosylation is not a universal apical sorting signal. Indeed some proteins are apically sorted independent of the presence or absence of *N*-glycosylation chains (Delacour and Jacob, 2006). Yeaman et al. (1997) have shown that *N*-glycans are not required for the apical sorting of p75NTR, which is a *N*-and *O*-glycosylated transmembrane protein. Other examples for *N*-glycan independent apical sorting are hepatitis B surface antigen (HBAgs) and Oesteopontin in MDCK cells (Marzolo et al., 1997; Trischler et al., 2001), and ecto-nucleotide pyrophosphates/phosphodiesterase NPP3 in CaCo-2 cells (Meerson et al., 2000). In addition, an apical sorting mechanism independent of N-glycosylation has been described for GPI-linked endolyn (Potter et al., 2004). Consistent with this data, our previous studies using glycosylation mutants and chimeric proteins have shown that neither N-glycans nor O-glycans are necessary for apical sorting of GPI-APs in MDCK cells (Catino et al., 2008). An interesting point is that some basolateral proteins are *N*-glycosylated but nevertheless they are not delivered to the apical membrane. This could be explained by the simultaneous presence of basolateral

targeting determinants with a high affinity for adaptor complexes that override apical sorting determinants present on the extracytosolic part of the protein (Simons and Ikonen, 1997; Delacour and Jacob, 2006).

O-linked glycosyl chains may also acts as an apical sorting signal. P75NTR and the hydrolase sucrase isomaltase (SI) contains heavily *O*-glycosylated stalk domains in close proximity to the membrane and deletion of these domains induced mistargeting of both p75NTR and SI from the apical to basolateral domain of plasma membrane (Yeaman et al., 1997; Jacob et al., 2000). Moreover, it has been shown that the position of *O*-glycans is crucial for apical sorting of human neurotrophin receptor and SI (Jacob et al., 2000; Breuza et al., 2002). Further evidence supporting the role of *O*-glycosylation in apical sorting is that addition of *O*-glycosylated stalk domain of SI to rat growth hormone (rGH) results in secretion of this hormone from the apical side (Spodsberg et al., 2001). Conversely, treatment of cells with a competitive inhibitor of glycosyl transferases (GalNAc α-O-benzyl), which resulted in the formation of truncated *O*-glycans, leads to trafficking defects of apical polypeptides by disturbing their association with lipid rafts (Alfalah et al., 1999; Gouyer et al., 2001; Naim et al., 1999; Slimane et al., 2000; Delacour et al., 2003).

Current studies have attempted to define which part of the *N*-glycan chain is involved in apical targeting. Weiss and coworkers proposed that terminal oligosaccharides constitute apical determinants of endolyn (Potter et al., 2004). On the other hand it was shown that the apical sorting signals are located in the core region of *N*-glycans of the H, K-ATPase beta subunit and in this case the terminal

region functions to stabilize the protein at the apical plasma membrane (Vagin et al., 2004).

Although these described findings highlight an involvement of *N*- or *O*-glycosylation in the apical delivery of proteins, the underlying mechanisms by which glycosylation mediating the apical trafficking is not known (Delacour and Jacob, 2006). Indeed, it remains to be determined whether glycans motifs are directly involved in apical targeting or they contribute indirectly to apical protein sorting. It was postulated that glycans can act either by stabilizing proteins, allowing access to a hidden apical sorting signal or by fixation of a competent conformation for apical sorting. Alternatively, glycosylation could have an impact on the lateral mobility of the protein in the membrane, which could affect the incorporation into membrane microdomains and delivery into apical carrier vesicles (Scheiffele et al., 1995; Simons and Ikonen, 1997; Rodriguez-Boulan and Gonzalez, 1999).

Apart from the *N*- and *O*-glycosylation, in some cases, it was shown that apical signals reside in the transmembrane domain of the proteins and mediate the association of the proteins with lipid rafts (Delacour and Jacob, 2006). Deletion of transmembrane domain of influenza virus neuraminidase (NA) and transferrin receptor demonstrated that transmembrane domain acts as a determinant for apical sorting and transport in MDCK cells (Kundu et al., 1996). Later Nayak and co-workers showed that several distinct regions of transmembrane domain of NA are involved in the apical delivery of NA and this apical sorting is independent of raft association (Barman and Nayak, 2000). In addition, it was shown that the primary amino acid sequence of transmembrane domain and its length are important for

determining the affinity of the protein for raft microdomains and therefore for apical sorting (Munro, 1995; Nezil and Bloom, 1992; Schuck and Simons, 2004).

In addition, two independent groups have found a putative apical sorting signal in the cytoplasmic tail of two seven-transmembrane-spanning proteins, rhodopsin (Chuang and Sung, 1998; Mellman et al., 2008) and the apical Na+- dependent bile acid transporter (Sun et al., 1998). In fact, it was shown that 39 and 40 amino acids, respectively, of the cytoplasmic tail of these two proteins were able to redirect these basolateral proteins to the apical surface in MDCK cells. Interestingly, these sequences are peculiar to these specific classes of proteins and there are no conserved residues between the cytoplasmic tails of these proteins (Rodriguez-Boulan, 2005).

Another specific case of apical sorting signal exits in megalin, which is the main endocytic receptor of the proximal tubule in the kidney, responsible for reabsorption of many filtered proteins (Kerjaschki and Farquhar, 1988). The cytoplasmic tail of megalin contains three NPXY motifs, YXXØ, SH3, and dileucine motifs in addition to PDZ-binding motif at its -COOH terminus.

Takeda et al., by deletion analysis, found that amino acids 107-136 of the cytoplasmic tail of megalin containing the second NPXY-like motif are critical for apical sorting and targeting, whereas the regions containing the first and third NPXY motifs are required for efficient endocytosis (Takeda et al., 2003). These studies indicate that cytoplasmic sorting machinery analogous to the one described for basolateral proteins might also exists for apically targeted proteins.

Apart from all these described structural apical sorting signals, the functional signals for apical transport have been recently reported (Delacour and Jacob, 2006). Paladino et al. showed that only apical and not the basolateral GPI-APs are able to oligomerize into high molecular weight complexes (Paladino et al., 2004; 2007). Interestingly, it was shown that protein oligomerization begins in the medial Golgi, concomitantly with DRM association (Paladino et al., 2004) and impairment of oligomerization leads to protein missorting indicating that protein oligomerization is an essential step for apical sorting of GPI-APs (Paladino *et al.*, 2004; 2007). Oligomerization could act by stabilizing the proteins into small lipid rafts which could then coalescence to form a larger functional sorting platform in the TGN from where apical vesicles emerge (Helms and Zurzolo, 2004; Paladino et al., 2004; 2007).

II.1.2.3.1 Apical receptors and protein sorting:

In analogy to the involvement of adaptor proteins in basolateral protein sorting, apical receptors might identify apical sorting signals to ensure proper apical protein delivery. Until now a variety of potential receptors have been discussed. Among one of them is the vesicular integral protein (VIP36). VIP36 has been isolated in the detergent resistant fractions of MDCK cells by Simons and coworkers (Fiedler et al., 1994). This lectin is present in the Golgi apparatus, at the apical plasma membrane and in endosomal or vesicular structures, and it was predicted to be involved in the glycoprotein trafficking from TGN to the plasma membrane (Fiedler et al., 1994). Later further studies demonstrated that VIP36 in fact involved in the early trafficking steps, such as trafficking from the ER to the Golgi apparatus and may not represent

an essential sorting factor for apical glycoproteins (Fullekrug et al., 1999; Hara-Kuge et al., 2002).

Another candidate that is also present in the lipid microdomains is VIP17/MAL. This protein has been showed to be involved in the apical delivery of HA, p75 NTR and some of GPI-APs such as GD1-DAF and PLAP in MDCK and FRT cells, respectively (Cheong et al., 1999; Martin-Belmonte et al., 2000; Puertollano et al., 1999).

In addition, recent data have highlighted the role of new lectin family, galectins, in the apical trafficking of proteins. Galectin-4, a 36-kDa protein is a major component of lipid rafts in enterocytes of the pig intestine (Danielsen and van Deurs, 1997) and in HT-29 cells (Delacour et al., 2005). This lectin interacts with high affinity with a specific variant of glycosphingolipids, the sulfatides with long-chain hydroxylated fatty acids, which are enriched in the lipid rafts in HT-29 cells. Depletion of galectin-4 by RNA interference impaired raft formation and accumulation of apical markers intracellularly (Delacour et al., 2005). This data suggests that the interaction between galectin-4 and sulfatides plays an important role in the organization of lipid rafts for efficient apical surface delivery (Delacour et al., 2005). More recently, another lectin, Galectin-3, a 29-kDa protein, has been identified in raft-independent apical carrier vesicles in MDCK cells. Depletion of Galectin-3 led to the mistargeting of LPH, p75NTR and gp114, suggesting that galectin-3 could play a role as apical sorting receptor and direct non-raft associated proteins into apical post-Golgi carriers (Delacour et al., 2006). The underlying mechanism of galectin-3 could be based on the formation of large galectin-3 containing clusters that direct the apical glycoproteins into newly formed apical vesicles (Delacour and Jacob, 2006). Even more recently, another galectin, galectin-9, has been shown to be interacting with

Forssman glycosphingolipid (FGL), a surface receptor in MDCK cells (Mishra et al., 2010). It has been proposed that the FGL-galectin couples play a role in the apical sorting of proteins and lipids by forming a circuit between Golgi apparatus and to the apical cell surface (Mishra et al., 2010).

These adaptor proteins binds to raft-associated ligands and generates a curvature in the vesicular membrane. This phenomenon may favor vesicle budding (Simons and Van Meer, 1988). Recent data indicates the involvement of novel coat proteins for apical carrier formation. PtdIns(4)P, a product of phosphati- dylinositol 4-kinase [PI(4)K] activity, is localized in the Golgi apparatus and regulates constitutive transport from the Golgi complex to the plasma membrane (De matteis et al., 2004). Moreover, FAPP1 and FAPP2 have been identified as effectors of PtdIns (4)P at the TGN level, indicating that they might control carrier vesicle formation, budding and fission (Godi et al., 2004). In addition, Simons and coworkers have shown that FAPP2 was required for apical carrier formation in MDCK cells (Vieira et al., 2005).

The apical and basolateral carrier vesicles that are emerging from TGN and recycling endosomes travel across the cytosol to specific areas of cell surface.

II.2. Transport of carrier vesicles to the plasma membrane:

The sorting of apical and basolateral proteins at the TGN and common recycling endosomes must be coordinated with their incorporation into specific carrier vesicles and the transport of these carriers across the cytosol to specific areas of cell surface. These different processes are coordinated by various factors including elements of the actin and microtubular cytoskeletons and their associated proteins.

II.2. 1 The microtubules cytoskeleton:

Many lines of evidences showed that there is a change in the arrangement of microtubules (MTs) depending on the polarity of the cell (Weisz and Rodriguez-Boulan, 2009). In contrast to the non-polarized MDCK cells, polarized MDCK and Caco-2 cells showed epithelial specific arrangement of MTs, with their minus ends facing the apical membrane and plus ends facing the basolateral membrane (Bacallao et al., 1989; Gilbert et al., 1991). Musch and collaborators have shown that the polarity protein kinase Par 1b is involved in the regulation of epithelial specific reorganization of MTs (Cohen et al., 2004a; 2004b). Four years ago, Kreitzer and co-workers have shown that in polarized MDCK cells there is a small population of very dynamic MTs emerging from the MT-organizing center (MTOC) that preferentially grow their plus ends towards the apical surface (Jaulin et al., 2007). It seems likely that this dynamic population of MTs originates the stable MTs by capturing of their negative ends to the junctional region of the lateral plasma membrane (Chausovsky et al., 2000; Shtuman et al., 2008).

Many evidences have shown that microtubules have a role in the apical-basolateral trafficking. Rindler et al. first showed that the depolymerization of the microtubules with chemical agents significantly reduces the polarity of apical protein HA, but did not affect the polarity of basolateral VSVG (Rindler et al., 1987). Subsequent pharmacologic experiments showed that both dynein and kinesin were involved in the apical delivery of the apical viral glycoprotein, influenza HA (Lafont et al., 1994; Tai et al., 2001). However other reports found no effect of MT disassembly on the virus budding polarity or found a slight retardation in the apical trafficking of

influenza virus HA without disruption of it's polarized delivery by the disassembly of MTs (Salas et al., 1986; van Zeijl and Matlin, 1990).

More recent work demonstrates that different MT motors are involved in the transport of specific apical cargos. The work of Chuang and sung demonstrated that in MDCK cells the transport of an apical protein rhodopsin was mediated by the interaction between cytoplasmic determinants with the microtubule motor protein dynein (Chuang and Sung, 1998). Later the work of Kreitzer and colleagues using antibodies to block the function of kinesin and dominant negative form of dynamin showed that kinesin and dynamin are required for different stages of post Golgi trafficking. Especially the Golgi exit of an apical chimeric protein P75-NTR-GFP is mediated by kinesin, while post Golgi vesicular trafficking of P75-NTR-GFP is mediated by both kinesin and dynamin (Kreitzer et al., 2000). More recently it was showed that KIF5B involved in the apical transport of P75-NTR-GFP (Jaulin et al., 2007). Furthermore, another microtubule motor protein KIFC3, was showed to be involved in the transport of apical protein HA and annexin-13b (Noda et al., 2001). It has been proposed that specific MT motors or myosin motors are involved in the cargo-clustering event, which takes place at the exit of sorting compartments and finally involved in the delivery of the vesicles to the apical plasma membrane (Weisz and Rodriguez-Boulan, 2009). Some MT motors, for example KIF 1A are thought to acquire the ability to interact with the lipid rafts upon dimerization (Klopfenstein et al., 2002). Similarly, some myosin motors such as myosin 1, 5 and 6, have the ability to oligomerize upon physiological stimuli and perform variety of unconventional functions such as tethering vesicles, organizing actin to bend membranes or regulating the activity of MT motors (Woolner and Bement, 2009). These activities

could contribute to the assembly of functional sorting rafts, recruitment of sorting machinery and the formation of sorting vesicles at the TGN or common recycling endosomes (CREs) (Weisz and Rodriguez-Boulan, 2009).

II.2. 2 The actin cytoskeleton:

Beneath the apical and lateral surface of the epithelial cells there is a dense network of actin filaments and the transport carriers destined for these domains must penetrate this dense network of actin. Their different organization is regulated by variety of proteins such as ezrin, villin and a CD317-Rich 2 complex for the subapical network and the E-cadherin system for the lateral network (Fievet et al., 2007; Rollason et al., 2009). The small G proteins of the mammalian Rho GTPase family (Rac, Rho and Cdc 42) are key regulators of actin dynamics at various intracellular sites. It was shown that the over expression of the small G protein, Rac 1 selectively inhibits the transport of newly synthesized and transcytosing proteins to the apical surface due to retention of apically destined proteins in a subapical aggregate that also contained various endocytic markers (Jou et al., 2000). Additionally expression of Cdc42 mutants in polarized MDCK cells inhibited the TGN export, biosynthetic delivery and polarity of the basolateral proteins VSVG and LDLR without affecting the polarity of the apical protein gp114 (Cohen et al., 2001; Kroschewski et al., 1999; Musch et al., 2001). The differential regulation of apical and basolateral routes by Rho GTPases might be explained by the use of different downstream effectors. For example, Rho isoforms regulate the activation of LIM kinase, a Golgi resident enzyme that inactivates the actin-severing protein cofilin, whereas Cdc42 is known to stimulate N-WASP-Arp2/3 mediated actin comet formation (Weisz and Rodriguez-Boulan, 2009).

Furthermore, the apical delivery of raft associated apical protein HA was found to be regulated by N-WASP-Arp2/3-dependent actin comet formation (Guerriero et al., 2006) whereas the non-raft associated apical protein P75 has been shown to be regulated by LIM Kinase 1 and cofilin, which probably regulate p75 by promoting a specific actin organization in the perinuclear region (Salvarezza et al., 2009). Interestingly, in non-polarized cells the TGN export of GPI- anchored proteins is not affected by LMK1 and, unlike p75, is not inhibited by the actin depolymerizing drugs (Jacob et al., 2003; Lazaro-Dieguez et al., 2007). Lazaro et al. showed that in non-polarized COS cells the exit of p75-GFP was inhibited by the treatment of cells with latrunculin, whereas GFP-FR was not affected by the same treatment (Lazaro-Dieguez et al., 2007). In contrast to this, recent work from our laboratory showed that the actin dependency of the same proteins (p75-GFP and GFP-FR) was different between polarized and non-polarized cells. By using the Fluorescence Recovery After Photobleaching (FRAP) analysis, Lebreton et al. (2008) showed that the Golgi organization is different between polarized and non-polarized cells. Interestingly, they showed that the segregation between apical and basolateral GPI-APs at the level of the Golgi complex is sensitive to the actin rearrangements in polarized MDCK cells (Lebreton et al., 2008).

Lastly some growing evidences shows that myosins also play a specific role in apical and basolateral protein trafficking. It was shown that myosin 5b involved in the trafficking between apical recycling endosomes (ARE) and the apical plasma membrane and Rab 11 regulates this process. Fath and Burgess in 1993, by using cell fractionation assays, showed that myosin-1 is present in apical post Golgi

vesicles suggesting a role in the apical sorting in biosynthetic pathway of intestinal cells (Fath and Burgess, 1993). Specifically myosin-1A has been showed to be involved in the apical trafficking of sucrose-isomaltase (Heine et al., 2005).

Myosins have also been involved in basolateral sorting. Both myosin 2b and myosin 5b was shown to be involved in the Golgi exit of the basolateral proteins and the transport of basolateral cargo proteins that require AP1B (Au et al., 2007).

In summary the microtubules and actin cytoskeletons have important regulatory roles in sorting of apical and basolateral proteins in the TGN, recycling endosomes and at the plasma membrane. The exact mechanisms underlying their regulation and the role of microtubules and actin cytoskeletons in the generation of transport intermediates are still largely unknown.

Once the apical and basolateral vesicles arrive at the plasma membrane, they will fuse with the membrane to unload their cargo. This process is called vesicle fusion.

II. 3. Vesicle fusion and role of SNARES:

Docking and fusion of carrier vesicles is based on protein tethering at the plasma membrane, and results in the secretion of the transported material from the cell. The earlier observation that membrane fusion has a role in the secretion came by the work of Novick and colleagues (Novick et al., 1980) on the yeast temperature sensitive mutant for secretion called sec. The electron microscopy studies of the sec mutant cells revealed that there is an accumulation of temperature dependent membrane enclosed secretory organelles (Novick et al., 1980). Further analysis of

these mutants revealed that in these mutants the secretion is blocked at the post-Golgi stage and a small GTPase binding protein that shares the homology with the Ras family regulates this process (Salminen and Novick, 1987).

Later Balch et al, in 1984, by using an *in vitro* assay, were able to reconstitute the transport between successive compartments of Golgi in mammalian cells. They showed that different cytosolic factors regulate the transport between donor and receptor compartments of the Golgi (Balch et al., 1984). These findings revealed that yeast and mammals shared conserved vesicular transport machinery. These earlier observations further led to many findings and to date we have a quite detailed molecular picture of mechanisms of trafficking in secretory pathway.

II. 3. 1 Discovery of SNARES:

An early contribution in understanding the molecular mechanisms of vesicle fusion came by the identification of membrane fusion of an N-ethylmaleimide-sensitive transport component (NSF), which could exists in cytosolic or membrane bound forms (Glick and Rothman, 1987). The electron microscopic studies by Lelio Orci demonstrated that when NSF was inactivated uncoated vesicles were accumulated on Golgi membranes, implying that NSF was required for membrane fusion (Malhotra et al., 1988). Further cloning of the corresponding gene revealed that NSF was the mammalian ortholog of yeast Sec18p, which had been implicated from ER to Golgi transport (Wilson et al., 1989; Eakle et al., 1988). Later many studies demonstrated that NSF acts in a wide range of membrane fusion steps in the secretory and endocytic pathways (Beckers et al., 1989; Diaz et al., 1989).

A crucial step in understanding the function of NSF came from the identification of its partner protein called SNAP (soluble NSF association protein), which binds NSF to the membrane (Clary et al., 1990). In addition, Thomas Sollner and colleagues, by the fractionation of brain lysate using NSF and SNAP as an affinity reagent, identified three membrane- associated SNAP receptors or SNAREs (Sollner et al., 1993). One of these proteins, known as VAMP or synaptobrevin, was shown to be associated with synaptic vesicles. The other two proteins known as syntaxin and SNAP-25 had been localized to the presynaptic plasma membrane. These same proteins have been shown to be involved in linking the synaptic vesicles to the plasma membrane (Walch-Solimenta et al., 1993).

II. 3. 2 SNARE hypothesis: The discovery of the SNAREs in the membrane fusion led to the proposal of the SNARE hypothesis. According to this hypothesis each type of transport vesicle carries a specific “V-SNARE” that bind to a cognate “t-SNARE” on the target membrane (Rothman, 1994). This hypothesis fits with the observation that cells contain families of proteins related to the synaptic SNAREs and that various SNAREs localized to different intracellular compartments (Bennett and Scheller, 1993; Weimbs et al., 1998; Chen and Scheller, 2001). Most of the SNAREs are C-terminally anchored transmembrane proteins containing a heptad repeat “SNARE motif” of 60-70 amino acids that can participate in coiled-coil formation (Bock et al., 2001). An exception is SNAP-25, which contains two SNARE motifs and binds to the membrane via covalently linked palmitate groups attached to the central part of the protein.

An alternative classification uses the terminology 'R' and 'Q' SNAREs depending on the presence of arginine and glutamine residues respectively, at the characteristic region of SNARE (Fasshauer et al., 1998). The R-SNAREs are always transmembrane proteins whereas Q-SNAREs can be either transmembrane or intrinsic membrane proteins (anchored by a myristate).

The different steps involved in the fusion process are represented in figure 7.

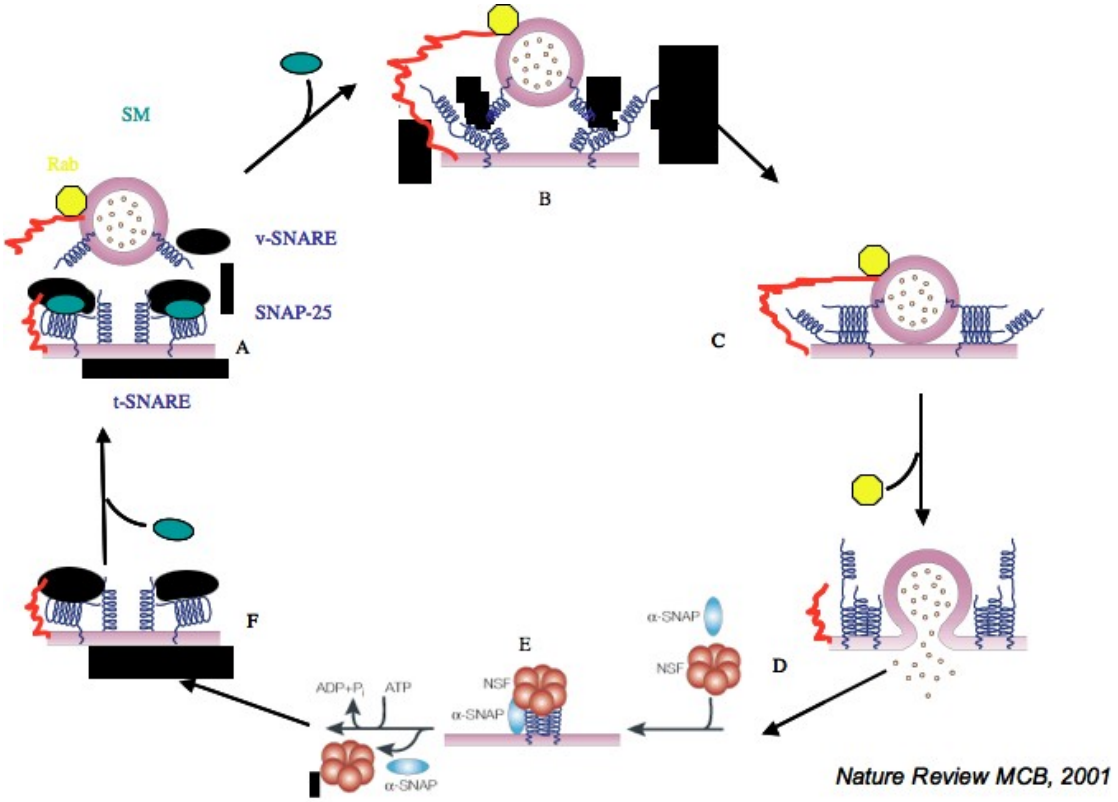


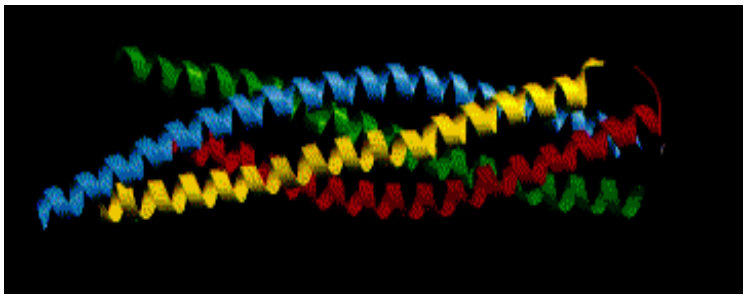
Figure 7: Molecular model of vesicle exocytosis.

