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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, Madin–Darby canine kidney (MDCK) and Fisher rat thyroid (FRT) cells, but whether the mechanism is conserved is 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. It is striking that 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.**

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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 neighboring cells, the basal membrane, and the internal milieu. These two domains differ markedly in their functions and in their protein and lipid composition due 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, 2010; 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 segregates 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 posttranslational modifications (Matter, 2000; Rodriguez-Boulan and Musch, 2005; Mellman and Nelson, 2008; Gonzalez and Rodriguez-Boulan, 2009), such as lipid

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Abbreviations used: DAF, decay accelerating factor; DRMs, detergent-resistant membranes; FB1, fumonisin B1; FR, folate receptor; FRT, Fisher rat thyroid; GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol; GPI-APs, GPI-anchored proteins; HMW, high molecular weight; LDL, low-density lipoprotein; MDCK, Madin-Darby canine kidney; PLAP, placental alkaline phosphatase; PrP, prion.

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 glycosylphosphatidylinositol (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 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). On the basis of their ability to segregate proteins and lipids in specific membrane compartments, rafts are believed to be involved in many cellular functions, such as protein sorting, endocytosis, signaling, bacterial 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, 2007), indicating that additional factors should be involved. Epithelial cells have apical sorting pathways that are either dependent or independent of glycan 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.*, 1999; Potter *et al.*, 2004; Catino *et al.*, 2008). Our previous studies using glycosylation mutants and chimeric proteins showed that neither N-glycans nor O-glycans are necessary for apical sorting of GPI-APs in Madin–Darby canine kidney (MDCK) cells (Catino *et al.*, 2008). We also showed 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, 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 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). On the basis of this and other evidence, we proposed that a specific membrane environment enriched