A. Tethering: the two tethering proteins (in red) combine after the removal of SM (green) allowing the priming. **B. Priming**: association of t-SNARE with SNAP-25.
C. Docking: association of t-SNARE/ SNAP-25 with v-SNARE and formation of functional SNARE complex. **D. Fusion**. **E. Disassembly of SNARE complex** by NSF (orange)/ α-SNAP (blue), F. The SM binds with the t-SNARE and SNAP-25 until the next round of fusion.

Nature reviews Molecular Cell Biology 2;98-106 (2001).

In the first step, a trans SNARE complex assembly takes place when a monomeric V-SNARE on the vesicle binds to an oligomeric T-SNARE on the target membrane,

forming a stable four-helix bundle that promotes fusion. This results in a cis-SNARE complex in the fused membrane. Later α-SNAP binds to the complex and recruits NSF, which hydrolyzes ATP to dissociate the complex. Unpaired V-SNAREs can then be packed into vesicles.



t-SNARE
v-SNARE
SNAP-25 (Nter SNARE motif)
SNAP-25 (Cter SNARE motif)

Figure 8: SNARE complex

Studies on polarized epithelial cells showed that different SNAREs are involved in the sorting of polarized vesicles to the apical and to the basolateral domains (Lafont et al., 1999; Low et al., 1998; Schroeder et al., 1998; Steegmair et al., 2000; Weimbs et al., 2003). Later it was showed that different syntaxins are involved in the apical and basolateral vesicle fusion. The syntaxin-3 was found in the apical membrane, where as syntaxin-4 predominantly localized at the basolateral plasma membrane in MDCK cells (Low et al., 1998). Interestingly, SNAP23 and syntaxin 2 were found on both domains of the plasma membrane (Schroeder et al., 1998). These findings suggest existence of specific mechanisms in polarized sorting of apical and basolateral vesicles.

Many lines of evidences in the literature showed that SNAREs are involved in sorting of proteins, especially in the polarized transport of vesicles. For example, VAMP-2 that specifically interacts with syntaxin-4 has been shown to be involved in basolateral sorting. Two apical SNAREs, Syntaxin-3 and TI-VAMP, have been demonstrated to be involved in the regulation of apical sorting of transmembrane proteins and secretory proteins. In CaCo2 cells (human origin) Syntaxin-3 and TI-VAMP are predominantly localized at the apical domain of plasma membrane and they form a complex by interacting with SNAP23. Overexpression of the Syntaxin-3 in MDCK cells inhibits the biosynthetic transport from TGN to the plasma membrane and the recycling apical endosmal pathway, without affecting the transport to the basolateral membrane (Low et al., 1998). This finding was also proved in another cell system. In CaCo2 cells, overexpression of the Syntaxin-3 strongly inhibited the apical targeting of Sucrase Isomaltase (SI, transmembrane protein) and alpha-glucosidase (secretory protein), but there was no affect on the basolateral transport pathway (Breuza et al., 2000). The blocking of TI-VAMP by using antibodies inhibits the transport of apical protein HA and there was no effect on the basolateral transport pathway (Lafont et al., 1999). All the data obtained by the overexpression studies and the blocking of SNAREs by using antibodies demonstrated that there is a slowdown in the apical transport pathway without affecting the apical transcytosis. This can be explained by assuming the formation of inactive SNARE complex by the large number of SNAREs present on the surface of the vesicle, which finally results in decrease in the number of vesicles fusing with the plasma membrane. Recently it has been shown from our laboratory by using knockdown studies in FRT and CaCo2 cells that TI-VAMP mediates the direct apical delivery of both raft and non-raft associated proteins. On the contrary transcytosis pathway was not affected by TI-

VAMP knockdown but appears to be regulated by VAMP8. All these data illustrates a higher degree of specificity of V-SNARE function in polarized cells (Pocard et al., 2007).

II. 3. 3 Accessory and regulatory proteins: Cytosolic factors such as GATE-16 and LMA1 have been shown to be involved in the regulation of SNARE complex formation. These factors bind to the individual V-and t-SNAREs and help them to be separate until the next round of fusion takes place (Elazar et al., 2003). In some cases, the SNARE complex formation is regulated by the phosphorylation of SNAREs or it's interacting components (Gerst et al., 2003). In another cases the regulatory elements are present in the SNAREs itself. For example, the N-terminal three helix bundle of syntaxin and the N-terminal Longin domain are shown to be involved in the auto inhibitory function of SNAREs, by binding internally to the SNARE motif to generate a closed conformation (Misura et al., 2002; Dletrich et al., 2003). Another accessory protein, Ca^{2+} sensor synaptogamin has been shown to interact with SNAREs and promote synaptic vesicle fusion in response to Ca^{2+} influx (Jahn et al., 2003).

II.4. Routes to the surface (direct and indirect delivery of the proteins in epithelial cells):

After the segregation in the TGN apical and basolateral proteins are delivered to the respective apical and basolateral plasma membrane following either a direct

route from the Golgi apparatus or may pass through the intermediate sorting station, recycling endosomes (Ang et al., 2004) (See Fig 9).

In addition certain epithelial cells posses an alternative pathway by which proteins and lipids reach the final destination by an indirect route called transcytosis. The most common transcytosis pathway refers to the proteins first reaching the basolateral surface and then recycling back to the apical surface passing through the early endosomes and then through the recycling endosomes (Rodriguez-Boulan and Powell, 1992; Mostov et al., 2000). Many studies have been shown that the proteins, which follow transcytosis route, have specific sorting signals (Casanova et al., 1990; Mostov et al., 2000). Interestingly, not all the apical proteins follow the transcytotic route. This depends on the type of the protein and the type of the origin of cells. For example the apical proteins, dipeptidyl peptidase (DPPIV) and HA follow direct pathway from the Golgi to plasma membrane in MDCK cells, whereas in intestinal cells and hepatocytes they follow the transcytosis route (Casanova et al., 1991b; Bonilha et al., 1997). In hepatocytes, in contrast to MDCK cells, all proteins first go to the basolateral (sinusoidal) domain, from where apical proteins are transcytosed to the cananicular pole (apical pole) (Wilton and Matthews, 1996). Interestingly, it was shown that in another epithelial cells, FRT (Fisher Rat thyroid cells), the apical protein DPPIV changes its sorting from indirect to the direct pathway during the maturation of the epithelium (Zurzolo et al., 1992a). In contrast to the apical proteins, most of the basolateral proteins follow a direct route from the Golgi to the plasma membrane.

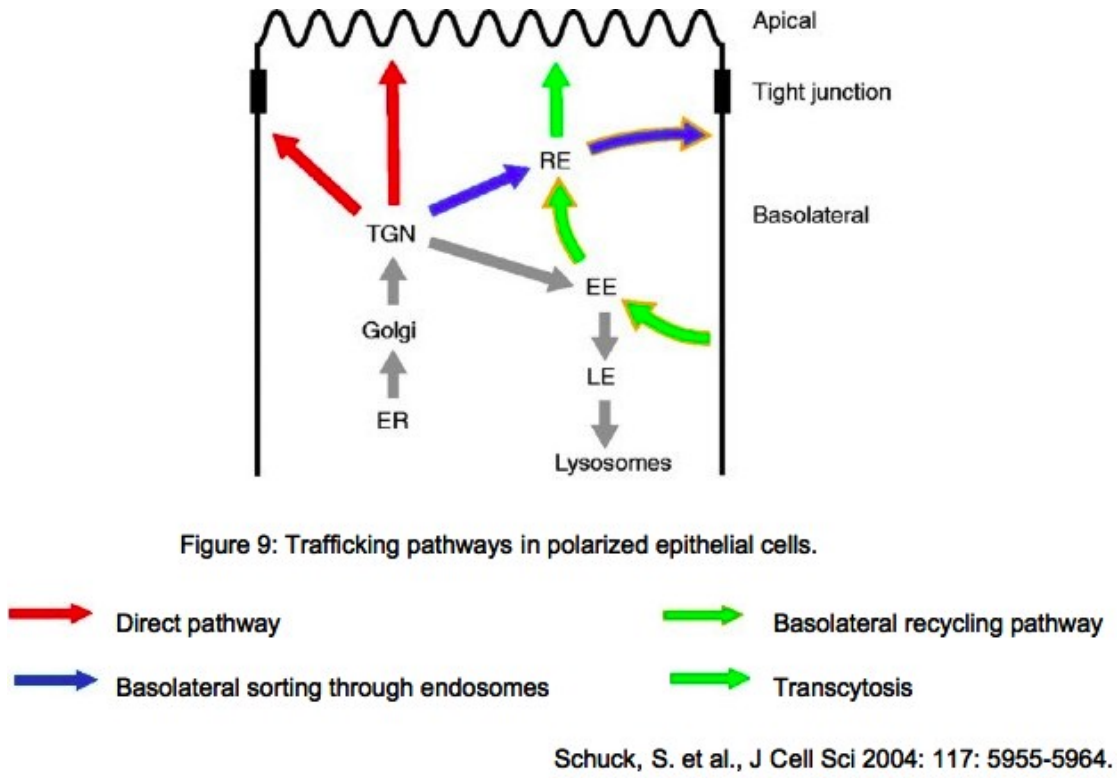


Figure 9: Schematic representation of different sorting pathways that exists in polarized epithelial cells.

From the TGN, cargo can be transported directly to the apical or basolateral membrane (red).
Basolateral cargo can also reach the plasma membrane through endosomes (blue).
Following intial transport to the membrane, apical cargo can be sorted to the apical membrane via the transcytotic route (green). The arrows marked with a gold outline together constitute the basolateral recycling pathway. EE, early endosomes; ER, endoplasmic reticulum; LE, late endosomes; RE, recycling endosomes; TGN, trans-Golgi network.

Thus these studies indicate that there are different sorting routes to plasma membrane depending on the type of the protein, cell type and state of differentiation of the cell (Zurzolo et al., 1992b; Rodriguez-Boulan et al., 2005).

In my PhD I became interested in understanding the sorting mechanisms of the GPI-anchored proteins in different model epithelial cells such as MDCK and FRT. These

two model cell lines have the property to sort GPI-APs differently. But interestingly oligomerization mediated apical sorting of GPI-APs is conserved in both cell lines. Therefore during my PhD I focused on the factors promoting the oligomerization, HMW complex formation and therefore apical sorting of GPI-APs in both MDCK and FRT cells.

III. GPI-ANCHORED PROTEINS:

Many membrane proteins are covalently linked to various lipids as fatty acids (Schmidt et al., 1979), isoprenoids (Glomset et al., 1990), diacylglycerols (Hantke and Braun, 1973) and glycosylphosphatidylinositols (GPI) (Ferguson et al., 1985) through post-translational modifications. All these modifications help to target the proteins to the membrane and in some peculiar cases to the membrane microdomains or rafts. Most of these modifications such as addition of isoprenoids or fatty acids take place in the cytoplasm, whereas the addition of palmitate and cholesterol takes place in the lumen of the endoplasmic reticulum (Mann et al., 2000; Mayor and Riezman, 2004). Another class of lipid modification such as addition of glycosylphosphatidylinositol (GPI) anchor occurs in the luminal side of the ER membrane (Mayor and Riezman, 2004).

GPI-anchored proteins are synthesized as precursors with a cleavable, hydrophobic amino- terminal signal sequence that targets the protein to the lumen of the ER and a cleavable, carboxy- terminal signal sequence that directs GPI anchoring (Figure 10a). The GPI-anchoring signal consists of a hydrophobic region, which is separated from the GPI-attachment site (ω-site) by a hydrophilic spacer

region (Figure 10b). The aminoacid with small side chains are highly preferred for the two amino acids that follow the ω-site. The glyco-lipid anchor is attached to the ω-site by the action of a GPI transamidase in the lumen of the endoplasmic reticulum (ER) (Mayor and Riezman, 2004). The core structure of GPI-anchor is conserved among all species that have so far been investigated (Ferguson, 1999; Ikezawa, 2002) and contained a phosphodiester linkage of phosphoethanolamine (PE) to a trimannosyl-glucosamine (Man3-GlcN) core (Figure 10c). The reducing end of GlcN is linked to phosphatidylinositol (PI). Phosphatidylinositol is then anchored through another phosphodiester linkage with diacylglycerol to the cell membrane through its hydrophobic region (Figure 10c). The core can be modified and indeed it is subjected to various remodeling reactions, during the secretion from the cell. Variations can occur by addition of extra sugars or ethanolamine phosphates to the mannose residues, acylation of the inositol ring, changes in the fatty acids (length, saturation, hydroxylation), or their types of linkage to the glycerol backbone (acyl to alkyl), or remodeling of the entire diacylglycerol to ceramide (Mayor and Riezman, 2004). At the end of the process of modification proteins bind to the membrane by the GPI-anchor (Figure 10).

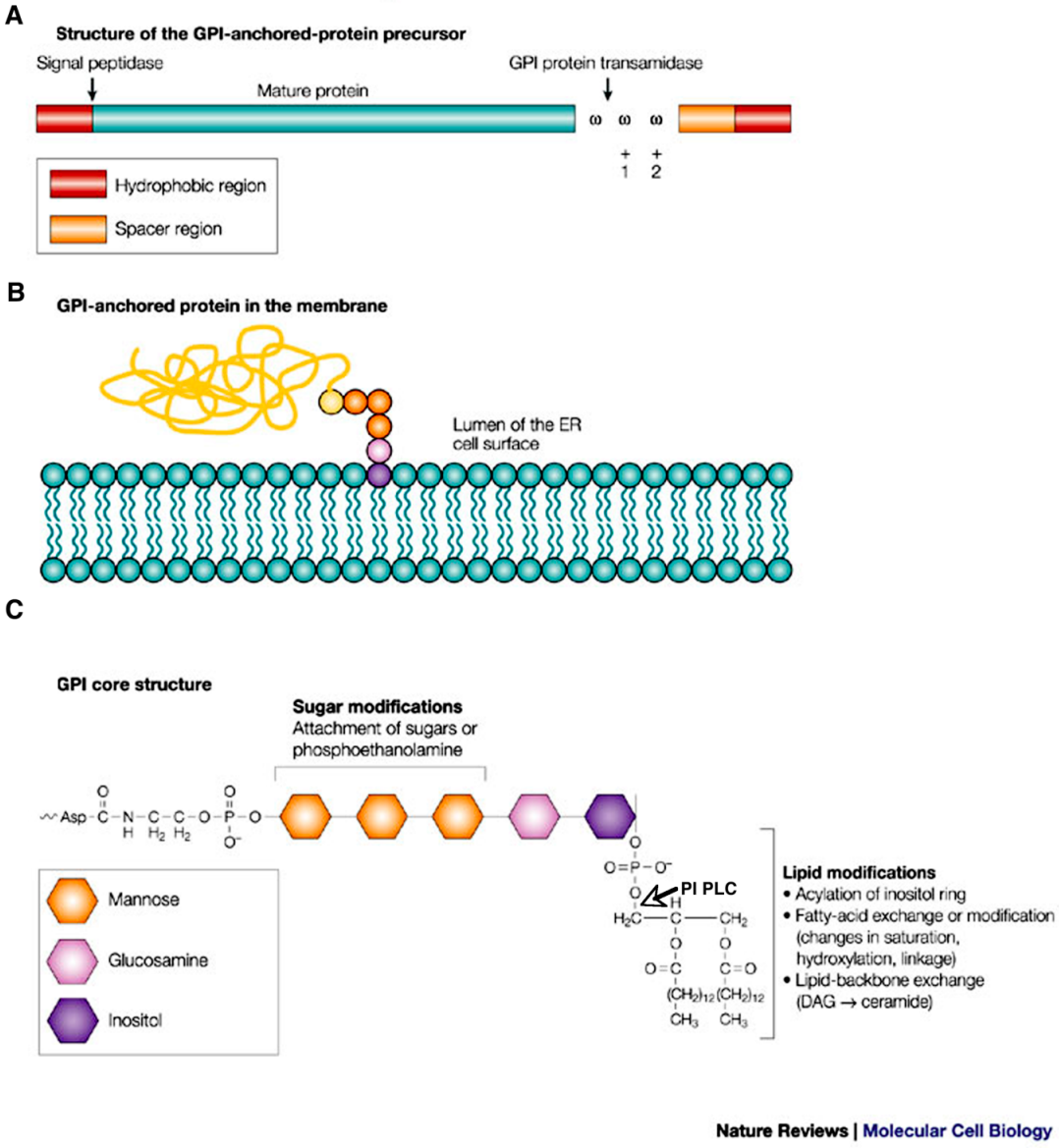


Figure 10 : GPI- anchored protein precursor and anchor structure: *(From Satyajit mayor & Howard Riezman, Nature reviews, Molecular Cell Biology 5, 110-120, 2004).*

A typical nacent GPI-AP contains a signal peptide fragment, ω-site for addition of GPI anchor, spacer and hydrophobic regions (A). GPI-anchored proteins are embeded in the extracellular or luminal leaflet of membrane through their glycolipid moieties and are not directly accessible from the cytosolic face of the membrane (B).
The conserved GPI consists of an ethanolamine phosphate in an amide linkage to the carboxy terminus of the protein, three mannose residues (orange), glucosamine (pink) and phosphoinositol (purple). GPI-anchor can be modified and is subjected to various remodelling reactions on the lipid moiety. Variations can be occur by the addition of extra sugars or ethanolamine phosphates to the mannose residues; acylation of the inositol ring; changes in the fatty acids (length, saturation, hydroxylation), or their types of linkage to the glycerol backbone (acyl to alkyl); or remodelling of the entire diacylglycerol to ceramide (C).

The functions of GPI-APs range from enzymatic to antigenic and adhesion properties. GPI-anchored proteins also play a critical role in a variety of receptor-mediated signal transduction pathways. In fungi synthesis of GPI anchors is essential for viability because their cell wall mannoproteins require a GPI anchor to be incorporated into the cell wall (Leidich et al., 1994; Bruel et al., 1997). In lower eukaryotes such as protozoa, GPI-anchored proteins play a role in the cell viability and defense against the host immune system. In animal cells GPI-anchored proteins are important in development. In animals, mutation in GPI synthesis leads to an embryonic–lethal phenotype (Kawagoe et al., 1996). For example, somatic mutation of the PIGA gene leads to paroxysmal nocturnal hemoglobinuria, an acquired hematopoietic stem cell disorder (Takeda et al., 1993). The larger number of functions supported by GPI-APs does not help to have a precise idea on the general role of GPI anchor. The stability and the modulation of these functions in cellular membrane could be dependent on many different parameters such as the nature of the surrounding lipids or cytoskeleton proteins and some other factors such as accessibility to phospholipases.

III. 1. Mechanism of polarized sorting of GPI-anchored proteins:

As mentioned before, sorting signals have been found along the entire length of transmembrane proteins and can be recognized by either luminal or cytosolic receptors. Instead, GPI-APs having no transmembrane domain or intracellular domain cannot interact directly with cytosolic components and were assumed to contain signals within the GPI anchor (in the hydrophobic/ or in the glycan portion), or

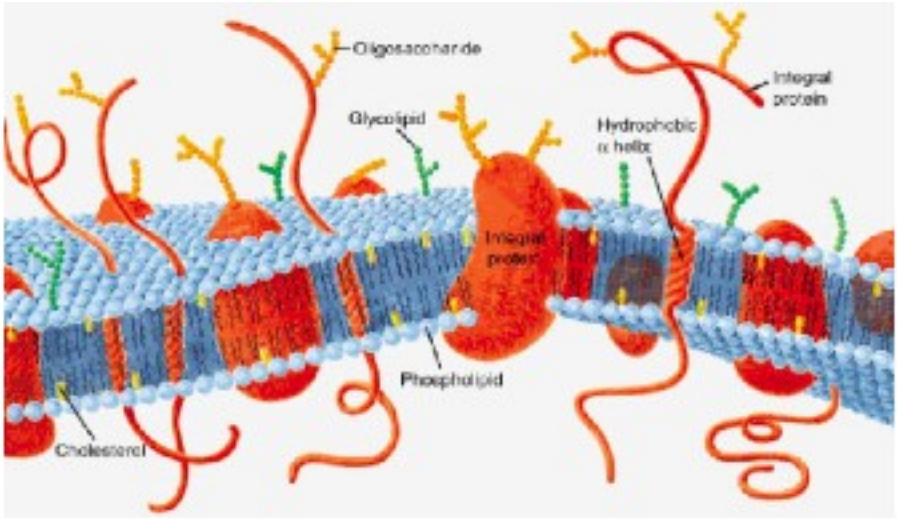
in the protein moiety or in N- or O-linked carbohydrates on the GPI-anchored proteins (Mayor, 2004).

An interesting hypothesis to explain the sorting mechanism of proteins has been proposed by Simons and Ikonen (Simons and Ikonen, 1997), which is called raft hypothesis.

III. 1. 1 The lipid-raft hypothesis:

According to the fluid-mosaic model (Figure 11), biological membranes are considered to be fluid structures in which the lipids and integral proteins are arranged in a mosaic manner. This model assumes that there is a continuous bilayer of phospholipid molecules in which globular proteins are embedded (Singer and Nicolson, 1972). The lipid raft theory proposed by Simons and Ikonen imposes that biological membranes contains segregated domains, which are enriched in cholesterol and sphingolipids (Brown, 2000; Brown and London, 1997). They are acting as functional units called rafts (figure 12). The relative insolubility of lipid rafts in cold non-ionic detergents is the most widely used method to purify raft components and membrane micro domains. The microdomains isolated by this method are called DRMs (Detergent Resistant microdomains) and are considered as a biochemical representation of rafts.

A



B

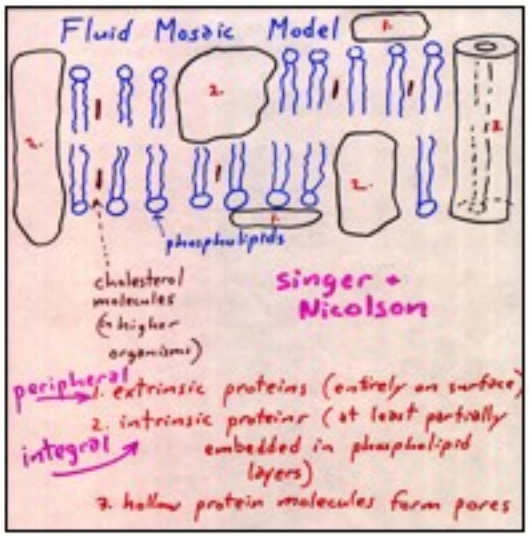


Figure 11: Fluid mosaic model

A. Fluid mosaic model of cell membrane. B. Original schematic representation of fluid mosaic model from Singer and Nicolson.

Singer and Nicolson, 1972, Science 173:720.

Because of their capacity to segregate proteins and lipids, lipid rafts have been implicated in several cell functions such as signaling and sorting (Simons and Ikonen, 1997; Simons and Toomre, 2000). In addition, it has been proposed that these lipid domains might also be able to affect the conformation of membrane proteins and thus their functions (London, 2005).

In the last years many studies, using different approaches and more sophisticated techniques, tried to prove directly the existence of lipid rafts and to determine their characteristics (size, composition, dynamics, etc.). Studies of domain formation in model membrane concluded that lipids could be present in three different phases: the gel phase (lb), the liquid ordered phase (lo), and the liquid crystalline phase or fluid phase (ld). Moreover, fluorescence quenching studies in model membrane vesicles showed that high cholesterol concentrations could promote ld/lo co-existence in mixtures of high and low Tm lipids including sphingolipid-containing mixtures (Ahmed et al., 1997). In addition, several studies on living cells using different approaches (from diffusion methods to probe partitioning, FRET and FRAP studies) suggest that lipid segregation in distinct domains also occurs in biological membranes (Maxfield, 2002; Ritchie *et al.*, 2003; Rao and Mayor, 2005; Meder *et al.*, 2006; Lebreton et al., 2008). All these findings indicate that lipid micro domains exist in the biological membranes.

In addition, many studies have showed that: i) GPI-APs do not exhibit Brownian motion but undergo random walks in a transient confinement zone (Fujiwara *et al.*, 2002); ii) single particle tracking experiments revealed that GPI-APs were found to be confined within 200-400 nm zones (Sheets *et al.*, 1997; Schutz *et al.*, 2000; Fujiwara *et al.*, 2002); iii) other studies using FRET, single particle tracking, or biochemical cross-linking have shown that GPI-APs are restricted to submicron sized domains on the cell surface (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998; Pralle *et al.*, 2000). All these data indicates that GPI-APs are present in restricted micro domains of the membrane.

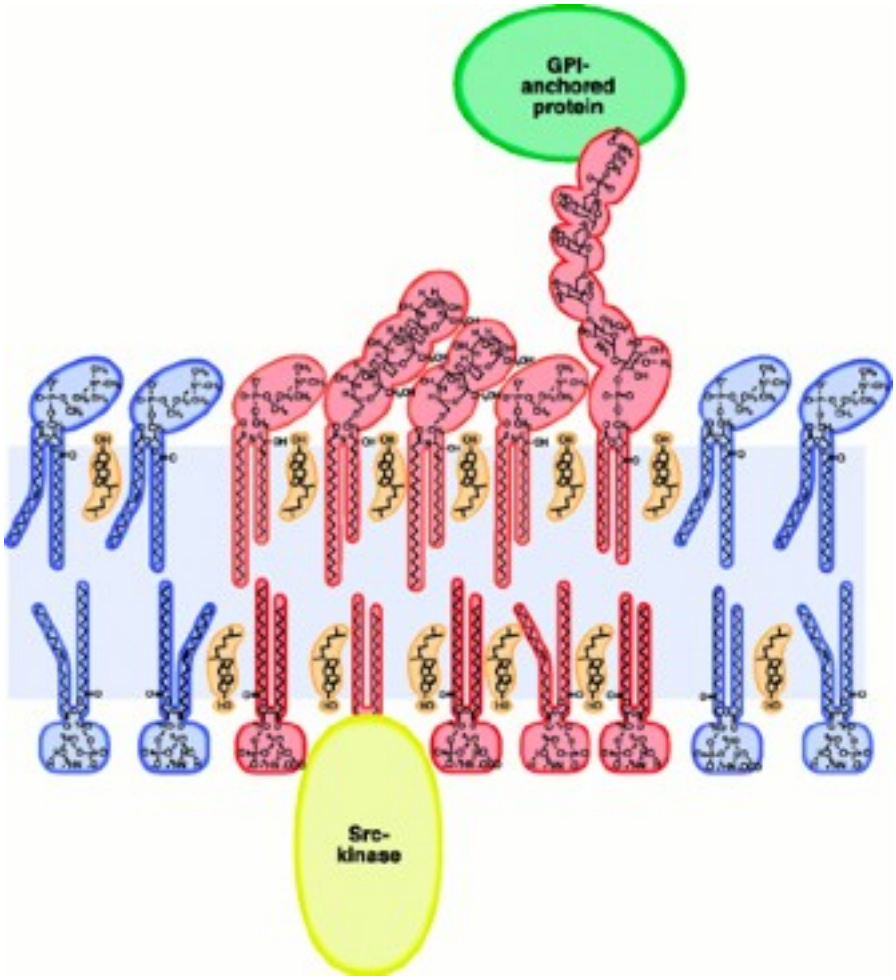


Figure 12: Lipid Raft.

Schematic representation of raft. Here unsaturated lipids were shown in blue, saturated lipids in red and cholesterol In orange.

Simons and Ikonen, 1997, Nature 387; 569-572.

According to the lipid raft hypothesis, rafts, because of their capacity to segregate specific classes of lipids and proteins and their enrichment in apical membranes, have been postulated to act as apical sorting platforms (Simons, 1997; 1998; Brown, 1998; Van Meer et al., 1987). This mechanism of sorting is particularly suitable for GPI-APs, which are apically sorted in several epithelial cell lines (Lisanti et al., 1989; Brown et al., 1989), and associated with DRMs (Brown et al., 1992;

Arreaza, 1995; Fiedler, 1993; Nosjean et al., 1997) during their passage through the Golgi apparatus (Brown et al., 1992; Lipardi et al., 2000; Paladino et al., 2004; 2007). Moreover, DRM perturbation by cholesterol and/ or sphingolipid depletion results in impaired trafficking to the PM or altered polarity (Lipardi et al., 2000; Ehehalt et al., 2008). These combined evidences led to the proposal that GPI anchor acts as an apical sorting signal by mediating raft association (Simons and Ikonen, 1997; Simons and Vanmeer, 1988). However this hypothesis has been challenged by the fact that in FRT cells majority of endogenous and some transfected GPI-APs are basolaterally sorted (Zurzolo et al., 1993) and some DRM associated GPI-APs are basolaterally delivered in MDCK cells (Benting et al., 1999; Paladino et al., 2004; 2006; 2007). This indicates that GPI anchor is not an apical sorting signal and the association of rafts is necessary but not sufficient for apical sorting. In addition the posttranslational modification of ecto domain has been proposed to be involved in the sorting of GPI-anchored proteins. But the exact mechanism how ectodomain involved in the sorting is still controversial.

An important finding came out from the work of Paladino and colleagues from our laboratory, by analyzing the sorting of two apical GPI-APs (PLAP and GFP-GPI) and two basolateral GPI-APs (PrP and GH-DAF). They demonstrated that both apical and basolateral GPI-APs were associated to DRMs, confirming the hypothesis that association of rafts is not sufficient for apical sorting (Paladino et al., 2004). In agreement with the hypothesis that affinity of the proteins to the lipid rafts depend on their state of oligomerization, they showed that in contrast to the basolateral GPI-APs only apical GPI-APs undergo oligomerization and form high molecular weight

complexes at the level of the Golgi concomitantly with the raft association (Paladino et al., 2004).

For sorting of GPI-anchored proteins they proposed a multistep model, which explains both apical and basolateral GPI-APs partitioned into the rafts because of the natural affinity of the GPI-anchor to these lipid microdomains. Oligomerization process leads to the stabilization of the apical GPI-APs in the lipid rafts and therefore allowing their incorporation in the apical sorting vesicle. In addition, they also showed that this oligomerization mechanism is conserved in different epithelial cells (Paladino et al., 2007). But what is the exact mechanism of oligomerization is still not clear.

III. 1. 2. Factors playing a role in the oligomerization:

Although oligomerization-driven apical sorting is an attractive hypothesis, the mechanism by which apical GPI-APs oligomerize is still unclear. Specially, it is not known what oligomerization depends on, and what is the nature of interactions that determines apical GPI-AP clustering prior to their sorting. It is still unclear whether these interactions involve the ectodomain, the GPI-anchor, or both.

III. 1. 2. 1. Protein ectodomain: It has been shown that GPI-AP oligomers, once formed, are resistant to conditions in which DRMs are disturbed (such as SDS extraction) (Paladino et al., 2004). This suggests that GPI-AP oligomers are maintained by protein-protein interactions. The analysis of a chimeric model GPI-AP, GFP-GPI in MDCK cells, showed that the non-covalent interactions between protein ecto domains are responsible for oligomerization of the protein (Paladino et al., 2004; 2007; 2008). Hence post translational modifications of the ectodomain such as *N*- and *O*- glycosylations could be involved in this process.

The role of glycosylation of the ectodomain in the sorting of GPI-APs is still controversial. It has been shown that addition of *N*-linked glycans to the GPI-anchored form of rat growth hormone confers its apical sorting (Benting et al., 1999). N-glycosylation is also shown to be involved in the apical localization of the native GPI-anchored protein dipeptidase (Pang et al., 2004). In contrast to this data, it has been shown that *N*-glycans are not required for the sorting of GPI-anchored form of endolyn (Potter et al., 2004). Whereas it was shown that *O*-glycosylation is important for apical delivery of several transmembrane proteins (Huet al., 1998; Alfalah et al., 1999; Yeaman et al., 1997). But there is no evidence for its role in the sorting of GPI-APs with the exception of CEA (Carcino Embryonic Antigen) (Huet et al., 1998). Interestingly, the recent evidences by Catino et al. from our laboratory demonstrated that mutagenesis of either *N*- or *O*- glycosylation site does not affect DRM association, oligomerization and apical sorting of native and chimeric GPI-APs (PLAP and P75GPI) in MDCK cells (Catino et al., 2008). These data indicate that *N*- and *O*-glycosylation do not have a direct role in GPI-AP oligomerization and apical sorting. All these evidences indicate that the role of the glycosylation in the sorting of GPI-APs might be different depending on the type of the protein and the origin of cells. To understand the exact role of glycosylation of ectodomain, during my thesis, I analyzed the role of *N*-glycosylation in the oligomerization and apical sorting of GPI-APs in polarized FRT cells, which have a peculiar tendency to sort most of the GPI-APs to the basolateral domain.

III. 1. 2. 2. Raft environment and GPI-anchor:

It has been shown that depletion of cholesterol impairs the oligomerization of

the protein in the Golgi apparatus (Paladino et al., 2004). This suggests that lipid rafts may constitute a favorable environment for the formation of HMW complex formation and therefore apical sorting. Furthermore, several reports suggests that differences in the lipid anchor (such as the length of acyl and alkyl chains; remodeling of glycan portion or inositol ring) are critical for their raft association and they could mediate a different affinity for different proteins (Benting et al., 1999; Fujita et al., 2007; Maeda et al., 2007; Jaquenoud et al., 2008). Interestingly, it was shown from our laboratory that different GPI-attachment signals (derived from an apical or basolateral native GPI-APs, FR and PrP respectively) affect the ability of the resulting GFP-fusion proteins (GFP-FR and GFP-PrP) to oligomerize and to be apically sorted (Paladino et al., 2008). Consistent with this data, using FRAP approach Lebreton and colleagues from our laboratory found that GPI-APs having same GFP ecto domain and different GPI-attachment signals display different apparent diffusion coefficient (D) at the level of the Golgi apparatus (Lebreton et al., 2008). More interestingly it was shown that addition of cholesterol is sufficient to promote the oligomerization and apical sorting of a basolateral GPI-AP, GFP-PrP, which in control condition exists as a monomer (Paladino et al., 2008). All together, these data suggests that a specific lipid environment is required for oligomerization and therefore for apical sorting of GPI-APs.

However, it was previously shown that, differently from the basolateral GFP-PrP, cholesterol addition does not affect the sorting of the S49/71 mutant, which has a double cys mutation in the GFP ectodomain (Paladino et al., 2004). This suggests that both specific lipid environment and permissive ectodomain are important for oligomerization and apical sorting.

To understand whether the cholesterol driven oligomerization is conserved in

different polarized epithelial cells, I studied the role of cholesterol in promoting the oligomerization and therefore apical sorting of GPI-APs in FRT cells.

III. 2. Segregation of GPI-anchored proteins: `

Many morphological and biochemical studies have shown that the segregation of apical and basolateral cargos occurs into distinct vesicles upon exit from the TGN (Wadinger-ness et al., 1990; Keitzer et al., 2003; Keller et al., 2001; Jacob and Naim, 2001). The TGN appears to be the site of sorting also for GPI-APs. Indeed, although GPI-APs progressively segregate from other cargo molecules through the Golgi stack, they have been shown to be completely segregated in the TGN, from where they exit into distinct vesicles (Keller et al., 2001; Rustom et al., 2002). In addition, it has been shown that GPI-AP carrying vesicles emerge from large Golgi domains with a spherical appearance in contrast to the elongated extensions from which basolateral carriers appear to arise (Rustom et al., 2002; Luini et al., 2005). Furthermore recently Lebreton and colleagues from our laboratory using a FRAP approach have shown that differently sorted GPI-APs have a different apparent diffusion coefficient in the Golgi apparatus, while they behave similarly at the level of the plasma membrane (Lebreton et al., 2008). Because the apparent diffusion coefficient of a protein is directly related to the surrounding environment, this data suggests that segregation between apical and basolateral GPI-APs occurs either in the Golgi apparatus or earlier.

Moreover, it was shown that addition of cholesterol leads to the decrease in the apparent diffusion coefficient (D) of a basolateral GPI-AP, GFP-PrP, which in control condition exhibits higher D value (Lebreton et al., 2008). This is in agreement with the data that this protein missorted to the apical upon cholesterol addition (Paladino et al., 2008). This data suggests that there is segregation between apical and

basolateral GPI-APs based on the surrounding lipid (raft) environment. On the other hand it is unknown where GPI-APs are segregated from other raft-associated proteins.

To understand whether cholesterol driven segregation is conserved in different epithelial cells, I analyzed the role of cholesterol in the apical sorting of GPI-APs in FRT Cells.

III. 3. Transport of GPI-anchored proteins:

It has been proposed that in MDCK cells the apical GPI-anchored proteins were directly sorted to the apical plasma membrane after exiting the TGN (Brown et al., 1989; Lipardi et al., 1999; Rodriguez-Boulan and Powell, 1992). In contrast to this data, recently it was shown that in MDCK cells the apical GPI-anchored proteins follow the trancytosis route, first to the basolateral domain and then they recycle back to the apical domain (Polishchuk et al., 2004). These results strongly suggests that the sorting of GPI-APs to the apical or basolateral domain takes place after they pass through the TGN, once the protein have reached the basolateral domain. In contrast to this data, we have shown that GPI-APs are directly sorted to the apical surface of MDCK cells without passing through the basolateral membrane (Paladino et al., 2006).

OBJECTIVES OF THE STUDY

OBJECTIVES OF THE STUDY:

During my PhD, I became interested in understanding the mechanisms involved in sorting of GPI-anchored proteins in two different polarized epithelial model cell lines, the MDCK (Madin Carby Kidney Cells) and FRT (Fisher Rat Thyroid cells), which exhibits substantial differences in the sorting of GPI-anchored proteins. In contrast to MDCK cells, which sort majority of GPI-APs apically, FRT cells address most of GPI-APs basolaterally (Zurzolo et al., 1993). Interestingly it was shown that in these two cell lines oligomerization is essential for apical sorting of GPI-APs. Indeed, all apical GPI-APs in FRT cells also oligomerize and form high molecular weight complexes differently from basolateral GPI-APs (Paladino et al., 2004; Paladino et al., 2007). We have recently shown that both the GPI-anchor and Golgi membrane environment (specifically the cellular cholesterol content) are involved in the regulation of oligomerization and therefore in apical sorting (Paladino et al., 2008; Lebreton et al., 2008). Addition of cholesterol is sufficient to drive the oligomerization and apical sorting of basolateral GPI-AP: GFP-PrP in MDCK cells (Paladino et al., 2008). Based on this and other evidences we have proposed that a specific membrane environment enriched in cholesterol is required to favor the oligomerization of GPI-APs in the Golgi and therefore ensure their apical sorting (Paladino et al., 2008; Lebreton et al., 2008). But the mechanism of cholesterol driven oligomerization is still unknown. In addition, we have also shown in MDCK cells that both N- and O- glycans are not directly involved in the apical sorting of GPI-APs (Catino et al., 2008). During my thesis, I focused in understanding the sorting differences of GPI-APs between MDCK and FRT

cells and I analyzed the role of cholesterol and of N-glycosylation in apical sorting of GPI-APs in FRT cells.

The main objectives of my thesis are to understand:

1) Whether the cholesterol-driven GPI-AP oligomerization is conserved in FRT cells.
2) What is the role of cholesterol enriched membrane domains segregation of apical and basolateral GPI-APs at the level of the Golgi in FRT cells.
3) Whether N-glycosylation is involved in apical sorting of GPI-APs in FRT cells.

To answer these questions I have used different approaches, which are described below.

In the first part of my thesis I mainly focused in understanding the role of cholesterol in promoting the oligomerization and apical sorting and asked whether this mechanism is conserved in different epithelial cell lines. For this study **I** used FRT cells as model epithelial cells and considered GFP-NO-GPI (GFP fused to the N glycosylation sequence (10 amino acid) and O- glycosylation sequence (56 amino acid) of LDL receptor followed by the GPI-signal attachment of the DAF (Decay Accelerating Factor) protein) and the native PLAP as apical GPI-APs, while GFP-FR (GFP is fused to the GPI-signal attachment of folate receptor) and GFP-PrP (GFP is fused to the GPI-signal attachment of prion protein) as basolateral GPI-APs. I then analyzed the behavior of these proteins regarding their capacity to be apically or basolaterally sorted and to be incorporated into oligomeric complexes in control condition and upon addition of cholesterol.

In the second part of my thesis, I analyzed the role of membrane environment in the organization of GPI-APs at the level of the Golgi complex in MDCK and FRT cells by using FRAP (Fluorescence Recovery After Photo Bleaching) experiments. For this study I considered two apical proteins (raft and non-raft); GFP-NO-GPI and P75-GFP, two basolateral GPI-APs (raft and non-raft); GFP-PrP and GFP-PIT in both MDCK and FRT cells. I analyzed the role of cholesterol and actin cytoskeleton in the segregation of apical and basolateral GPI-APs at the level of Golgi in FRT cells considering the previously published results in MDCK cells (Lebreton et al., 2008). In addition to this, by performing FRAP analysis at the level of the Golgi and by selective domain biotinylation in conditions that perturb rafts such as cholesterol depletion and sphingolipid, I analyzed the role of rafts in the segregation of apical and basolateral GPI-APs in FRT cells.

In the third part of my thesis, I focused on the role of glycosylation of the ectodomain in promoting the oligomerization and therefore apical sorting in FRT cells. For this study I used the FRT cells stably transfected with two apical GPI-APs; PLAP and GFP-NO-GPI and I analyzed the sorting behavior of these proteins upon tunicamycin treatment, which is an inhibitor of glycosylation. Later I used FRT cells stably expressing a mutant protein, PLAPΔN that lacks two N-Glycosylation sites of PLAP protein. I analyzed the oligomerization capacity in correlation with the sorting behavior and specific domain expression of this mutant protein using different biochemical approaches. The results of this work have been submitted recently to Molecular Biology of the Cell.

In addition to this work, I have started to analyze the role of phosphoinositols in

the regulation of Golgi morphology and mechanism of segregation between GPI-APs and transmembrane proteins in MDCK and FRT cells. I have described the preliminary results of these analyses in the perspectives.

MATERIALS AND METHODS

MATERIALS AND METHODS

Reagents and antibodies:

Cell culture media were purchased from Sigma Aldrich, Inc., (St Louis, USA) and Euroclone Spa (Pero (MI) Italy). Antibodies were purchased from the following companies: polyclonal α-GFP and monoclonal α-GFP from Invitrogen (Eugene, Oregan, USA), α-PLAP from Rockland (Gilbetsville, PA, USA) and monoclonal anti-flotilin-1 antibody from BD Transduction Laboratories, polyclonal giantin from Covance (1:5000 for WB and 1:500 for IF; Emeryville, California), polyclonal furin from Thermo scientific (1:1000 for WB and 1:100 for IF; Rockford, USA), polyclonal calnexin from Stressgene (1:1000; Ann Arbor, USA) and monoclonal N-cadherin from BD Transduction Laboratories (1:1000). Biotin and horseradish peroxidase (HRP)-linked streptavidin were from Pierce (Rockford, Il, USA). FB1, tunicamycin and all other reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Cell culture and transfections:

MDCK and FRT cells were grown in DMEM (Dulbecco's modified Eagle's medium) and F12 (F12 Coons modification medium) respectively containing 5% FBS. MDCK cells stably expressing GFP-PrP and FRT cells stably expressing PLAP, GFP-PIT and p75-GFP had been obtained previously (Paladino *et al.*, 2004; Catino *et al.*, 2008). FRT cells were transfected with sequences encoding GFP-PrP, GFP-NO-GPI (GFP fused to the N glycosylation sequence (10 amino acid) and O- glycosylation sequence (56 amino acid) of LDL receptor followed by the GPI-signal attachment of the DAF (Decay Accelerating Factor) protein) and PLAPΔN(Catino *et al.*, 2008) by using lipofectin (Invitrogen). Stable clones were selected according to the antibiotic resistance.

Modification of cholesterol content:

To deplete cellular cholesterol we used a previously published protocol (Lipardi et al., 2000; Lebreton et al., 2008). Briefly, FRT cells were plated on filters and 24h after plating mevinolin (10μM) and mevalonate (250μM) were added to the cells in F12 medium supplemented with 5% delipidated calf serum for 48 hours.

To load the cells with cholesterol we used a previously published protocol (Lebreton *et al.*, 2008). Cells were plated on filters for 4 days and we added water soluble cholesterol (10 mM in MβCD) to warm medium containing 20 mM Hepes, pH 7.5, and 0.2% bovine albumin for 45 min at 37 °C.

The water-soluble cholesterol is prepared with in MβCD and the ratio between the amount of two chemicals in the complex, which determine if it will act as a cholesterol donor (ratio 1 cholesterol: 6 in MβCD) or acceptor (Christian et al., 1997; Zidovetzki and Levitan, 2007). To determine the rate of cholesterol depletion or addition, we measured cellular cholesterol levels by a colorimetric assay (cholesterol/cholesteryl ester quantification; Calbiochem, Merck biochemical Ltd, Nottingham). In brief cells were washed with PBS containing CaCl2 and MgCl2, lysed with the appropriate lysis buffer for colorimetric detection according to the manufacturer's instructions.

Perturbation of the cytoskeleton meshwork:

In order to perturb the actin cytoskeleton we incubated the polarized MDCK and FRT cells with latrunculin A (Molecular probes, Invitrogen, California) (1μM) in DMEM and F12 respectively, containing 5%FBS for 5 minutes or 60 minutes at 37°C before FRAP analysis.

Velocity Gradients:

Velocity gradients were performed using a previously published protocol (Paladino et al., 2004; Scheiffele et al., 1997; Paladino et al., 2008). Cells were grown to confluency in 100-mm dishes, washed in phosphate-buffered saline containing $CaCl_2$ and $MgCl_2$ and lysed on ice for 30 min in 20 mM Tris, pH 7.4, 100 mM NaCl, 0.4% SDS, 0.2% Triton X-100. Lysates were scraped from dishes, sheared through a 26-gauge needle, and layered on top of a sucrose gradient (30 to 5%) after removal of nuclei by low speed centrifugation. After centrifugation at 45,000 rpm for 16 h in an ultracentrifuge (model SW 50; Beckman counter), fractions of 500 µL were harvested from the top of the gradient and TCA precipitated. Proteins were revealed by western blot using specific antibodies.

Biotinylation assay:

Cells grown on transwell filters for 4-5 days were selectively biotinylated and processed as previously described (Paladino et al., 2006; Paladino et al., 2008). Biotinylated proteins were immunoprecipitated with specific antibodies and revealed with HRP-conjugated streptavidin.

Sucrose density gradients:

Sucrose gradient analysis of TX-100-insoluble material was performed using previously published protocols (Paladino *et al.*, 2008). Cells (20-25 x 10^6) grown on 150 mm dishes to confluence were scraped in PBS containing $CaCl_2$ and $MgCl_2$, and resuspended in 1mL of lysis buffer containing 1% TX-100, 10 mM Tris buffer (pH 7.5), 150 mM NaCl, 5 mM EDTA and sheared through a 23 G needle. The lysate was mixed with an equal amount of 85% sucrose and run on discontinuous sucrose

gradients (40-5%) for 16h at 4°C. 12 fractions were collected from top to bottom of the gradient. Proteins were TCA precipitated and detected by western blotting using anti-GFP antibody and anti-flotilin antibody (a typical raft-marker).

Fluorescence Microscopy:

FRT cells were grown on transwell filters for 4 days, washed with phosphate-buffered saline containing $CaCl_2$ and $MgCl_2$, fixed with 4% paraformaldehyde, and quenched with 50 mM NH_4Cl. Cells were stained with anti-GFP antibody for GFP-PrP and GFP-NO-GPI (Molecular Probes, Invitrogen) and with anti-PLAP for PLAP and PLAPΔN followed by Alexa-conjugated secondary antibody in non-permeabilized conditions. The images were acquired using a laser scanning confocal microscope (LSM 510; Carl Zeiss Microimaging Inc.) equipped with a plan Apo 63X oil immersion (NA 1.4) objective lens.

Cell Fractionation Assay:

FRT cells (12 plates of 150-mm) were homogenized by 10 strokes in an isobiotec cell homogenizer with a tungsten-carbide ball in 500ul of 20mM HEPES/KOH, Ph 7.3 and 120mM sucrose. A post nuclear supernatant fraction was obtained by centrifugation at 600X g for 5min in an eppendorf tube. The post-nuclear supernatant was loaded on the top of a discontinuous sucrose gradient (0.6ml of each of 15, 20, 25, 30, 35, 40 and 45% with 0.5ml of 60% on the bottom) made up in the same buffer. The gradient was spun in an SW 50.1 rotor for 1h at 45,000 rpm in a Beckman centrifuge and 14 fractions were collected from the top of the tube and 1/20 of each fraction was loaded on 12% poly acrylamide gels. Western blots were performed using different antibodies specific for ER, plasma membrane, cis/medial and trans Golgi markers.

FRAP Measurements and Analysis:

FRAP experiments allow to access two parameters, the mobile fraction, M*f,* and the apparent diffusion coefficient, D. Here the mobile fraction corresponds to the fraction of the studied protein able to repopulate into the bleached area, whereas the apparent diffusion coefficient indicates the speed with which the protein diffuse. FRAP analysis was developed on a LSM 510 META from Zeiss using the plan Apo x63 oil-immersion (NA 1.4) objective lens. For FRAP analysis we monitored the fluorescence of our fused GFP protein using low-intensity laser excitation (488nm) (pre bleach scans). We always kept the airy unit of 1. As a preliminary analysis to define the mathematical model to apply for the proteins, different sizes of region of interest (ROI) from 280nm to 6.2μm were selectively bleached (Sprague and McNally, 2005). From those analyses it clearly appears that our model proteins were following a diffusion-coupled FRAP recovery (Sprague and McNally, 2005). Furthermore, since we used a circular ROI, we analyzed our raw data using the previously described soumpasis mathematical equation (Meder et al., 2006; Soumpasis, 1983). In order to compare our raw data with published studies we opted for a bleaching ROI of 1,4μm for all our experiments. Therefore, a circular defined region of 1,4μm was photo bleached with the same laser excitation at high intensity (decrease of the fluorescence into the ROI by 60% to 80%) and then we followed the recovery of fluorescence into the bleached region over time by recording with low-intensity laser (post-bleach scans) as before the photo bleaching point. This recovery reflects the ability of unbleached fluorescent proteins around the ROI to repopulate into the photo bleached ROI. For each FRAP acquisition we considered two internal controls, one which indicates over time the natural bleaching of the sample and the

other one which gives the level of fluorescence background (Sprague and McNally, 2005). The raw data were fitted with the Igor Pro software with an application developed in EMBL using soumpasis mathematical equation. FRAP recordings were obtained in CO_2-independent medium (NaCl 3M, KCl 0.1M, CaCl2 0.1M, MgCl2 0.1M and Hepes 0.5M) at 37°C.

Temperature block:

In order to achieve an almost complete protein block in the TGN, we used a previously published protocol (Paladino et al., 2006 ; Paladino et al., 2004). Confluent MDCK cells grown on coverslips were incubated at 19.5°C for 2h in areal medium (F12 Coon's modified medium without $NaHCO_3$ and with 0.2% BSA and 20 mM Hepes, pH 7.4) along with 150 µg/ml cycloheximide. Cells were fixed with PFA (time 0) and alternatively cells were warmed at 37°C for 10min, in order to release from the block before fixation. Serial confocal images were collected.

EXPERIMENTAL RESULTS

Article: N-glycosylation instead of cholesterol mediates oligomerization and apical sorting of GPI-APs in FRT cells.

Introduction:

The important characteristic of epithelial cells is that they have the ability to establish polarity. The plasma membrane of polarized epithelial cells is asymmetric and divided into two domains, an apical domain facing the external environment and the basolateral domain that mediates the contacts with neighboring cells, the basal membrane and the internal milieu. These two domains differ in their function and in their protein as well as lipid composition. This asymmetric distribution of these domains is achieved by continuous sorting of newly synthesized proteins and lipids to either of the two surfaces and their regulated internalization (Mellman et al., 1996 and Matter et al., 2000). Evidences from biochemical and live imaging studies have shown that apical and basolateral proteins segregate into distinct vesicles upon exit from the TGN, supporting the hypothesis that the TGN is the major sorting station during exocytosis of newly synthesized proteins (Wandinger-Ness et al., 1990; Keller et al., 2001; Kreitzer et al., 2003; Griffiths et al., 1986; Paladino et al., 2006; Rodriguez-Boulan 2005). However it was also shown that protein sorting could also occur in recycling endosomes (REs) after their exit from the TGN (Ellis et al., 2006; Matter et al., 2000; Mellman et al., 1996; Ang et al., 2004; Folsch et al., 2003). From the TGN, proteins can be addressed to apical or basolateral surfaces either directly or by first traversing recycling endosomes (Ang et al., 2004; Cancino et al., 2007; Cresawn et al., 2007; Gravotta et al., 2007).

Intracellular sorting of newly synthesized proteins at the TGN or in REs is based upon recognition of specific apical and basolateral sorting signals present on the proteins by the sorting machinery (Mostov et al., 2000) that mediates their incorporation into specific sorting vesicles (Wandinger-ness et al., 1990; Keller et al., 2001; Rodriguez-Boulan et al., 2005). As a result of these events, proteins can be transported either to apical or to basolateral surface using direct or an indirect route (transcytosis- see introduction part II-4**).**

Basolateral sorting is mediated by discrete domains in the cytosolic protein tail frequently containing tyrosine or dilucine motifs (Bonifacino and Traub, 2003), which are recognized by the clatherin adaptor complex (Folsch et al., 1999; Sugimoto et al., 2002) (See introduction part II.1.2.1). However the situation is more complicated for apical proteins (Weisz et al., 2009). In contrast to the basolateral sorting signals, apical sorting signals are highly variable, including peptide sequences and post translational modifications such as lipid and sugar moieties and can present in the extracellular, tansmembrane or intracellular domains of the cargo proteins (see introduction part II.1.2.3) (Weisz et al., 2009). Furthermore, in contrast to the basolateral sorting, apical recognition is not only mediated by protein-protein interactions but also on lipid-lipid and lipid-protein interactions (Paladino et al., 2007). In particular, it has been postulated that sphingolipid and cholesterol rich micro domains, because of their capacity to segregate proteins and lipids (Simons and van Meer, 1988; Brown and London, 1998) has been postulated as apical sorting platforms (Simons and Ikonen, 1997). The best example of raft mediated apical sorting is represented by glycosylphosphotidyl (GPI-anchored proteins (GPI-APs), which associate into detergent- resistant microdomains (DRMs) during their passage

through the Golgi apparatus (Simons and Ikonen, 1997; Brown and Rose, 1992). Therefore, it was proposed that GPI anchor itself acts as an apical sorting signal by mediating raft association (Simons and Ikonen, 1997; Simons and van Meer, 1988; Lisanti et al., 1989; Brown et al., 1989; Harder and Simons, 1997). However the role of GPI-anchor and of lipid rafts as apical determinants has been questioned by the finding that both apically and basolaterally sorted GPI-APs associate with the DRMs in MDCK and in FRT cells (Zurzolo et al., 1993; Lipardi et al., 2000; Paladino et al., 2004; Paladino et al., 2007), indicating that additional factors should be involved.

In has been shown that N-glycosylation play a role in the apical sorting of secretory and transmembrane proteins (Rodriguez-Boulan and Gonzalez, 1999; Potter et al., 2006). But the role of N-glycans in apical sorting of GPI-APs has been controversial (Benting et al., 1999a; Potter et al., 2004; Catino et al., 2008). Benting et al. (1999a) showed that addition of a GPI anchor attachment signal to non-glycosylated rat growth hormone did not result in apical sorting of this protein, however addition of N-glycans resulted in efficient apical targeting of this protein. Similarly, removal of N-glycans from GPI-anchored dipeptidase resulted in preferential localization of the protein to the basolateral cell surface in both MDCK and Caco2 cells (Pang et al., 2004). In contrast to this data, an apical sorting mechanism independent of N-glycosylation has been described for GPI-linked endolyn (Potter et al., 2004). Consistent with this data, our previous studies using glycosylation mutants and chimeric proteins have shown that neither N-glycans nor O-glycans are necessary for apical sorting of GPI-APs in MDCK cells (Catino et al., 2008). We have previously shown that although both apical and basolateral GPI-APs are raft associated in the Golgi apparatus, apical sorting is mediated by the capacity

of GPI-APs to form high molecular weight (HMW) complexes and impairment of oligomer formation results in basolateral missorting (Paladino et al., 2004; Paladino et al., 2007). But the exact mechanism of oligomerization mediated apical sorting is still not known. In addition, we have recently shown that both the GPI-anchor and Golgi membrane environment (specifically the cellular cholesterol content) are involved in the regulation of oligomerization and therefore in apical sorting (Paladino et al., 2008; Lebreton et al., 2008). Moreover, addition of cholesterol promoted the oligomerization and apical sorting of basolateral GPI-AP: GFP-PrP in MDCK cells (Paladino et al., 2008). Based on this and other evidences we have proposed that a specific membrane environment enriched in cholesterol is required to favor oligomerization of GPI-APs in the Golgi and therefore ensure their apical sorting (Paladino et al., 2008; Lebreton et al., 2008).

However it is not known whether this cholesterol driven oligomerization is conserved in different epithelial cells, what are the factors involved and what is the mechanism of oligomerization is still not known. In order to answer to these questions, we decided to study the role of cholesterol in apical sorting of GPI-APs in Fisher Rat Thyroid (FRT) cells, which also possess cholesterol and sphingolipid enriched microdomains (rafts) (Zurzolo et al., 1994), but exhibits different sorting properties compared to MDCK cells (Paladino et al., 2004). In contrast to MDCK cells, FRT cells sorts most of the GPI-APs basolaterally (Zurzolo et al., 1994). Interestingly in this cell line also oligomerization is essential for apical sorting of GPI-APs (Paladino et al., 2007).

Over all biochemical data along with FRAP analysis at the level of the Golgi, our data indicate that in FRT cells cholesterol is not a key regulator in the segregation of apical and basolateral GPI-APs in the Golgi. Strikingly, in contrast to MDCK cells, N-glycosylation is required to determine oligomerization and apical sorting of GPI-APs in FRT cells.

Summary of results:

We studied the role of cholesterol in promoting the oligomerization and apical sorting of GPI-APs in FRT cells by considering two model proteins, GFP-NO-GPI as an apical GPI-AP and GFP-PrP as a basolateral GPI-AP. Using biochemical analysis (selective domain biotinylation, sucrose density gradients and velocity gradients) and confocal microscopy analysis we showed that, in contrast with MDCK cells, addition of cholesterol does not promote the oligomerization and apical missorting of basolateral GPI-AP: GFP-PrP that is normally monomeric / dimeric and basolaterally sorted. This data was further confirmed by another basolateral GPI-AP: GFP-FR.
These results indicate that there might be another cholesterol-independent mechanism that promotes the oligomerization and of apical sorting of GPI-APs in FRT cells.

Then we decided to analyze the membrane environment in the Golgi of FRT cells using a FRAP approach. By measuring FRAP behavior of all the studied proteins at the level of Golgi, we showed that, in contrast with MDCK cells, all our studied proteins (GFP-NO-GPI, P75-GFP, GFP-PrP and GFP-PIT) are restricted in their apparent diffusion coefficient (D) at the level of the Golgi in control condition, independent of their raft association and polarized sorting. Furthermore, addition of cholesterol does not affect the apparent diffusion coefficient of apical and basolateral GPI-APs (both raft and non-raft). Next, performing FRAP analysis along with selective domain biotinylation in conditions that disturbs lipid rafts (cholesterol depletion and sphingolipid depletion) we showed that in FRT cells neither the

segregation between apical and basolateral GPI-APs at the level of the Golgi nor the apical sorting is driven by association with cholesterol enriched membrane domains.

In addition to this, we also showed that the Golgi membranes in FRT cells are more enriched with cholesterol than in MDCK cells. Further, it is likely that the Golgi membranes in FRT cells do not incorporate the uptaken cholesterol because they are already saturated with cholesterol. All these data indicate that in FRT cells cholesterol is not a key regulator in the segregation of apical and basolateral GPI-APs at the Golgi level.

The third part of the results is focused in understanding the role of N-glycosylation in apical sorting of GPI-APs in FRT cells. By combined studies of tunicamycin, an inhibitor of glycosylation, and using a PLAPΔN mutant, which lacks the two N-glycosylation sites, we demonstrated that, strikingly in contrast with MDCK cells, N-glycosylation is required for oligomerization and apical sorting of GPI-APs in FRT cells.

All our combined data indicate that in FRT cells oligomerization and apical sorting of GPI-APs are mediated by N-glycosylation, independently of raft association and of the cholesterol content in the Golgi.

ARTICLE

N-GLYCOSYLATION INSTEAD OF CHOLESTEROL MEDIATES OLIGOMERIZATION AND APICAL SORTING OF GPI-APs IN FRT CELLS.

Naga Salaija Imjeti[#1], Stéphanie Lebreton[#1], Simona Paladino[2], Erwin de la Fuente[3], Alfonso Gonzales[3] and Chiara Zurzolo[1*]

1, Unité de traffic membranaire et pathogenèse, Institut Pasteur, 25 rue du docteur roux, 75015 Paris; 2, Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università Federico II, Napoli, Italy; 3, Departamento de Inmunología Clínica y Reumatología, Facultad de Medicina and Centro de Envejecimiento y Regeneración, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

*Corresponding author: Chiara Zurzolo, Unité de traffic membranaire et pathogenèse, Institut Pasteur, 25 rue du docteur roux 75015 Paris, tel:+33145688277; fax:+33140613238; chiara.zurzolo@pasteur.fr

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[#] These authors contributed equally to this work.

Abstract

Sorting of Glycosylphosphatidyl-inositol anchored proteins (GPI-APs) in polarized epithelial cells is not fully understood. Oligomerization in the Golgi complex has emerged as the crucial event driving apical segregation of GPI-APs in two different kind of epithelial cells, MDCK and FRT cells, but whether the mechanism is conserved remains unknown. In MDCK cells cholesterol promotes GPI-AP oligomerization as well as apical sorting of GPI-APs. Here we show that FRT cells lack this cholesterol-driven oligomerization as apical sorting mechanism. In these cells both apical and basolateral GPI-APs display restricted diffusion in the Golgi likely due to a cholesterol-enriched membrane environment. Strikingly, N-glycosylation is the critical event for oligomerization and apical sorting of GPI-APs in FRT cells, but not in MDCK cells. Our data indicate that at least two mechanisms exist to determine oligomerization in the Golgi leading to apical sorting of GPI-APs. One depends on cholesterol and the other depends on N-glycosylation and is insensitive to cholesterol addition or depletion.

INTRODUCTION

Polarized epithelial cells possess an asymmetrical plasma membrane divided in an apical surface facing the external environment and a basolateral domain that contacts the neighbouring cells, the basal membrane and the internal milieu. These two domains differ markedly in their functions and in their protein and lipid composition, thanks to a selective sorting machinery that directs specific proteins and lipids to each domain. Several lines of evidence have shown that the Golgi complex and recycling endosomes cooperate to segregate apical and basolateral proteins to their corresponding cell surfaces (Welling and Weisz; Rodriguez-Boulan and Musch, 2005; Gonzalez and Rodriguez-Boulan, 2009). Early experiments highlighted the *trans*-Golgi network (TGN) as the first sorting station for polarized sorting of newly synthesized proteins (Rindler *et al.*, 1984; Fuller *et al.*, 1985; Griffiths and Simons, 1986). From the TGN proteins can be directed to apical or basolateral cell surfaces either directly or indirectly through recycling endosomes (Ang *et al.*, 2004; Cancino *et al.*, 2007; Cresawn *et al.*, 2007; Gravotta *et al.*, 2007). Polarized sorting at the TGN and recycling endosomes is directed by specific sorting signals present in cargo proteins, which are decoded by a yet not well understood sorting machinery that segregate the specified cargo into either apical or basolateral carrier vesicles (Wandinger-Ness *et al.*, 1990; Matter, 2000; Ellis *et al.*, 2006; Mellman and Nelson, 2008). All known basolateral sorting signals have been located within the cytosolic tails of transmembrane proteins encompassing discrete peptidic sequences and at least in part are decoded by a clathrin-mediated pathway, including the clathrin adaptor AP1B (Matter, 2000; Rodriguez-Boulan *et al.*, 2005; Deborde *et al.*, 2008; Mellman and Nelson, 2008). In contrast, apical sorting signals are of variable nature, including peptide sequences and post-translational modifications (Matter, 2000; Rodriguez-Boulan and Musch, 2005; Mellman and Nelson, 2008; Gonzalez and Rodriguez-Boulan, 2009), such as lipid and sugar moieties, and can reside in the extracellular, transmembrane or intracellular domains of the cargo proteins (Weisz and Rodriguez-Boulan, 2009). The machinery that decodes the variety of apical sorting is less well understood. The glycosylphosphatidyl-inositol (GPI) anchor was one of the first postulated apical sorting signals (Brown *et al.*, 1989; Lisanti *et al.*, 1989; Harder and Simons, 1997) but the underlying mechanism is still unclear. A common characteristic of GPI-anchored proteins (GPI-APs), mediated by the GPI anchor, is their association to specific membrane domains called rafts. Rafts are lipid ordered membrane microdomains enriched in cholesterol and sphingolipids that accommodate different proteins such as GPI-APs, transmembrane proteins and acylated proteins

having high affinity for raft lipids (Harder and Simons, 1997). Based on their ability to segregate proteins and lipids in specific membrane compartments, rafts are thought to be involved in many cellular functions like protein sorting, endocytosis, signalling, bacteria infection and virus budding (Manes *et al.*, 2000; Cherukuri *et al.*, 2001; Nguyen and Taub, 2004; Leser and Lamb, 2005; Howes *et al.*, 2010; Simons and Gerl, 2010). In particular, apical sorting of GPI-APs has been postulated to be mediated by their association with rafts. However, raft-associated GPI-APs can be sorted either apically or basolaterally (Zurzolo *et al.*, 1993; Lipardi *et al.*, 2000; Paladino *et al.*, 2004; Paladino *et al.*, 2007), indicating that additional factors should be involved. Epithelial cells have apical sorting pathways that are either dependent or independent of glycans moieties in cargo proteins, well documented for some secretory and transmembrane proteins (Rodriguez-Boulan and Gonzalez, 1999; Potter *et al.*, 2006). However the role of N-glycosylation in apical sorting of GPI-APs is controversial (Benting *et al.*, 1999a; Potter *et al.*, 2004; Catino *et al.*, 2008). Our previous studies using glycosylation mutants and chimeric proteins have shown that neither N-glycans nor O-glycans are necessary for apical sorting of GPI-APs in MDCK cells (Catino *et al.*, 2008). We have also shown that although both apical and basolateral GPI-APs are raft associated in the Golgi apparatus, apical sorting is mediated by the capacity of GPI-APs to form high molecular weight (HMW) complexes and impairment of oligomer formation results in basolateral missorting (Paladino *et al.*, 2004; Paladino *et al.*, 2007). Nevertheless, the mechanism that promotes clustering of apical GPI-APs in HMW complexes and segregation from basolateral GPI-APs at the Golgi level is still unknown. We recently proposed that both the GPI anchor and the Golgi membrane environment (specifically the cellular cholesterol content) are involved in the regulation of these events (Lebreton *et al.*, 2008; Paladino *et al.*, 2008). We showed in MDCK cells that addition of cholesterol promotes the oligomerization and consequently the apical sorting of a GPI-AP that is normally monomeric and basolaterally sorted (Paladino *et al.*, 2008). Based on this and other evidences we have proposed that a specific membrane environment enriched in cholesterol is required to favour oligomerization of GPI-APs in the Golgi and consequently ensure their apical sorting (Lebreton *et al.*, 2008; Paladino *et al.*, 2008).

In order to further understand the molecular events determining oligomerization linked to apical sorting we decided to study the role of cholesterol in Fisher rat thyroid (FRT) cells, which possess membrane domains enriched in cholesterol and sphingolipids (Zurzolo *et al.*, 1994) but exhibit different sorting properties compared to MDCK cells (Paladino *et al.*, 2004). In contrast to

MDCK cells, FRT cells direct most GPI-APs basolaterally (Zurzolo *et al.*, 1993). However, the few GPI-APs that form HMW complexes are apically sorted, thus supporting our hypothesis that oligomerization at the Golgi level is necessary for apical sorting of GPI-APs in different epithelia (Paladino *et al.*, 2004; Paladino *et al.*, 2007). Here, we show that differently from MDCK cells, addition of cholesterol in FRT cells is not sufficient to determine oligomerization and apical sorting of basolateral GPI-APs. We found that the Golgi membranes in FRT cells are enriched in cholesterol compared to MDCK cells, and do not incorporate uptaken cholesterol exogenously added into the culture medium. The higher cholesterol level in the Golgi of FRT cells is likely to increase the membrane rigidity, resulting in a restricted diffusion of both apical and basolateral GPI-APs, observed in FRAP experiments.

All our combined data indicate that in FRT cells cholesterol is not a key element regulating the segregation of apical and basolateral GPI-APs at the Golgi level. Strikingly, in contrast with MDCK cells, we demonstrate that N-glycosylation is required to determine oligomerization and apical sorting of GPI-APs in FRT cells. In conclusion our data confirm that oligomerization is the mechanism that segregate apical and basolateral GPI-APs in the Golgi, leading to their differential sorting. The data also indicate that the oligomerization mechanism of apical GPI-APs present important variations in different epithelial cells, depending preferentially on either cholesterol or N-glycosylation.

RESULTS

Cholesterol overload does not affect sorting of apical and basolateral GPI-APs

Lipid rafts enriched in cholesterol and sphingolipids have been proposed as apical platforms for apical sorting (Schuck and Simons, 2004). However, we have previously shown that MDCK cells direct a model GPI-AP protein (GFP-PrP) to the basolateral domain despite its association with lipid rafts (Paladino et al., 2008). Furthermore cholesterol addition to the media re-routes GFP-PrP to the apical domain, and leads to its oligomerization in the Golgi (Lebreton et al., 2008; Paladino et al., 2008). This observation provides a mechanistic link between cholesterol contents in Golgi membranes, oligomerization and apical sorting of raft-associated GPI-APs. In order to study whether this apical sorting mechanism also operates in FRT cells, which unlike MDCK cells sort most GPI-APs

to the basolateral domain, we analyzed the behaviour of different apical and basolateral model GPI-APs.

As an apical model protein we constructed a chimeric protein consisting of GFP fused to N- and O-glycosylation sequences of the LDL receptor followed by the GPI-anchor attachment signal of DAF protein, which we called GFP-NO-GPI. This and the previously described basolateral model protein GFP-PrP, which results from the fusion of GFP to the GPI anchor attachment signal of the prion protein (Lebreton *et al.*, 2008; Paladino *et al.*, 2008) were stably transfected in FRT cells. Several clones expressing comparable levels of proteins were selected. In order to define the polarized distribution and Detergent Resistant Membrane (DRM) association of these proteins we performed confocal microscopy analyses (Figure 1A) and selective biotinylation assays (Figure 1B) on polarized FRT cells seeded on transwell filters as well as sucrose density gradient assays (Figure 1C). At steady state, confocal analysis and selective biotinylation showed GFP-NO-GPI localizing almost exclusively at the apical membrane of polarized FRT cells (93.5%+/-6.3) (Figure 1A and B left panels), while GFP-PrP was mainly basolateral (76%+/-2.8) (Figure 1A and B right panels). As expected from being GPI-anchored, both proteins migrated to lighter fractions on sucrose density gradients suggesting association to DRMs (Figure 1C). These results reproduce previous observations in MDCK cells (Paladino *et al.*, 2004; Paladino *et al.*, 2007), they indicate that apical and basolateral GPI-APs cannot be distinguished by the property of association with DRMs since both are associated to raft domains.

In agreement with our hypothesis on the critical role of oligomerization in apical sorting, analysis by velocity gradients showed that GFP-NO-GPI form HMW complexes in FRT cells, both in control conditions (36.1%+/-3.5 of total) and upon cholesterol addition (31%+/-5.2) (Figure 2A). However, while in MDCK cells cholesterol addition results in the oligomerization and apical sorting of basolateral GFP-PrP (Lebreton *et al.*, 2008; Paladino *et al.*, 2008), in FRT cells it remained in a monomeric/dimeric form (Figure 2A) and continued to be basolaterally sorted (Figure 2B and C right panels) upon cholesterol addition. Such cholesterol-insensitive behaviour was not restricted to these two model proteins. Indeed PLAP and GFP-FR, respectively apically and basolaterally sorted in FRT cells (Paladino *et al.*, 2007), showed a similar behaviour and did not change their oligomerization status and sorting upon cholesterol addition (Figure S1). Overall these data suggests that although

FRT cells conserve the requirement for oligomerization of GPI-AP apical sorting (Paladino *et al.*, 2007) they must have another cholesterol-independent mechanism, that promotes oligomerization of apical GPI-APs.

Apical and basolateral GPI-APs display restricted diffusion in FRT cells that does not change upon cholesterol addition

In the Golgi membranes of polarized MDCK cells, apically sorted GPI-APs form HMW complexes and by FRAP analysis appear restricted in their apparent diffusion coefficient (D). On the contrary basolaterally sorted GPI-APs do not form HMW complexes and exhibit a higher D (Lebreton *et al.*, 2008). Interestingly, the addition of exogenous cholesterol results in the restriction of the apparent diffusion coefficient of the basolateral protein GFP-PrP. This correlates with its incorporation into HMW complexes and subsequent apical sorting (Lebreton *et al.*, 2008; Paladino *et al.*, 2008). Because in FRT cells GFP-PrP appears to be insensitive to cholesterol addition, we performed a similar confocal FRAP analysis in order to obtain information on the membrane environment of GPI-APs in the Golgi of FRT cells. We analysed both GFP-NO-GPI and GFP-PrP, as well as two transmembrane non-raft proteins, the apical P75-GFP and the basolateral GFP-PIT (Lebreton *et al.*, 2008) (Figure 3A). Surprisingly, in the Golgi of FRT cells, all studied proteins showed a restricted apparent diffusion coefficient of <0,015 (Figure 3B), irrespectively of their raft association and polarized sorting. This contrasted with MDCK cells in which we previously reported a significantly higher apparent diffusion coefficient of basolateral GFP-PrP compared to apical GPI-APs (Lebreton *et al.*, 2008). Interestingly, in both MDCK and FRT cells, GFP-PrP exhibited a similar D of 0,05 $\mu m^2/s$ at the level of the basolateral plasma membrane of both MDCK and FRT cells (Figure S2), validating our measurements in the Golgi. Thus, in FRT cells the membrane of the Golgi seems to restrict the diffusion capacity not only of GPI-APs but also of transmembrane proteins, independently of their oligomerization state or raft association. This is different from MDCK cells, in which the capacity of GPI-AP to form HMW complexes correlates with a low apparent diffusion coefficient (Lebreton *et al.*, 2008).

Next, we repeated the FRAP analyses upon addition of cholesterol. In contrast to MDCK cells (Lebreton *et al.*, 2008) FRT cells did not show any change in the apparent diffusion coefficient of

apical and basolateral GPI-APs (Figure 3B). This correlates with the observation that cholesterol addition does not have any effect on the oligomerization and sorting of GPI-APs in these cells (Figure 2). In addition, we analyzed whether the FRAP property of our model proteins is sensitive to cholesterol depletion. By using an inhibitor of cholesterol synthesis (HMG-COA reductase) that allows to reduce by 30% the cellular cholesterol content (Lebreton *et al.*, 2008) we observed that all apical proteins (raft- and non-raft-associated) equally exhibited an increased D ($p<0.05$), while basolateral proteins (raft- and non-raft-associated) remained restricted (Figure 3C). Because both apical and basolateral GPI-APs are associated with DRMs, their different responses might reflect segregation into membrane domains with different sensitivity to cholesterol depletion. However the fact that all apical proteins independently of their raft association were affected suggests that the effect of cholesterol depletion does not derive from perturbations of lipid rafts. Consistently, the increased diffusion of apical GPI-APs induced by cholesterol depletion does not result in basolateral missorting contrary to what was previously shown in MDCK cells (Paladino *et al.*, 2004; Lebreton *et al.*, 2008; Paladino *et al.*, 2008). Overall these data suggest that in FRT cells neither segregation between apical and basolateral GPI-APs in the Golgi membranes nor the apical sorting is driven by association with cholesterol enriched domains.

This hypothesis is further supported by the findings that inhibition of sphingolipid synthesis by FB1 does not disrupt the lipid raft association of PLAP despite provoking its missorting (Lipardi et al., 2000). Here we confirmed and extended these findings to GFP-NO-GPI (Figure 4B) suggesting that missorting of GPI-APs in FRT cells is unrelated to lipid raft disruption. In order to support this hypothesis we tested whether FB1 affects apical sorting of non-raft associated transmembrane proteins such as P75-GFP and endogenous DPPIV. Strikingly, in FRT cells both transmembrane proteins were missorted to the basolateral domain upon FB1 treatment (Figure 4 D, E) indicating that inhibition of sphingolipid synthesis affects apical sorting through some yet unknown mechanism that does not correlate with lipid raft association. These data also show that GPI-APs can be apically sorted or basolaterally missorted while maintaining their raft association. Therefore raft association is not sufficient to ensure apical sorting of GPI-APs, strengthening the notion that oligomerization is the most important driving element.

Addition of cholesterol does not affect the Golgi structure of FRT cells

Both MDCK and FRT cells upon loading of cholesterol show an increase in total cellular cholesterol content between 60 and 100% (see material and methods). Because in FRT cells addition of cholesterol does not have any effect on the apparent diffusion coefficient and sorting of GPI-APs the question arises as to whether the cholesterol up-taken by these cells becomes incorporated into the Golgi membranes. It has been reported that increases in the cholesterol content induces vesiculation of the Golgi compartment (Stuven *et al.*, 2003; Ying *et al.*, 2003). Therefore, we monitored the distribution of markers of the cis/medial Golgi (e.g; giantin) (Linstedt and Hauri, 1993) and the Trans Golgi Network (TGN) (e.g; furin) (Takahashi *et al.*, 1995). MDCK or FRT cells stably expressing GFP-PrP were grown to high confluency on coverslips, stained with either anti-giantin or furin antibodies and imaged by confocal microscopy in control conditions and upon cholesterol addition. On the acquired images we applied a robust image analysis that allowed us to define the physical space (e.g; number of pixels) occupied by these two markers relative to the size of the cell and the nucleus (see material and methods). In control conditions in MDCK cells the staining of giantin and furin was restricted to the central part of the cell, occupying respectively 3.25% (+/-2.4) and 3% (+/-2.4) of the total cell surface (Figure 5 A, C upper panels and 5E). Cholesterol addition induced a scattering of both Golgi compartments, which exhibited an expanded surface (respectively 11.1%+/-3.8 for giantin and 9% +/-3.05 for furin of the cell surface ($p<0.0001$)) (Figure 5 A, C lower panels and 5E). Similar to MDCK cells giantin occupies 4.2% +/-2.6 of the cell surface of FRT cells, while furin displayed a more scattered distribution (16.1% +/- 4.8 of the cell surface) (Figure 5 B, D and F). Surprisingly, upon cholesterol addition we did not observe any significant change in the surface occupied either by giantin or furin (6%+/-4 and 17.5%+/-3.5 respectively). Therefore, in contrast with MDCK and other cells (Stuven *et al.*, 2003; Ying *et al.*, 2003) the Golgi of FRT cells seems relatively insensitive to cholesterol loading, showing no changes in the distribution of *cis* and TGN markers and no vesiculation. One possible explanation is that the Golgi membranes of FRT cells are enriched in cholesterol and therefore unable to incorporate the uptaken cholesterol after exogenous addition.

To verify this hypothesis we performed subcellular fractionation and quantified the amount of cholesterol in Golgi enriched fractions. The cholesterol contents found in Golgi membranes of FRT cells was significantly higher than in MDCK cells and showed no increase upon cholesterol addition to

the culture medium (Figure S3). Thus, FRT cells are able to uptake cholesterol from the medium but do not incorporate it into Golgi membranes, likely because they are already saturated with this lipid.

N-glycosylation is critical for apical sorting and oligomerization of GPI-APs

Having excluded a role for cholesterol, we investigated other mechanisms that might mediate oligomerization and apical sorting of GPI-APs in FRT cells. Although the role of N-glycosylation in apical sorting of GPI-APs in MDCK cells has been controversial (Lisanti *et al.*, 1989; Benting *et al.*, 1999a; Catino *et al.*, 2008) and our previous data in MDCK cells argued against a direct role in the apical sorting of PLAP (Catino *et al.*, 2008). However, considering the differences in the apical sorting machinery already disclosed in FRT cells we decided to study the role of N-glycosylation in these cells using different model proteins. Inhibition of N-glycosylation with tunicamycin resulted in basolateral missorting of both PLAP and GFP-NO-GPI proteins, as shown by confocal immunofluorescence and domain selective biotinylation (Figure 6A-D). We previously showed that tunicamycin has a milder effect upon apical sorting of PLAP in MDCK cells, which is likely due to an indirect effect as its N-glycosylation mutant (PLAPΔN) oligomerizes and is efficiently addressed to the apical domain in these cells (Catino *et al.*, 2008). Strikingly, in FRT cells this mutant was totally missorted to the basolateral domain (Figure 6E and F) and consistently with our oligomerization model, it did not form HMW complexes in these cells (Figure 6G). These data disclose a previously unexpected role of N-glycosylation in the oligomerization of GPI-APs and indicate that different mechanisms may assure this essential event for apical sorting of GPI-APs in different epithelia.

Discussion

The mechanism(s) of GPI-APs sorting in polarized epithelial cells remains unclear and includes some controversial aspects regarding the roles of rafts and glycosylation. We have previously shown that sorting of apical, but not basolateral GPI-APs depends on their clustering into HMW complexes, which occurs in the Golgi concomitantly with their association with DRMs (Paladino *et al.*, 2004; Paladino *et al.*, 2007). Interestingly, the requirement of clustering in the Golgi complex for GPI-APs apical sorting is conserved between MDCK and FRT cells, even though they sort the majority of GPI-APs to different domains (Paladino *et al.*, 2004; Paladino *et al.*, 2007). However, while in MDCK cells cholesterol is a critical element regulating apical sorting here we show that FRT cells direct GPI-

APs to the apical surface through a N-glycosylation-dependent, rather than cholesterol-dependent oligomerization mechanism. We further show that such sorting mechanism is insensitive to cholesterol addition or depletion presumably due to the cholesterol-enriched Golgi membranes of these cells.

The role of cholesterol in polarized protein trafficking has been evoked in many cases but is still not well understood (Paladino *et al.*, 2004; Simons and Gerl, 2010). The hypothesis that raft cargo proteins can exit the Golgi in specialized carriers has been recently supported by direct evidence obtained in yeast (Klemm *et al.*, 2009). Conversely apical and basolateral protein segregation in the TGN can involve association with rafts that cluster before forming a transport carrier (Paladino *et al.*, 2004; Schuck and Simons, 2004). Previous studies in MDCK cells showed that cholesterol depletion decreases the clustering of apical GPI-APs in the Golgi compartment and causes missorting of these proteins to the basolateral surface (Paladino *et al.*, 2004). Consistently, addition of cholesterol was shown to be necessary and sufficient to induce oligomerization and revert sorting of basolateral GFP-PrP towards the apical domain (Lebreton *et al.*, 2008; Paladino *et al.*, 2008). These findings highlight the fundamental role of cholesterol in regulating oligomerization and apical sorting of GPI-APs in MDCK cells (Lebreton *et al.*, 2008; Paladino *et al.*, 2008). However, both apical PLAP and basolateral GFP-PrP are associated to DRMs in MDCK and FRT cells, indicating that raft association is not sufficient to segregate apical and basolateral GPI-APs in the Golgi (Paladino *et al.*, 2004; Paladino *et al.*, 2007). Cholesterol depletion has been also reported to induce basolateral-to-apical transcytosis of a transmembrane protein that associates with DRMs in MDCK cells (Burgos *et al.*, 2004). Therefore, cholesterol plays roles in both apical and basolateral sorting depending on the kind of cargo and its location, and additional elements besides association with lipid rafts can be key determinants of sorting behaviours. Our results point to oligomerization as a crucial requirement for apical sorting of GPI-APs which is maintained among different epithelial cells despites important variations in GPI-APs sorting (Zurzolo *et al.*, 1993; Paladino *et al.*, 2004; Paladino *et al.*, 2007).

In order to get further insights in the mechanism of apical sorting we asked whether the cholesterol-dependent oligomerization mechanism of GPI-APs operating in MDCK cells was conserved in FRT cells, which opposite to MDCK cells, direct the majority of GPI-APs to the basolateral surface (Zurzolo *et al.*, 1993). MDCK and FRT cells contain the same amount of cholesterol (Lipardi *et al.*, 2000) and uptake similar amounts of exogenously added cholesterol (see methods). Nevertheless, in FRT cells addition of cholesterol does not affect basolateral sorting of

GFP-PrP (figure 2 and S1), as it does in MDCK cells (Paladino *et al.*, 2008). Interestingly, FRAP experiments revealed that in the Golgi of FRT cells, all studied proteins (apical, basolateral, raft- and non-raft-associated) exhibit restricted diffusion, which is not affected by cholesterol addition (figure 3B). Furthermore, the TGN of FRT cells appears already scattered and differently from other cells the Golgi apparatus does not vesiculate following cholesterol addition (Stuven *et al.*, 2003; Ying *et al.*, 2003) (figure 5 B, D, F). Overall our data indicate that the Golgi membranes of FRT cells are saturated in cholesterol (Figure S2) resulting in the inability to incorporate uptaken cholesterol and in a rigidified membrane environment, which restricts protein diffusion (figure 3B).

Contrary to MDCK cells (Lebreton *et al.*, 2008) in FRT cells there is no correlation between formation of HMW complexes and the restricted diffusion of apical GPI-APs. Both GPI-AP monomers and oligomers are restricted (figure 3B), and depletion of cholesterol results in the increase in the apparent diffusion coefficient of all apical membrane proteins irrespectively of their raft-association. The increased diffusion of a non-raft apical transmembrane protein (P75NTR) by cholesterol depletion suggests that this effect is not directly due to disruption of raft domains (figure 3C). Consistently the increased diffusion of apical GPI-APs following cholesterol depletion does not result in their basolateral missorting, as it happens in MDCK cells (Lipardi *et al.*, 2000; Lebreton *et al.*, 2008; Paladino *et al.*, 2008). A likely possibility is an alteration of the underlying actin cytoskeleton (Lazaro-Dieguez *et al.*, 2007; Goswami *et al.*, 2008), as we observed a similar increased diffusion under latrunculin A treatment (not shown). Interestingly, in contrast with MDCK cells (Paladino *et al.*, 2004), in FRT cells cholesterol depletion neither affects oligomerization (data not shown) nor apical sorting of GPI-APs, even though it reduces their association to DRMs (Lipardi *et al.*, 2000). All these evidences suggest that in FRT cells apical and basolateral GPI-APs are segregated at the level of the Golgi complex independently of their association with membrane domains enriched in cholesterol. This is also supported by the findings that depletion of sphingolipids does not alter the DRM association of GPI-APs but induces missorting of all apical proteins (raft- and non raft associated) (Lipardi et al., 2000) (figure 4). These data indicate that FB1 causes missorting through some unknown mechanism that does not rely on lipid raft disruption providing further evidence that apical sorting of GPI-APs in FRT cells occurs independently of raft association.

Having excluded a role for cholesterol in the oligomerization of GPI-APs in FRT cells we explored the involvement of N-glycosylation. In MDCK cells, N-glycosylation has been involved in

apical sorting of secretory and transmembrane proteins (Scheiffele *et al.*, 1995; Gut *et al.*, 1998; Su *et al.*, 1999; Pang *et al.*, 2004; Potter *et al.*, 2006) while O-glycosylation is required for apical sorting of certain transmembrane proteins (Yeaman *et al.*, 1997; Alfalah *et al.*, 1999). However, these cells also possess apical pathways independent of any glycosylation as demonstrated for certain secreted and transmembrane proteins (Alonso *et al.*, 1997; Marzolo *et al.*, 1997; Rodriguez-Boulan and Gonzalez, 1999; Bravo-Zehnder *et al.*, 2000), as well as for GPI-APs (Potter *et al.*, 2004; Paladino *et al.*, 2008). The role of N-glycans in apical sorting of GPI-APs has been controversial. When modified by a GPI linker, growth hormone, normally secreted unpolarized (Gottlieb *et al.*, 1986; Scheiffele *et al.*, 1995), has been reported to become either apically sorted (Lisanti *et al.*, 1989), or sorted unpolarized unless N-glycosylation sites were added (Benting *et al.*, 1999b). An apical sorting mechanism independent of N-glycosylation has been described for GPI linked endolyn (Potter *et al.*, 2004) and, consistent with these data, we found no changes in the apical polarity of a PLAP mutant lacking its two N-glycosylation sites (PLAPΔN) (Catino *et al.*, 2008; Paladino *et al.*, 2008). Strikingly, here we found that in FRT cells PLAPΔN is missorted to the basolateral membrane (figure 6 E, F) and does not form oligomers (figure 6G). Furthermore, inhibition of N-glycosylation with tunicamycin leads to basolateral missorting of both PLAP and GFP-NO-GPI, which remains O-glycosylated (Figure 6 A-D). All together these findings indicate that in FRT cells N-glycosylation is the critical event for oligomer formation and apical sorting of GPI-APs, independently of the cholesterol content of the surrounding membrane environment in the Golgi.

Our results show a fundamental difference in the role of N-glycans in FRT and MDCK cells whose mechanistic elucidation might shed light on the debated role of sugars in apical sorting (Rodriguez-Boulan and Gonzalez, 1999; Potter *et al.*, 2006). It is still unknown whether N-and O-glycans play an indirect role ensuring the acquisition of a transport-competent conformation or constitute sorting moieties directly recognized by sugar binding receptors. Lectins of the galectin family that bind β-galactosides are attractive candidates for such binding receptors (Delacour *et al.*, 2009). Galectin-3, -4, and -9 have been involved in apical sorting, can be expressed preferentially in distinct epithelial cells and can be distinctly required for delivery of raft or non raft apical proteins (Huet *et al.*, 2003; Delacour *et al.*, 2005; Mishra *et al.*, 2010). Galectins are secreted via a nonconventional mechanism, as they lack a signal peptide, but can reach the sorting compartments of the biosynthetic route, including endosomal compartments (Schneider *et al.*, 2010) and TGN (Mishra *et al.*, 2010), via

endocytosis. Gal-3 has been shown to promote glycoprotein clustering (Delacour *et al.*, 2007). Thus, it is tempting to speculate that certain galectins could predominate in FRT cells acting as mediators of the N-glycan-dependent oligomerization of apical GPI-APs.

Our observations in FRT cells provide further evidence that oligomerization of apical GPI-APs is an essential step for their segregation from basolateral GPI-APs and possibly from other apical proteins as well (transmembrane and non-raft associated). Whether apical GPI-APs and raft associated transmembrane proteins exit the Golgi in the same carriers or are previously segregated at the level of the Golgi membranes remains unknown. Oligomerization of transmembrane proteins has been proposed as a mechanism for retention in the Golgi complex (Weisz *et al.*, 1993). Our observations indicate that only apical GPI-APs and not transmembrane proteins form HMW oligomers at the Golgi complex (Paladino *et al.*, 2004; Paladino *et al.*, 2007), suggesting that they are segregated at this level. Live cell imaging experiments, currently underway in our laboratory, will test this hypothesis.

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MATERIAL AND METHODS

Reagents and antibodies

Cell culture media were purchased from Sigma Aldrich, Inc, (St Louis, USA) and Euroclone Spa (Pero (MI) Italy). Antibodies were purchased from the following companies: polyclonal α-GFP and monoclonal α-GFP from Invitrogen (Eugene, Oregan, USA), α-PLAP from Rockland (Gilbetsville, PA, USA) and monoclonal anti-flotilin-1 antibody from BD Transduction Laboratories, polyclonal giantin from Covance (1:5000 for WB and 1:500 for IF; Emeryville, California), polyclonal furin from Thermo scientific (1:1000 for WB and 1:100 for IF; Rockford, USA), polyclonal calnexin from Stressgene (1:1000; Ann Arbor, USA) and monoclonal N-cadherin from BD Transduction Laboratories (1:1000). Biotin and horseradish peroxidase (HRP)-linked streptavidin were from Pierce (Rockford, Il, USA). FB1, tunicamycin and all other reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Cell culture and transfections

MDCK and FRT cells were grown in DMEM (Dulbecco's modified Eagle's medium) and F12 (F12 Coons modification medium) respectively containing 5% FBS. MDCK cells stably expressing GFP-PrP and FRT cells stably expressing PLAP, GFP-PIT and p75-GFP had been obtained previously (Paladino *et al.*, 2004; Catino *et al.*, 2008). FRT cells were transfected with sequences encoding GFP-PrP, GFP-NO-GPI (GFP fused to the N glycosylation sequence (10 amino acid) and O- glycosylation sequence (56 amino acid) of LDL receptor followed by the GPI-signal attachment of the DAF (Decay Accelerating Factor) protein) and PLAPΔN (Catino *et al.*, 2008) by using lipofectin (Invitrogen). Stable clones were selected according to the antibiotic resistance.

Biotinylation assay

Cells grown on polycarbonate filters for 4 days were selectively biotinylated from the apical or the

basolateral side using sulpho-N-hydroxysulfosuccinimide-long chain (-NHS-LC)-biotin. Lysates were immunoprecipitated using a monoclonal anti-GFP antibody (Molecular Probes, Invitrogen) for GFP-PrP and GFP-NO-GPI and with a polyclonal anti-PLAP antibody for PLAP and PLAP∆N and analyzed by western blotting using horseradish peroxidase (HRP)-streptavidin (Pierce).

Modification of cholesterol content

To deplete cellular cholesterol we used a previously published protocol (Lipardi *et al.*, 2000; Lebreton *et al.*, 2008). Briefly, FRT cells were plated on filters and 24h after plating mevinolin (10μM) and mevalonate (250μM) were added to the cells in F12 medium supplemented with 5% delipidated calf serum for 48 hours.

To load the cells with cholesterol we used a previously published protocol (Lebreton *et al.*, 2008). Cells were plated on filters for 4 days and water soluble cholesterol (10 mM in MβCD) was added to warm medium containing 20 mM Hepes, pH 7.5, and 0.2% bovine albumin for 45 min at 37°C. To determine the rate of cholesterol depletion or addition, we measured cellular cholesterol levels by a colorimetric assay (cholesterol/cholesteryl ester quantification; Calbiochem) according to the manufacturer's instructions. Following cholesterol addition FRT and MDCK cells uptake similar amount of cholesterol ranging from 60% to 100% of total cellular cholesterol.

Velocity Gradients

Velocity gradients were performed using a previously published protocol (Paladino *et al.*, 2008) The cells were grown to confluency in 100-mm dishes, washed in phosphate-buffered saline containing $CaCl_2$ and $MgCl_2$ and lysed on ice for 30 min in 20 mM Tris, pH 7.4, 100 mM NaCl, 0.4% SDS, 0.2% Triton X-100. The lysates were scraped from dishes, sheared through a 26-gauge needle, and layered on top of a sucrose gradient (30 to 5%) after removal of nuclei by low speed centrifugation. After centrifugation at 45,000 rpm for 16 h in an ultracentrifuge (model SW 50; Beckman counter), fractions of 500 μl were harvested from the top of the gradient and trichloroacetic acid-precipitated. The proteins were revealed by western blot using specific antibodies.

Sucrose density gradients

Sucrose gradient analysis of TX-100-insoluble material was performed using previously published protocols (Paladino *et al.*, 2008). Cells were grown on 150 mm (20-25 x 10^6) dishes to confluence, were scraped from dishes in PBS containing $CaCl_2$ and $MgCl_2$, and resuspended in 1mL of lysis buffer containing 1% TX-100, 10 mM Tris buffer (pH 7.5), 150 mM NaCl, 5 mM EDTA and sheared through a 23 G needle. The lysate was mixed with an equal amount of 85% sucrose and run on discontinuous sucrose gradients (40-5%) for 16 hours at 4°C. 12 fractions were collected from top to bottom of the gradient. Proteins were TCA precipitated and detected by western blotting using anti-GFP antibody and anti-flotilin antibody (a typical raft-marker).

FRAP Measurements and Analysis

FRAP analysis was performed as described previously (Lebreton *et al.*, 2008) on a confocal LSM 510 META from Zeiss using the plan apo 63X oil immersion (NA 1.4) objective lens. We monitored the fluorescence of the GFP fused proteins using low intensity laser excitation (488 nm) (prebleach scans). For analysis we always kept an airy unit of 1. In all the experiments a circular region (ROI) of 1.4 µm was photobleached with the same laser excitation at high intensity (decrease of the fluorescence into the ROI by 60-80%), and then the recovery of fluorescence into the bleached region over time was monitored. This recovery reflects the ability of unbleached fluorescent proteins around the ROI to repopulate the photobleached ROI. For each FRAP acquisition, we considered two internal controls, one that indicates the natural bleaching of the sample over time and second one is the level of fluorescence background. The raw data were fitted with the Igor Pro software and an application developed in EMBL with the Soumpasis mathematical equation. We grew the cells on filter upside down in polarized conditions as previously reported and performed all our analysis at 37°C in order to reproduce as closest possible the physiological conditions (Lebreton *et al.*, 2008). FRAP recordings were obtained in CO_2-independent medium (0.15 M NaCl, 0.1 M KCl, 0.1 M $CaCl_2$, 0.1 M $MgCl_2$, 0.2 M Hepes).

Fluorescence Microscopy

FRT cells were grown on transwell filters for 4 days, washed with phosphate-buffered saline containing $CaCl_2$ and $MgCl_2$, fixed with 4% paraformaldehyde, and quenched with 50 mM NH_4Cl. Cells were stained with anti-GFP antibody for GFP-PrP and GFP-NO-GPI (Molecular Probes, Invitrogen)

and with anti-PLAP for PLAP and PLAPΔN followed by Alexa-conjugated secondary antibody in non-permeabilized conditions. The images were acquired using a laser scanning confocal microscope (LSM 510; Carl Zeiss Microimaging, Inc.) equipped with a plan apo 63X oil immersion (NA 1.4) objective lens.

Equal number of MDCK and FRT cells stably expressing GFP-PrP were plated on cover slips and grown until they reached very high confluency. Cells were untreated (control) or loaded with 10 mM cholesterol (+cholesterol) for 45 min and then stained either with cis-medial Golgi marker, giantin, or trans Golgi marker, furin convertase, followed by secondary antibody coupled to Alexa 546 in permeabilized conditions. Serial confocal sections of 1μm were collected from top to bottom of cell monolayers by using zeiss confocal microscope (LSM 510; Carl Zeiss Microimaging, Inc.) equipped with a plan apo 63X oil immersion (NA 1.4) objective lens and Images were analyzed by using Quia-ICY software (www.bioimageanalysis.org) . For the quantification we considered GFP staining of GFP-PrP at the level of the plasma membrane to measure the total number of pixel associated to the cell surface. The DAPI, giantin and furin staining is used to define the number of pixel associated respectively to the nucleus, the cis/medial or trans Golgi compartments. Then the number of pixel associated to the Golgi sub-compartment is normalized to cell surface. Here DAPI staining is used as internal control to see that we have the same cell density for both cell lines and allow us to check the accuracy of our experiments. For the quantification we kept the same threshold of fluorescence intensity (red and blue pixels) for all the images both in control conditions and upon loading of cholesterol.

Figure Legends

Figure 1: GFP-NO-GPI and GFP-PrP are respectively localized on the apical and basolateral domain in FRT cells

A) FRT cells stably expressing GFP-NO-GPI or GFP-PrP were grown in polarized condition on transwell filters for 4 days. Cells were fixed, quenched and serial confocal sections of 1μm were

collected from top to bottom of cell monolayers using 488 nm laser. Scale bars 10 μm. B) FRT cells stably expressing GFP-NO-GPI or GFP-PrP were incubated with long chain (LC)-biotin added to the apical (Ap) or the basolateral (Bl) surfaces. After immunoprecipitation with specific antibodies, samples were run on SDS-PAGE and biotinylated proteins revealed using HRP-streptavidin. Amount of labelled proteins was quantified by using ImageJ software by considering three independent experiments and the error bars are the mean ± SD. The white and black bars indicate the percentage of protein labelled respectively at the apical and basolateral domain. C) FRT cells stably expressing GFP-NO-GPI or GFP-PrP were lysed in a buffer containing 1% TX-100 at 4°C and separated by centrifugation at the equilibrium on 5-40% sucrose density gradients to purify TX-100-insoluble microdomains. Fractions of 1 mL were collected from top (fraction 1) to bottom (fraction 12) of the gradient. Proteins were precipitated with TCA and detected by western blotting using anti-GFP antibody and anti-flotilin antibody used as a DRM marker.

Figure 2: Addition of cholesterol affects neither the oligomeric state nor the polarized distribution of both apical and basolateral GPI-APs in FRT cells.

A) FRT cells stably expressing GFP-NO-GPI or GFP-PrP were grown to confluency in control conditions or incubated with 10 mM cholesterol solution (+cholesterol) for 45 min. Cells **lysate were** ran through a non-linear 5-30% sucrose gradients. Fractions of 500 µl were collected from the top (fraction 1) to the bottom (fraction 9) of the gradients. TCA precipitated **proteins were** detected by western blotting using anti-GFP antibody. The molecular weight of the monomeric form of each protein is indicated. The position on the gradients of molecular weight markers is indicated on top of the panel. B), C) FRT cells stably expressing GFP-NO-GPI or GFP-PrP were grown in polarized condition on transwell filters for 4 days. The plasma membrane localization of proteins was determined by analyzing the natural fluorescence of GFP with 488 nm laser **in** control cells untreated or cells loaded with 10mM cholesterol (+cholesterol) (B) or by double immunofluorescence in non-permeabilized conditions by adding anti-GFP antibody followed by Alexa 546- conjugated secondary antibody to the apical and basolateral side of the cells (C). Serial confocal sections of 1 μm were collected from the top to bottom of the cell monolayers. Scale bars 10 μm. D) FRT cells stably expressing GFP-NO-GPI or GFP-PrP were incubated with long chain (LC)-biotin added to the apical (Ap) or the basolateral (Bl)

surfaces in control conditions or upon addition of cholesterol. After immunoprecipitation with specific antibodies, samples were run on SDS-PAGE and biotinylated proteins revealed using HRP-streptavidin. Amount of labelled proteins was quantified by using ImageJ software by considering three independent experiments and the error bars are the mean ± SD. The white and black bars indicate the percentage of protein labelled respectively at the apical and basolateral domain.

Figure 3: Diffusional mobilities of raft- and non-raft-associated associated proteins at the Golgi membranes of polarized FRT cells

A) Scheme of fluorescent model proteins: Two raft-associated proteins, GFP-NO-GPI and GFP-PrP respectively apically and basolaterally sorted, and two transmembrane non-raft associated proteins, P75-GFP and GFP-PIT respectively apically and basolaterally sorted. All proteins are fused to the GFP (green cylinder) and stably expressed independently in FRT cells. B) Apparent diffusion coefficients (D) of all studied proteins in control condition (colored bars) and upon addition of cholesterol (white bars) at the level of Golgi complex in polarized FRT cells. C) Apparent diffusion coefficients of all studied proteins in control condition (colored bars) and upon depletion of cholesterol (white bars) at the level of Golgi complex in polarized FRT cells. Experiments have been performed at least two independent times, n> 15. The error bars are the means ± S.D with *, p< 0.05.

Figure 4: FB1 treatment induces basolateral missorting of both apical GPI-APs and transmembrane non raft-associated proteins in polarized FRT cells

FRT cells stably expressing PLAP (A), GFP-NO-GPI (B), GFP-PrP (C) and P75-GFP (D), DPPIV (E) were grown on transwell filters in the absence (-) or presence (+) of FB1 (10μg/ml for 20 hours). Cells were incubated with (-LC)-biotin added either on the apical (AP) or on the basolateral (BL) surface. After immunoprecipitation with specific antibodies, samples were run on SDS-PAGE and biotinylated proteins were revealed using HRP-streptavidin. Amount of labelled proteins was quantified by using ImageJ software by considering three independent experiments and the error bars are the mean ± SD. Light grey bars represent the percentage of protein labelled at the apical domain and black bars represent the percentage of protein labelled at the basolateral domain.

Figure 5: Addition of cholesterol does not affect Golgi morphology in polarized FRT cells

Equal number of MDCK and FRT cells stably expressing GFP-PrP were plated on the coverslips and grown until they reach high confluency. Cells were untreated (control) or loaded with 10 mM cholesterol (+cholesterol) for 45 min, permeabilized and then stained either with giantin antibody (cis-medial Golgi marker) or with furin convertase antibody (trans Golgi marker) followed by secondary antibody coupled to alexa 546. Serial confocal sections of 1 μm were collected from top to bottom of cell monolayers. Images were analysed by using Quia software. (A-D) middle panels show the green mask of the cell used to measure the total number of pixel of cell surface. DAPI staining is used to evaluate the number of pixel of the nucleus. (A), (B) Giantin staining in MDCK (A) or in FRT (B) cells stably expressing GFP-PrP in control condition (figure A or B upper panels) and upon loading of cholesterol (figure A or B lower panels). C), D) Furin staining in MDCK (C) or in FRT (D) cells stably expressing GFP-PrP in control condition (figure C or D upper panels) and upon loading of cholesterol (figure C or D lower panels). E), F) Number of pixel associated to Golgi marker (giantin and furin) normalized to cell surface and expressed as percentage in MDCK (E) and FRT (F) cells, in both conditions (giantin white bars and furin dark grey bars). Experiment have been performed at least two independent times ($n \geq 60$ cells) and the error bars are the means ± S.D with *, $p < 0.0001$.

Figure 6: N-glycosylation is essential for apical sorting of GPI-APs in polarized FRT cells.

FRT cells stably expressing PLAP (A) or GFP-NO-GPI (B) were plated on filters and after 48h tunicamycin (10ug/ml) was added to the cell culture medium for 20h. Cells were fixed, permeabilized and labelled with specific primary antibody (anti-PLAP) for PLAP and (anti-GFP) for GFP-NO-GPI followed by alexa-546 conjugated secondary antibody. Serial confocal sections were collected from the top to bottom of cell monolayers. FRT cells stably expressing PLAP (C) or GFP-NO-GPI (D) polarized for 4 days on filters were incubated with (-LC)-biotin added either on the apical (AP) or on the basolateral (BL) surface. After immunoprecipitation with specific antibodies, samples were run on SDS-PAGE and biotinylated proteins were revealed using HRP-streptavidin. Amount of labelled proteins was quantified by using ImageJ software by considering three independent experiments and the error bars are the mean ± SD. The white and black bars indicate the percentage of protein labelled

respectively at the apical and basolateral domain E) FRT cells stably expressing PLAP or PLAP∆N were fixed, permeabilized and labelled with specific primary antibody (∝-PLAP) and alexa- 488 conjugated secondary antibody. Serial confocal sections were collected from top to bottom of cell monolayers. Scale bar 10 μm. F) FRT cells stably expressing PLAP or PLAP∆N polarized for 4 days on filters were incubated with (-LC)-biotin added either on the apical (AP) or on the basolateral (BL) surface. After immunoprecipitation with specific antibodies, samples were run on SDS-PAGE and biotinylated proteins were revealed using HRP-streptavidin. Amount of labelled proteins was quantified by using ImageJ software by considering three independent experiments and the error bars are the mean ± SD. The white and black bars indicate the percentage of protein labelled respectively at the apical and basolateral domain. G) FRT cells stably expressing PLAP or PLAP∆N were lysed and ran through a non-linear 5-30% sucrose gradients. Fractions of 500 µl were collected from the top (fraction 1) to the bottom (fraction 9) of the gradients. Proteins were precipitated with TCA and detected by western blotting using ∝-PLAP antibody. The molecular weight of the monomeric form of each protein is indicated. The position on the gradients of molecular weight markers is indicated on top of the panel.

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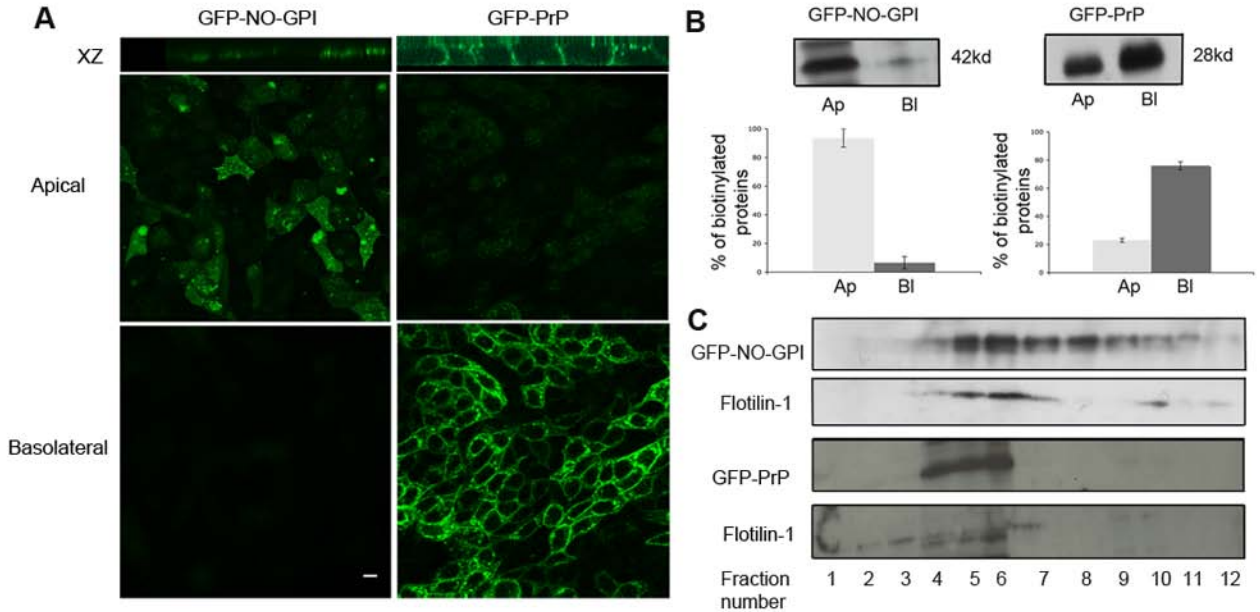

A
GFP-NO-GPI
GFP-PrP
XZ
Apical
Basolateral
B
GFP-NO-GPI
GFP-PrP
42kd
28kd
Ap
Bl
Ap
Bl
% of biotinylated proteins
% of biotinylated proteins
Ap
Bl
Ap
Bl
C
GFP-NO-GPI
Flotilin-1
GFP-PrP
Flotilin-1
Fraction number
1 2 3 4 5 6 7 8 9 10 11 12

Figure 1

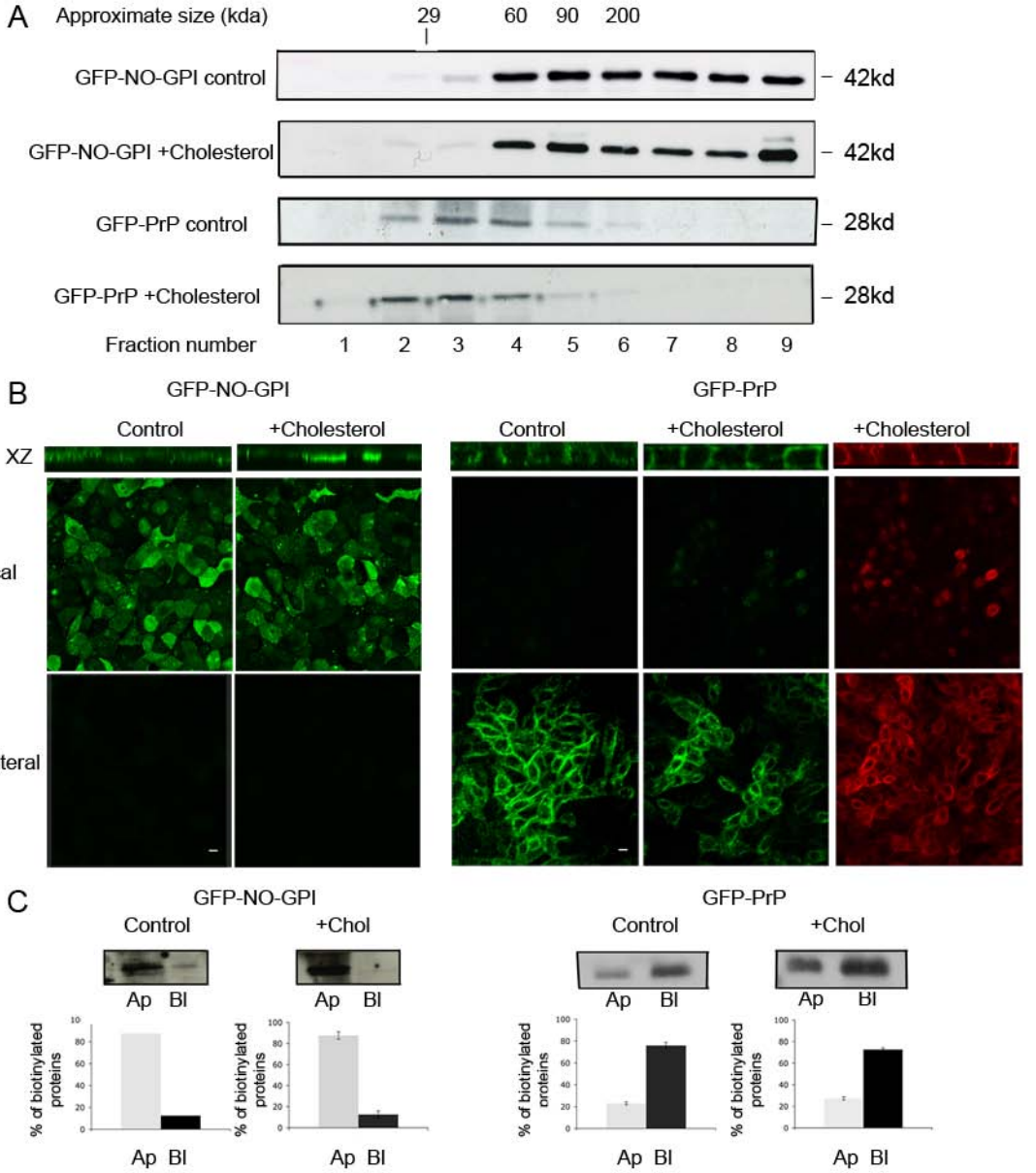

A
Approximate size (kda)
29
60
90
200
GFP-NO-GPI control
42kd
GFP-NO-GPI +Cholesterol
42kd
GFP-PrP control
28kd
GFP-PrP +Cholesterol
28kd
Fraction number
1 2 3 4 5 6 7 8 9
B
GFP-NO-GPI
GFP-PrP
Control
+Cholesterol
Control
+Cholesterol
+Cholesterol
XZ
Apical
Basolateral
C
GFP-NO-GPI
GFP-PrP
Control
+Chol
Control
+Chol
Ap Bl
% of biotinylated proteins
Ap Bl

Figure 2

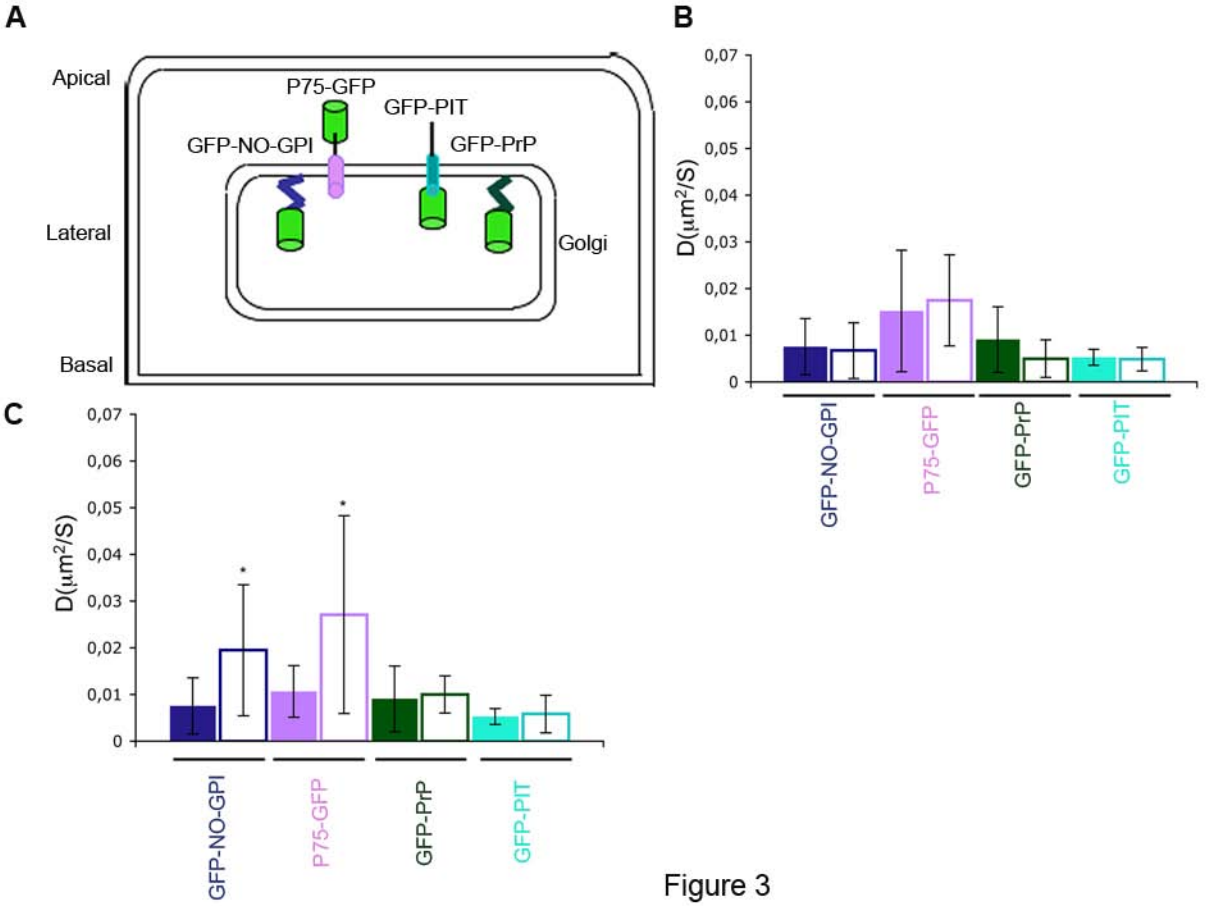

A
Apical
Lateral
Basal
P75-GFP
GFP-PIT
GFP-NO-GPI
GFP-PrP
Golgi
B
D(µm²/S)
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0
GFP-NO-GPI
P75-GFP
GFP-PrP
GFP-PIT
C
D(µm²/S)
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0
*
*
GFP-NO-GPI
P75-GFP
GFP-PrP
GFP-PIT

Figure 3

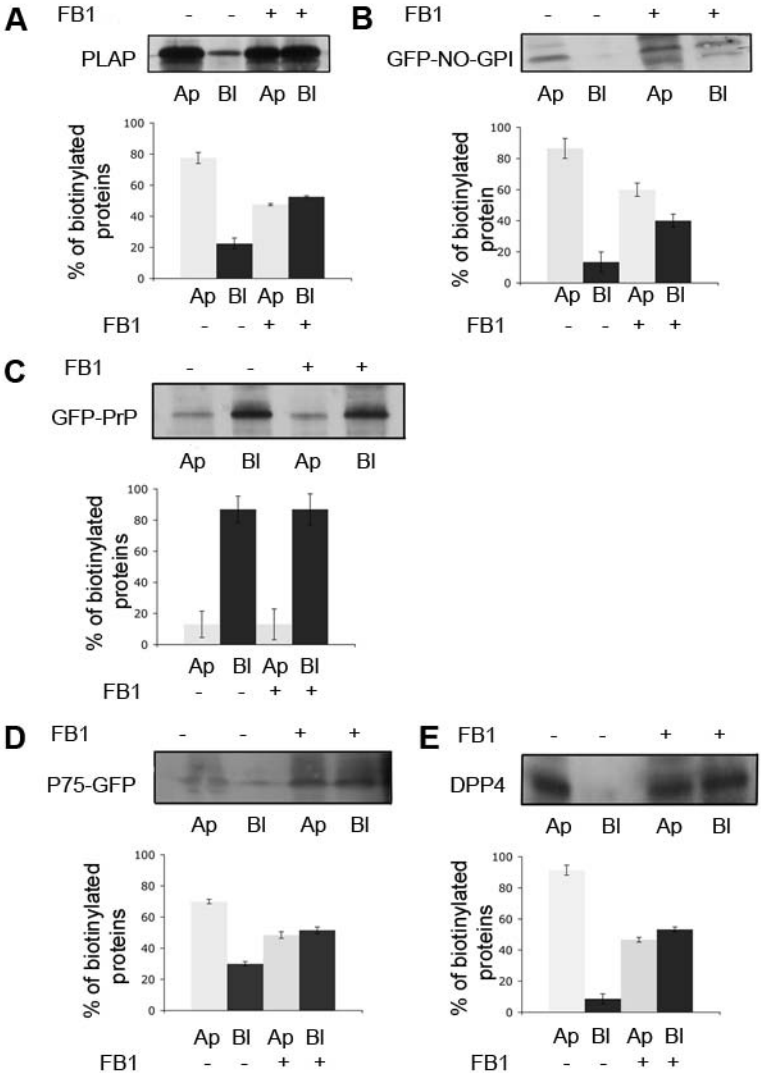

A
FB1 - - + +
PLAP
Ap Bl Ap Bl
% of biotinylated proteins
100
80
60
40
20
0
Ap Bl Ap Bl
FB1 - - + +
B
FB1 - - + +
GFP-NO-GPI
Ap Bl Ap Bl
% of biotinylated protein
100
80
60
40
20
0
Ap Bl Ap Bl
FB1 - - + +
C
FB1 - - + +
GFP-PrP
Ap Bl Ap Bl
% of biotinylated proteins
100
80
60
40
20
0
Ap Bl Ap Bl
FB1 - - + +
D
FB1 - - + +
P75-GFP
Ap Bl Ap Bl
% of biotinylated proteins
100
80
60
40
20
0
Ap Bl Ap Bl
FB1 - - + +
E
FB1 - - + +
DPP4
Ap Bl Ap Bl
% of biotinylated proteins
100
80
60
40
20
0
Ap Bl Ap Bl
FB1 - - + +

Figure 4

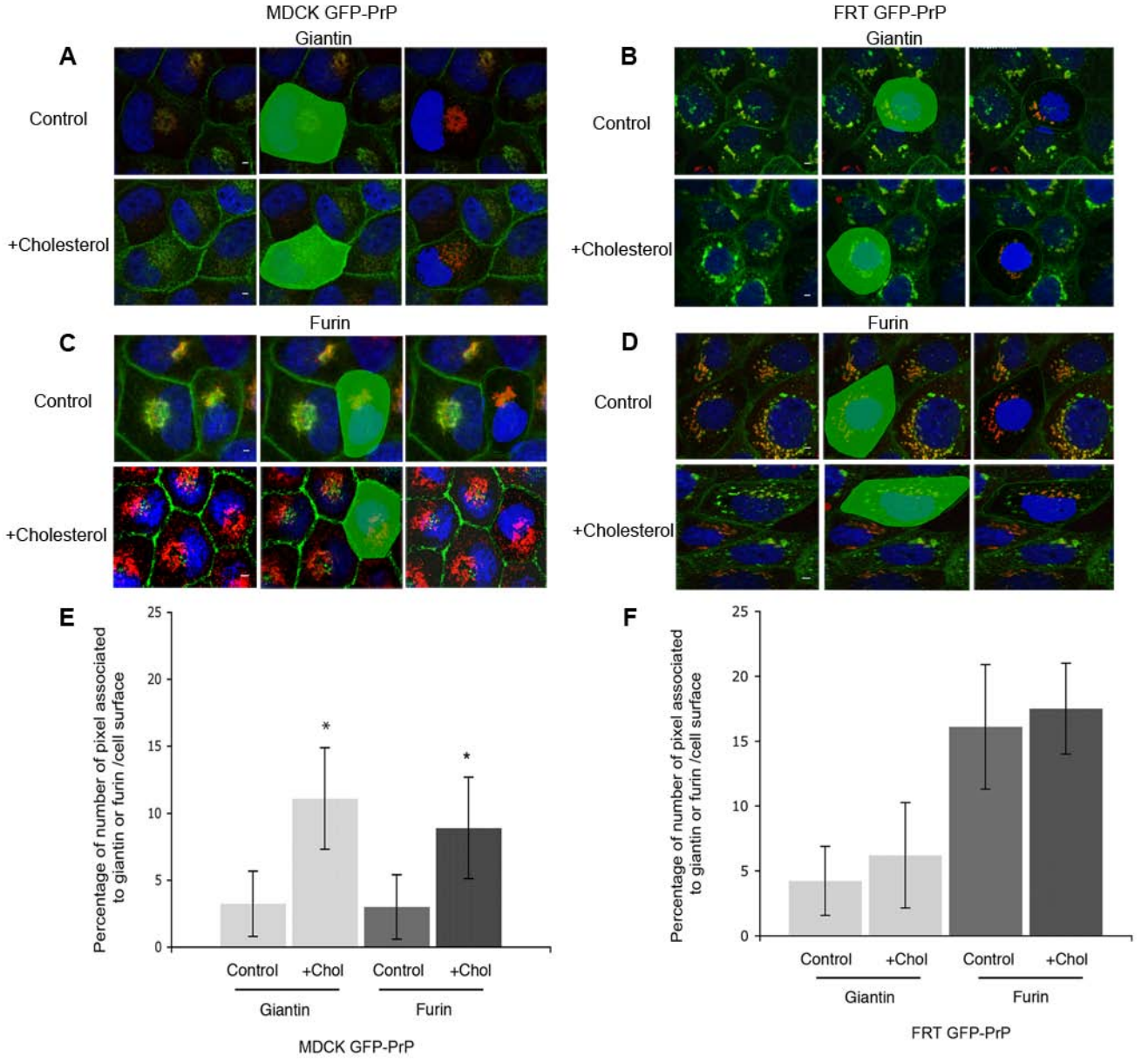

MDCK GFP-PrP
Giantin
A
Control
+Cholesterol
FRT GFP-PrP
Giantin
B
Control
+Cholesterol
Furin
C
Control
+Cholesterol
Furin
D
Control
+Cholesterol
E
Percentage of number of pixel associated to giantin or furin /cell surface
25
20
15
10
5
0
*
*
Control
+Chol
Control
+Chol
Giantin
Furin
MDCK GFP-PrP
F
Percentage of number of pixel associated to giantin or furin /cell surface
25
20
15
10
5
0
Control
+Chol
Control
+Chol
Giantin
Furin
FRT GFP-PrP

Figure 5

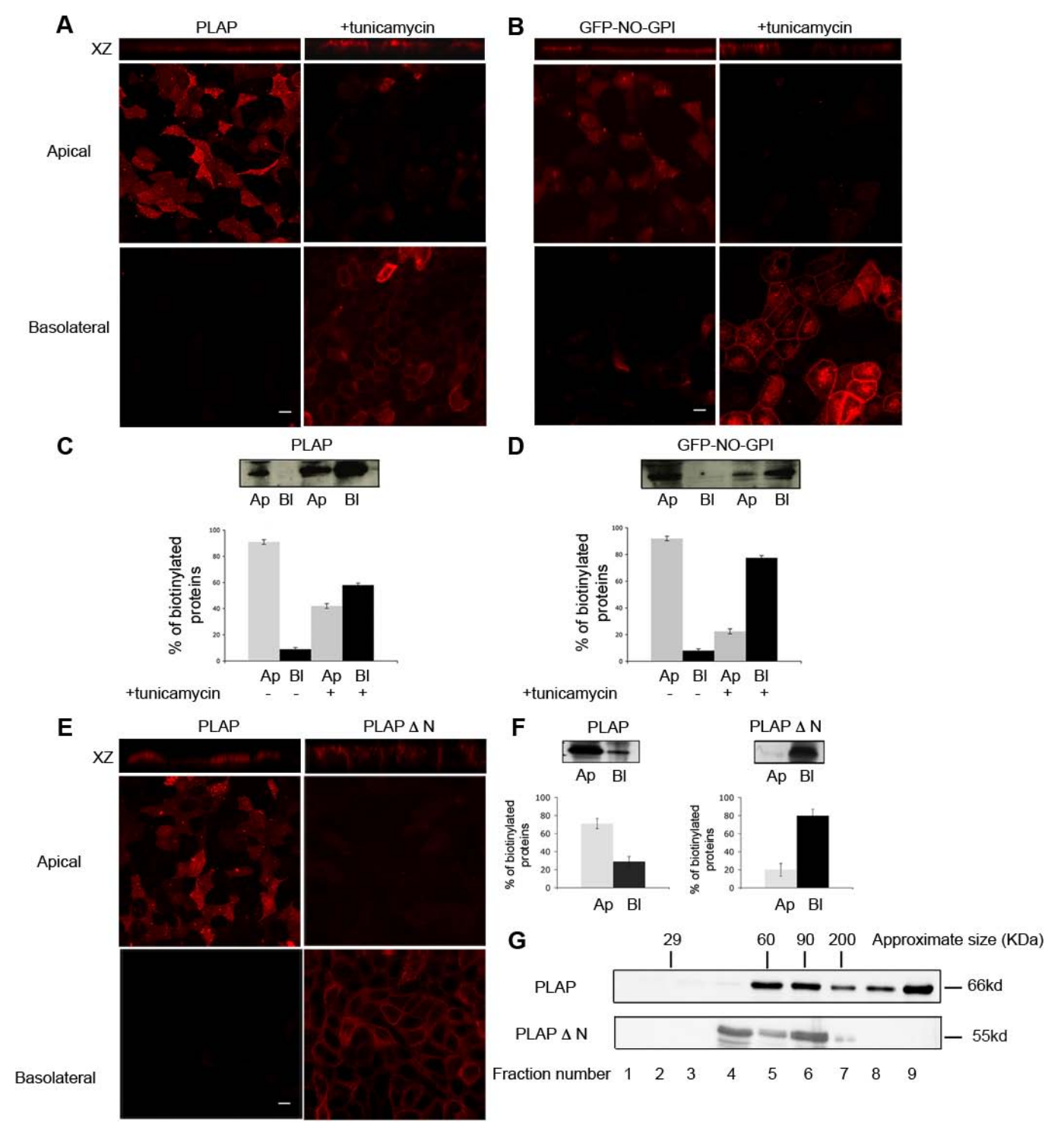

A
PLAP
+tunicamycin
XZ
Apical
Basolateral
B
GFP-NO-GPI
+tunicamycin
C
PLAP
Ap Bl Ap Bl
% of biotinylated proteins
Ap Bl Ap Bl
+tunicamycin - - + +
D
GFP-NO-GPI
Ap Bl Ap Bl
% of biotinylated proteins
Ap Bl Ap Bl
+tunicamycin - - + +
E
PLAP
PLAP Δ N
XZ
Apical
Basolateral
F
PLAP
PLAP Δ N
Ap Bl
Ap Bl
% of biotinylated proteins
% of biotinylated proteins
Ap Bl
Ap Bl
G
29 60 90 200 Approximate size (KDa)
PLAP
66kd
PLAP Δ N
55kd
Fraction number 1 2 3 4 5 6 7 8 9

Figure 6

Supplemental informations

Materials and methods for supplementary information:

Cell Fractionation:

FRT cells (12 plates of 150-mm) were homogenized by 10 strokes in an isobiotec cell homogenizer with a tungsten-carbide ball in 500µl of 20mM HEPES/KOH, pH 7.3 and 120mM sucrose. A postnuclear supernatant fraction was obtained by centrifugation at 600 × g for 5min in an eppendorf tube. The postnuclear supernatant was loaded on the top of a discontinuous sucrose gradient (0.6ml of each of 15, 20, 25, 30, 35, 40 and 45% with 0.5ml of 60% on the bottom) made up in the same buffer. The gradient was spun in an SW 50.1 rotor for 1h at 45,000 rpm in a Beckman centrifuge and 14 fractions were collected from the top of the tube and 1/20 of each fraction was loaded on to 12% polyacrylamide gels. Western blots were performed using different antibodies specific for ER, plasma membrane, cis/medial and trans Golgi markers.

Figure legends for supplementary figures:

Figure S1: Addition of cholesterol does not affect the oligomeric state and apical sorting of PLAP and GFP-FR in FRT cells.

A) FRT cells stably expressing PLAP and GFP-FR were grown to confluency and incubated in the absence (control) or presence of 10mM cholesterol (+cholesterol) for 45min. Cells lysates were run through a non-linear 5-30% sucrose gradient. Fractions of 500 µl were collected from the top (fraction 1) to bottom (fraction 9) of the gradients. Proteins were TCA precipitated and detected by western blotting using anti-PLAP and anti-GFP antibodies. The molecular weight of the monomeric form of each protein is indicated. The position on the gradients of molecular weight markers is indicated at the top of the panel. B) FRT cells stably expressing PLAP or GFP-FR were grown in polarized condition on transwell filters for 4 days. Cells were untreated (control) or loaded with 10mM cholesterol (+cholesterol) and plasma membrane localization of proteins was determined by using anti-PLAP antibody for PLAP in non permeabilized conditions and by analyzing the natural fluorescence of GFP with 488nm laser for GFP-FR. Scale bars 10µm.

Figure S2: Diffusional mobilities of GFP-PrP at the basolateral membrane of MDCK and FRT cells

Apparent diffusion coefficients of GFP-PrP in control conditions in MDCK cells (dark grey box) and in FRT cells (light grey box). Experiments have been performed three independent times, n>15. The error bars represent the means +/-SD.

Figure S3: Cholesterol quantification after subcellular fractionation of MDCK and FRT cells.

MDCK and FRT cells stably transfected with GFP-PrP were subjected to cell fractionation in control condition (control) or after addition of cholesterol (+Cholesterol). The distribution of ER, plasma membrane, cis/ medial and trans Golgi was analyzed along the gradient. A) Schematic representation of the distribution of ER, plasma membrane, cis /medial and trans Golgi along the 14 fractions of the gradient. The following proteins were quantified in each fraction and express as percentage of total: calnexin (ER maker), N-Cadherin (plasma membrane marker), giantin (cis/medial Golgi marker) and furin (TGN marker) both in MDCK and FRT cells stably expressing GFP-PrP in control condition and upon addition of cholesterol. (B) The amount of cholesterol in the Golgi enriched fractions (11-14 fractions) was quantified and normalized per μg of protein in control condition (white bars) and upon addition of cholesterol (black bars). This experiment has been performed two independent times and the error bars are the means ± S.D with *, $p < 0.05$.

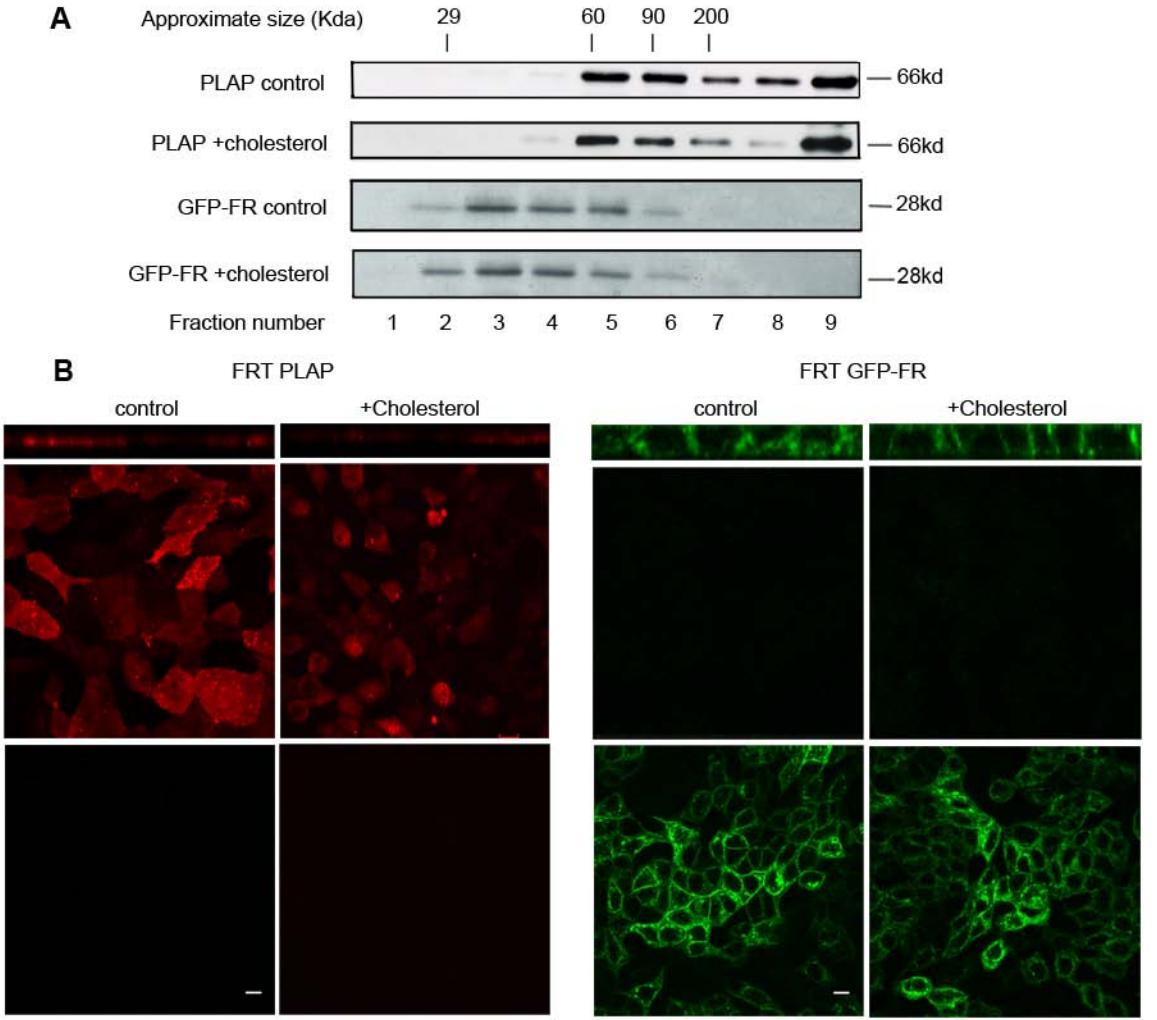

A
Approximate size (Kda)
29
60
90
200
PLAP control
66kd
PLAP +cholesterol
66kd
GFP-FR control
28kd
GFP-FR +cholesterol
28kd
Fraction number
1 2 3 4 5 6 7 8 9
B
FRT PLAP
control
+Cholesterol
FRT GFP-FR
control
+Cholesterol

Figure S1

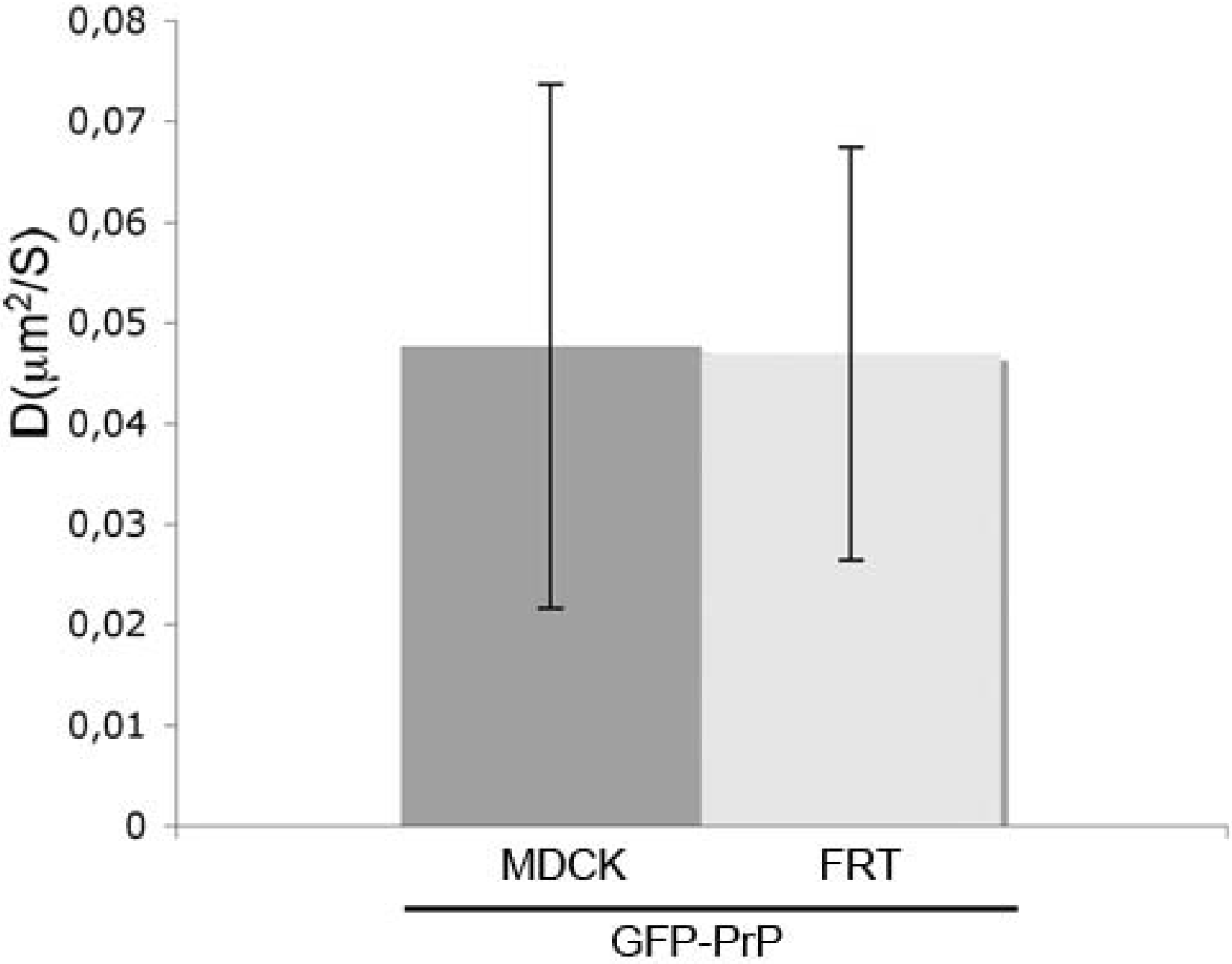


Figure S2

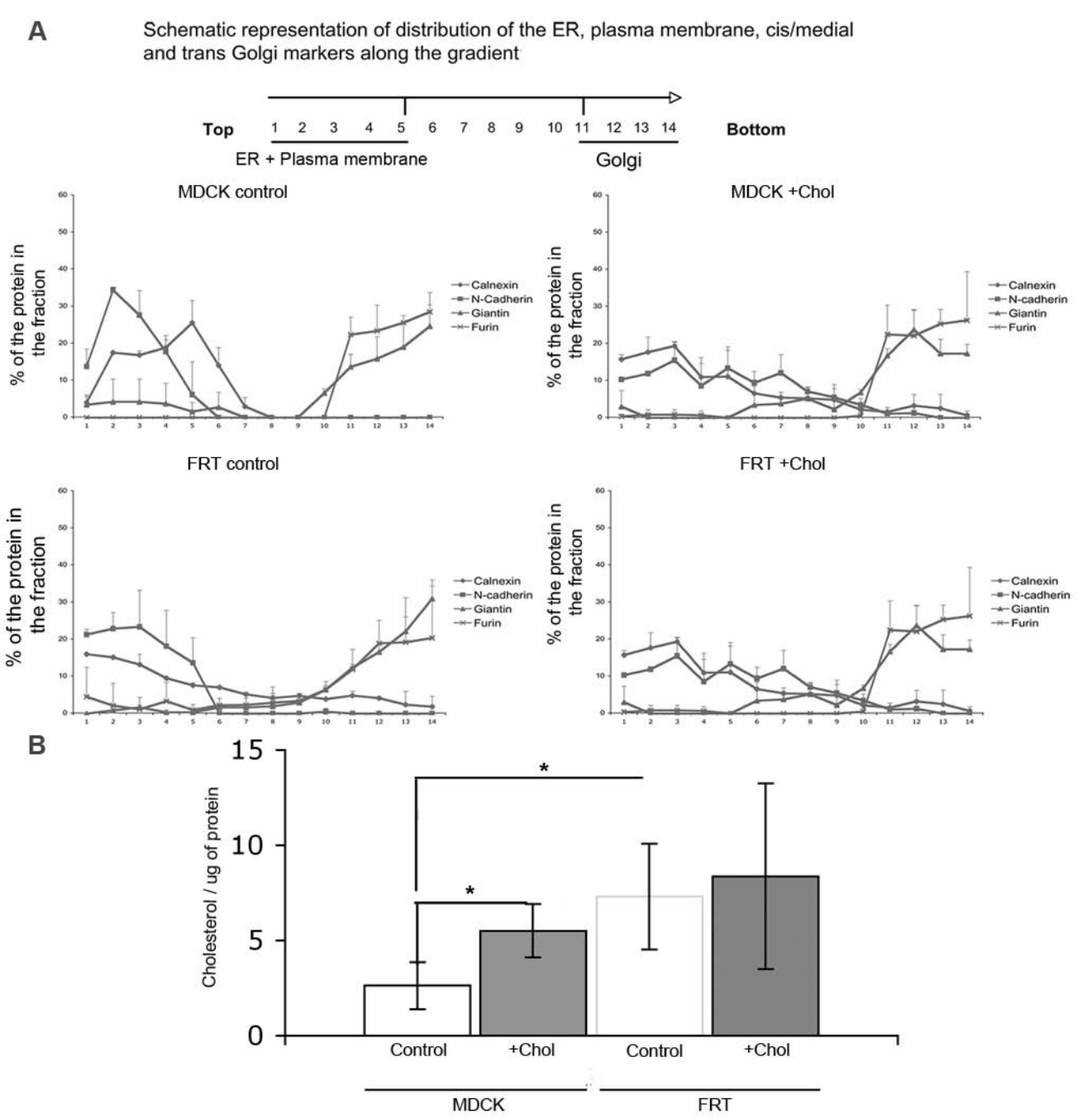

A
Schematic representation of distribution of the ER, plasma membrane, cis/medial and trans Golgi markers along the gradient
Top
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Bottom
ER + Plasma membrane
Golgi
MDCK control
MDCK +Chol
FRT control
FRT +Chol
% of the protein in the fraction
Calnexin
N-Cadherin
N-cadherin
Giantin
Furin
B
Cholesterol / ug of protein
Control
+Chol
MDCK
FRT

Figure S3

DISCUSSION

Discussion:

The sorting of GPI-APs in polarized epithelial cells is still not clear. As outlined in the introduction, the signals responsible for basolateral sorting of proteins have been clearly understood whereas the characterization of signals responsible for apical sorting appears to be more controversial. The important finding that helps to understand the mechanism of apical sorting of GPI-APs shown was that only apical GPI-APs undergo oligomerization and form high molecular weight complexes concomitantly to their association with detergent resistant domains in the Golgi prior to their sorting (Paladino et al., 2004; Paladino et al., 2007). Indeed impairment of the oligomers formation leads to the basolateral missorting (Paladino et al., 2004). This indicates that GPI-APs clustering at the level of the Golgi is an essential event for apical sorting of GPI-APs. Interestingly this clustering of GPI-APs is conserved in MDCK and FRT cells, even though they exhibit a difference in the sorting of GPI-APs (Paladino et al., 2004). MDCK cells sort most of the GPI-APs to the apical plasma membrane (Paladino et al., 2004; Paladino et al., 2006; Paladino et al., 2007), whereas FRT cells address most of the GPI-APs to the basolateral surface (Zurzolo et al., 1994). However the mechanism of oligomerization and the factors responsible for oligomerization are still not known.

In the present study using mutant PLAP protein, which lacks two N-glycosylation sites, and tunicamycin treatment we clearly demonstrated that N-glycosylation of the protein ectodomain is essential for clustering of GPI-APs in the Golgi and therefore for their apical sorting in FRT cells. This is in contrast with the data shown in MDCK cells that N-glycosylation is not involved in the apical sorting of GPI-anchored form of endolyn (Potter et al., 2004) and Placental alkaline protein

(PLAP) (Catino et al., 2008). It is likely that in FRT cells GPI-APs could interact with each other through the glycosylation of ectodomain to form GPI-AP clusters at the level of the Golgi prior to their apical sorting. The finding that mutant PLAP protein (PLAPΔN) does not oligomerize and is basolaterally sorted in FRT cells further supports this hypothesis. It is still unknown whether N-glycans are involved in the clustering event by directly promoting the interaction among GPI-APs to form clusters or indirectly by interacting with some sugar binding receptors, such as lectins. It has been shown that lectins of the galectin family, which binds with β-galactosides, are involved in the apical sorting of the proteins (Delacour and Jacob, 2006; Delacour et al., 2009). Until now galectins -3, -4 and -9 have been shown to be involved in the apical sorting of glycoproteins (Huet et al., 2003; Delacour and Jacob, 2006; Delacour et al., 2007; Mishra et al., 2010). Among them galectin-3 has been shown to act as an apical sorting receptor by promoting the clustering of non-raft associated proteins (Delacour et al., 2007; Delacour and Jacob, 2006). In addition, it was shown that even though galectins can be secreted via a non-conventional mechanism, they could reach the sorting compartments of the biosynthetic route including TGN (Mishra et al., 2010) and endosomal compartments via endocytosis (Schneider et al., 2010). Thus, it could be interesting to investigate whether certain galectins expressed in FRT cells mediate the N-glycan dependent oligomerization of GPI-APs.

In addition, our results in FRT cells showed that the N-glycan dependent apical sorting is insensitive to cholesterol addition and depletion. This is in contrast with the data shown in MDCK cells in which the addition of cholesterol promotes the oligomerization and induces the mistargeting of a basolateral GPI-AP: GFP-PrP to the apical surface (Paladino et al., 2008). It was shown that addition of cholesterol

was able to decrease the apparent diffusion coefficient of GFP-PrP at the level of the Golgi of MDCK cells, which correlates with its oligomerization and segregation in apical rafts that occurs at the Golgi level (Lebreton et al., 2008). Moreover it was shown that in MDCK cells cholesterol depletion leads to the decrease in the clustering of apical GPI-APs and causes the basolateral missorting of these proteins (Paladino et al., 2004). All these data indicates that the specific membrane environment (cholesterol content) plays a role in the segregation of apical and basolateral GPI-APs at the level of Golgi in MDCK cells.

In the present study we demonstrated that in contrast with MDCK cells, addition of cholesterol does not promote the apical sorting of two basolateral GPI-APs: GFP-PrP and GFP-FR in FRT cells. Interestingly using FRAP experiments performed at the level of the Golgi we demonstrated that the Golgi membrane environment of FRT cells is different from MDCK cells. In addition we showed that the Golgi in FRT cells is highly enriched in cholesterol and does not incorporate exogenously added cholesterol. It is likely that in FRT cells, the Golgi environment saturated with cholesterol induces the rigidification of the membrane and therefore causes the restricted diffusion of all the studied proteins independent of their association with cholesterol enriched membrane microdomains and their polarized sorting. Moreover, addition or depletion of cholesterol does not affect the polarity of both apical and basolateral GPI-APs in FRT cells. Overall this data indicate that cholesterol is not a key regulator in the apical sorting of GPI-APs in FRT cells.

Further, using high-resolution immunofluorescence we showed that the morphology of the Golgi in FRT cells is different from MDCK cells. The TGN of the FRT cells is

more scattered and does not vesiculate upon addition of cholesterol like in MDCK cells (Stuven et al., 2003) and in HeLa cells (Ying et al., 2003). It has been shown that several phosphoinositides, their down stream effectors and their associated proteins regulate the structure and functions of the Golgi apparatus (Peter Mayinger, 2010; Todd and Christopher, 2010). Golph3, which is a Golgi protein with PH domain (pleckstrin homology domain), has been shown to bind with sPtdIns(4)P and unconventional myosin MYO18A creating a tensile force to stretch the Golgi into the extended ribbon. Another PtdIns(4)P binding protein OSBP, Oxysterol-binding protein, has been shown to function according to the cholesterol level in the Golgi. An elevated cholesterol level in the Golgi activated the phosphorylation of OSBP and causes the fragmentation of Golgi apparatus (Nhek et al., 2010; Yang et al., 2007). In addition an integral membrane protein, SAC1 phosphatase has been shown to be involved in maintaining the structural integrity of Golgi membranes by regulating the PtdIns(4)P level in the Golgi (Yang liu et al., 2008). It is tempting to speculate that there might be overexpression of certain phosphoinositol binding proteins, which regulates the phosphoinositol levels in the Golgi there by regulating the Golgi morphology in FRT cells. We are now investigating the role of phosphoinositols in the regulation of Golgi morphology, which I described in the perspectives that is followed by the discussion in the manuscript.

It has been postulated that apical sorting of GPI-APs has been mediated by their association with rafts (Simons and Ikonen, 1997). But the role of rafts in the apical sorting of GPI-APs has been controversial. Our results clearly demonstrate that rafts are not involved in the apical sorting of GPI-APs in FRT cells. In these cells, depletion of cholesterol affects the apparent diffusion coefficient of both raft and non-

raft associated proteins without changing their polarity. In addition sphingolipid depletion affects the polarity of all apical proteins independent of raft association. Moreover both apical and basolateral GPI-APs are associated to DRMs. Overall these data suggests that in FRT cells neither segregation between apical and basolateral GPI-APs in the Golgi membranes nor the apical sorting is driven by association with cholesterol enriched microdomains.

Altogether our results clearly demonstrate that N-glycosylation of the GPI-AP ectodomain is essential for oligomer formation and therefore apical sorting of GPI-APs in FRT cells. These results further support the hypothesis that oligomerization of apical GPI-APs is an essential event for apical sorting of GPI-APs and segregation from basolateral GPI-APs and other apical proteins (transmembrane and non-raft associated). In addition this study opens the way to further questions such as at what level apical GPI-APs are segregated from apical transmembrane proteins (raft and non-raft), whether apical GPI-APs travel together with transmembrane proteins in the same post TGN-carrier vesicles or they segregated at the level of the Golgi or further in the endosomes and is there any adaptor proteins over expressed in FRT cells, which mediates the basolateral sorting of most of the GPI-APs in FRT cells. However, further biochemical and live imaging experiments, which I mentioned in the perspectives of my thesis, should answer these questions.

PRELIMINARY RESULTS
AND
PERSPECTIVES

Preliminary results and perspectives:

Analyze the transport of GPI-APs and transmembrane proteins to the apical and basolateral domains of polarized epithelial cells

The earlier evidence for segregation of apical and basolateral cargos at the level of the TGN came from the work of Wandinger-ness and colleagues. By immunopurification of TGN-derived vesicles they showed that two distinct classes of vesicles are responsible for apical and basolateral protein delivery (Wandinger-ness et al., 1990). Further, live imaging studies performed in non-polarized MDCK cells showed that apical and basolateral proteins GPI-AP and transmembrane, respectively, were sorted into distinct tubular and vesicular carrier vesicles that emanated from the TGN (Keller et al., 2001). In addition, several growing evidences show that apical transmembrane raft and non-raft associated proteins are transported to the apical surface in distinct carriers, which display different morphologies. Their formation occurs from distinct subdomain of the Golgi and involves different factors (Jacob et al., 2001; Jacob et al., 2004; Guerriero et al., 2006; Guerriero et al., 2008). Thus, it appears that multiple pathways exist from the TGN to the apical surface. On the other hand, it is still not clear whether and where GPI-APs are segregated from other raft-associated proteins.

Our laboratory has recently set up a protocol to visualize protein segregation at the level of the Golgi complex in living polarized MDCK cells grown on filters (Paladino et al., 2006). This will allow us further to understand the mechanism of segregation between GPI-APs and transmembrane proteins in living polarized cells. Our previous data in polarized MDCK cells indicates that apical and basolateral GPI-APs inhabit

different membrane microdomains (Lebreton et al., 2008). Whereas, my results in FRT cells suggest that both apical proteins (GPI-AP and transmembrane raft, non-raft) occupy the same environment at the level of the Golgi. In these cells depletion of cholesterol resulted in the increase of the apparent diffusion coefficient of apical GPI-APs and transmembrane proteins. In addition, sphingolipid depletion affected the polarity of all apical proteins (GPI-AP and transmembrane) independently of their association with cholesterol enriched membrane microdomains. These data indicate that apical GPI-APs are not segregated from transmembrane (raft and non-raft) proteins at the level of the Golgi in FRT cells. Therefore the next questions we would like to address are:

1) At which level apical GPI-APs are segregated from other apical transmembrane proteins in polarized epithelial cells?

2) Do they travel in the same carrier vesicles or in distinct carrier vesicles?

3) Whether they are segregated at the level of the Golgi or further along the secretory pathway in the endosomes?

To answer these questions I am collaborating with another student and a post-doc in Naples. We have established several stable clones expressing both apical proteins (GPI-AP and transmembrane) and clones expressing an apical GPI-AP and a basolateral transmembrane protein. Currently we are using these clones to set up the live experiments to visualize the segregation at the level of the Golgi in polarized and non-polarized MDCK and FRT cells.

Our preliminary results in fixed polarized MDCK cells expressing an apical GPI-AP: GFP-FR and a transmembrane apical protein: mcherry-P75 indicate that GFP-FR is

completely segregated from cherry-P75 at the level of the Golgi both in steady state and immediately after the temperature block (Figure 1). This indicates that at the level of the Golgi apical GPI-APs are segregated from transmembrane proteins in polarized MDCK cells. We are currently performing the live experiments to understand where specifically they segregate. Moreover, the results in fixed MDCK cells co-transfected with an apical GPI-AP: CFP-FR and a basolateral transmembrane protein: YFP-PIT indicate that these two proteins are partially segregated at the level of the Golgi and they are present in different post-TGN carriers (Figure 2).

These results in MDCK cells are preliminary evidences for the segregation between apical GPI-APs and transmembrane proteins. I am currently setting up experiments in live FRT cells in order to analyze the data with MDCK cells and to obtain more information regarding the segregation mechanisms that exists in different polarized epithelial cells.

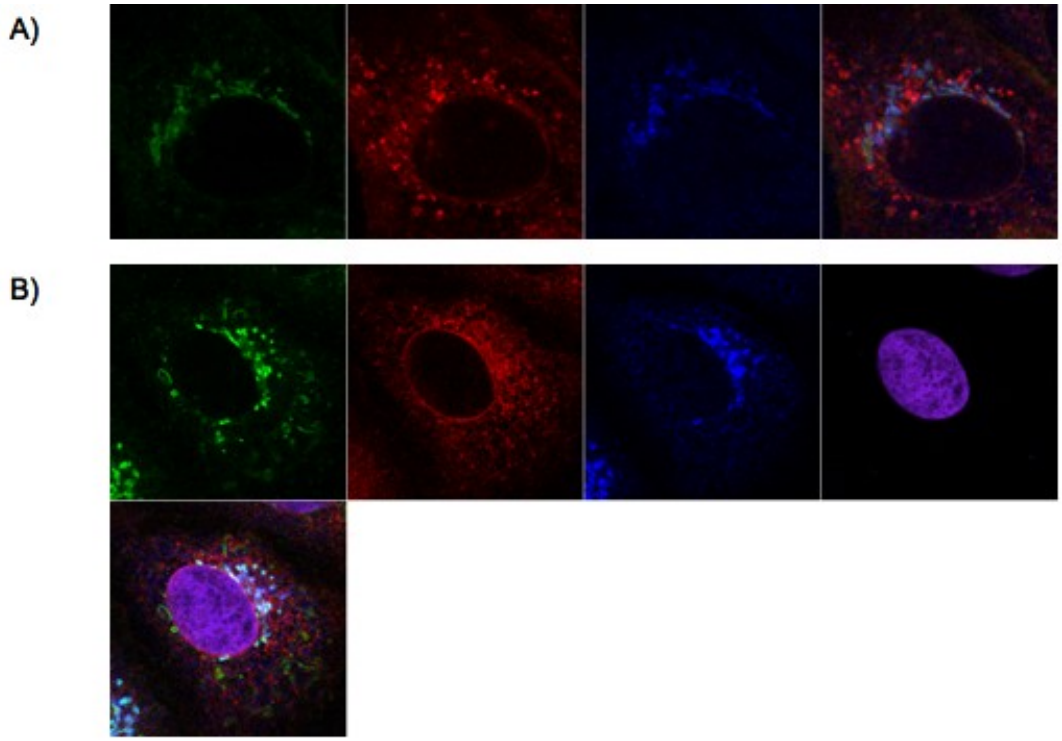


Figure 1: MDCK cells co-expressing GFP-FR and mcherry-P75 were grown on coverslips until they reach the confluency. Cells were subjected to temperature block (incubation 2h at 19.5°C) to accumulate proteins in the Golgi apparatus (B) or not (control) (A). Cells were fixed with PFA, permeabilized and then stained with giantin (cis/medial Golgi marker) followed by secondary antibody coupled to alexa-350. Serial confocal images were collected and the 3D reconstruction image was shown here.

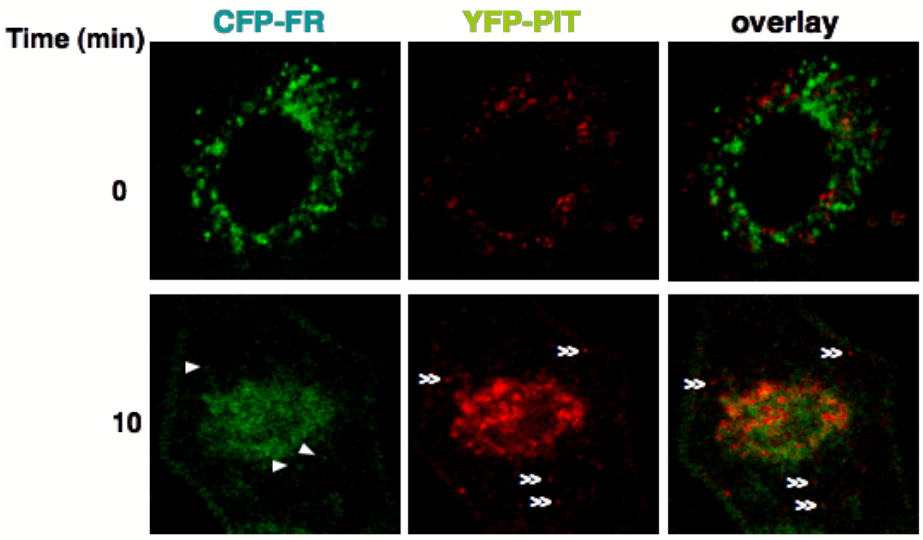


Figure 2: MDCK cells were transiently co-transfected with cDNA coding for the cyan variant of GFP-FR (CFP-FR) and the YFP-PIT (in which YFP is fused to the cytosolic tail of LDL receptor). Cells grown on coverslips were subjected to temperature block (incubation 2 h at 19,5°C) to accumulate proteins in the Golgi apparatus and were fixed with PFA (time 0). Alternatively cells were warmed at 37°C for 10 min (in order to release from the block) before fixation. Serial confocal images were collected and the 3D reconstruction image was shown here.

Analysis of the role of phosphoinositides and their binding proteins in the regulation of Golgi morphology in MDCK and FRT cells

My thesis work has shown that the Golgi morphology in polarized FRT cells is different from MDCK cells. The Golgi of FRT cells appear to be more scattered compared to the compact Golgi in polarized MDCK cells. It was shown that alteration in cholesterol level affects Golgi morphology and results in the fragmentation of Golgi apparatus (Stuven et al., 2003). Our results in FRT cells show that the Golgi of FRT cells is enriched in cholesterol, which might be responsible for its scattered appearance. Apart from cholesterol, it has been shown that some PtdIns(4)P binding proteins and phosphatases that regulate the Golgi phosphoinositides levels play a role in the regulation of Golgi morphology (Dippold et al., 2009; Liu et al., 2008).

Recently it was shown in HeLa cells that the Golgi protein GOLPH3 binds to PtdIns(4)P rich trans Golgi membranes and to an unconventional myosin-18A creating a tensile force that stretches the Golgi into an extended ribbon. It has been demonstrated that knockdown of GOLPH3 results in the condensation of Golgi complex (Dippold et al., 2009). In addition, the PtdIns(4)P phosphatase, SAC1 that shuttles between ER and the Golgi in response to nutrient levels and cell growth stages (Blagoveshchenskaya et al., 2008) has been shown to be involved in the regulation of Golgi morphology in HeLa cells (Liu et al., 2008). Golgi localization of SAC1 leads to the depletion of PtdIns(4)P level that induces the condensation of Golgi (Dippold et al., 2009). Therefore these two proteins are good candidates to analyze the role of phosphoinositides in producing the two different Golgi morphologies, compact and scattered, in polarized MDCK and FRT respectively. Furthermore, we would like to analyze whether different Golgi morphology is responsible for different sorting behaviors of these two polarized epithelial cells.

To understand the role of GOLPH3 and SAC1 in the regulation of Golgi morphology, as a first step we analyzed the endogenous expression of GOLPH3 and SAC1 in MDCK, FRT cells both in polarized and non-polarized conditions and we compared our results with previously published results in HeLa cells.

Our preliminary results (Figure 3) show that the expression of GOLPH3 appears to be lower in FRT cells in polarized condition in contrast with MDCK and HeLa cells. Whereas GOLPH3 expression did not change in both polarized and non-polarized conditions in MDCK and in HeLa cells (Figure 3A). In addition we observed higher expression of SAC1 phosphatase in FRT cells compared with MDCK and HeLa cells

(Figure 3B). Further, by immunofluorescence we could detect that SAC1 phosphatase was localized at the level of the Golgi in MDCK and FRT cells in polarized condition (Figure 3C). It has been reported that knockdown of SAC1 leads to the fragmentation of Golgi complex (Liu et al., 2008). Since FRT cells exhibit more SAC1 expression, we could expect to see the condensation of Golgi complex. Surprisingly in this cell line more expression of SAC1 does not induce the condensation of Golgi complex.

This preliminary data indicates that the regulation of PI4-P levels might be different in MDCK and FRT cells and likely it is linked with different Golgi cholesterol levels in both cell lines as explained before. It has been shown that endogenous cholesterol levels regulate the activity of PI4KII α kinase, which is involved in the PI4-P synthesis (Waugh et al., 2006; Minogue et al., 2010). Therefore, further analysis of the Phosphoinositides levels in the Golgi of FRT cells and the expression of kinases involved in the synthesis of PtdIns(4)P in FRT cells should provide the complete information regarding the role of phosphoinositides in the regulation of Golgi morphology in these cells. Once we gather all these information, we will analyze the effect of knock down or over expression of both GOLPH3 and SAC1 in the sorting and trafficking of GPI-APs and transmembrane proteins in MDCK and FRT cells in both polarized and non-polarized conditions.

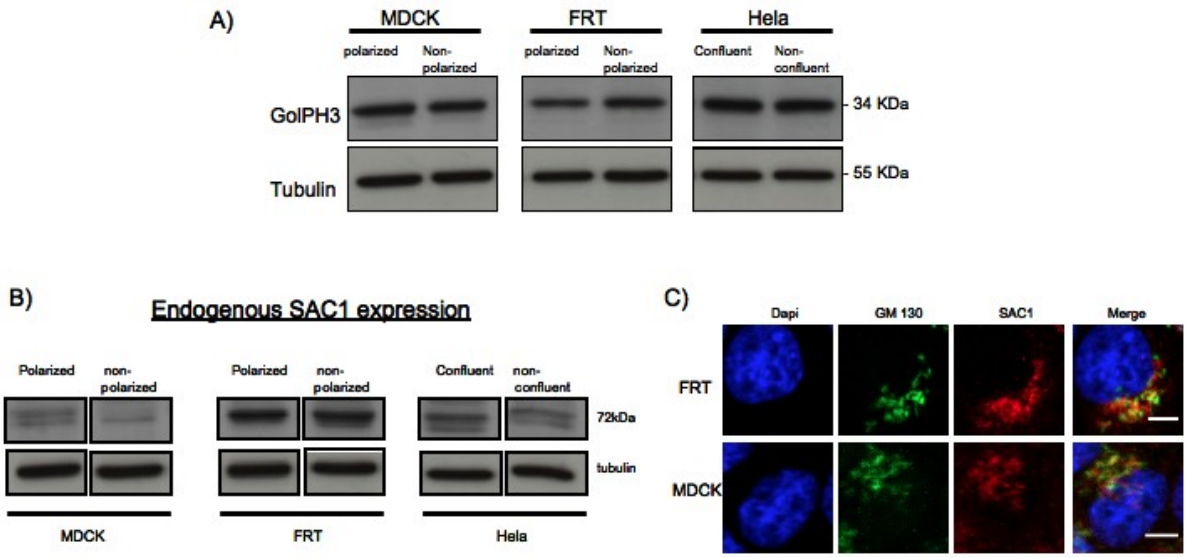


Figure 3: Analysis of the endogenous level of GOLPH3 and SAC1.

A). Analysis of the endogenus levels of GOLPH3 in MDCK, FRT and Hela cells in polarized and in non-polarized condition.
B) Analysis of the endogenous levels of SAC1 in MDCK, FRT (in polarized and non-polarized) and Hela cells (confluent and non-confluent condition)
C) Endogenous SAC1 Golgi localization in polarized FRT and MDCK cells.

Conclusions:

My PhD work is mainly focused in understanding the mechanism involved in apical sorting of GPI-anchored proteins in polarized epithelial cells.

Clustering of GPI-APs in the Golgi is a crucial event for the segregation and apical sorting of GPI-APs in MDCK and FRT cells. I have discovered that the factors promoting the oligomerization process are different in both cell lines. Previous work from our laboratory demonstrated that in MDCK cells cholesterol promotes the oligomerization as well as apical sorting of GPI-APs. In this present study I showed that in contrast with MDCK cells, cholesterol is not involved in the segregation and apical sorting of GPI-APs in FRT cells. In addition, I showed that the segregation of apical and basolateral GPI-APs in the FRT Golgi is independent of their association with cholesterol enriched membrane micro domains. Strikingly, N-glycosylation of the protein ectodomain is essential for the oligomerization and apical sorting of GPI-APs. Impairment of N-glycosylation leads to the basolateral missorting of apical GPI-APs in FRT cells.

Overall, this data indicate that at least two different mechanisms are involved in promoting the oligomerization as well as apical sorting of GPI-APs in polarized epithelial cells. They also reinforce our hypothesis that the critical event to sort GPI-APs to the apical membrane is their capacity to form oligomers in the Golgi apparatus, independently of the cell type.

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ABSTRACT

Epithelial cells represent the ability to polarize with an apical and basolateral domains which differ markedly in proteins, lipid composition and therefore in function. This asymmetry reflects the ability of epithelial cells to sort newly synthesized proteins and lipid to either cell surface. While the signals responsible for basolateral targeting of the proteins have been clearly understood, the situation regarding the apical sorting of proteins is more obscure.

We have previously shown that differently from basolateral GPI-APs oligomerization in the Golgi apparatus is necessary for apical sorting of Glycosylphosphatidylinositol-anchored proteins (GPI-APs). Interestingly this mechanism is conserved in two different kinds of epithelial cells, MDCK and FRT cells, which exhibits a difference in the sorting of GPI-APs. However the precise mechanism leading to this event is not understood.

Our previous data demonstrates that simple addition of cholesterol to MDCK cells is necessary and sufficient to induce the oligomerization and apical sorting of a basolateral GPI-AP. Whereas, in this present study in FRT cells we showed that in contrast with MDCK cells cholesterol is not an active player in the regulation of GPI-APs apical sorting. In addition, we also showed that apical and basolateral GPI-APs are not segregated in the Golgi on the bases of the cholesterol content of the surrounding membrane environment. Furthermore, we demonstrate that N-glycosylation of the protein ectodomain is critical for oligomerization and apical sorting of GPI-APs. Our data indicate that at least two mechanisms depending either on cholesterol or on N-glycosylation exist to determine oligomerization in the Golgi and sorting to the apical membrane of GPI-APs.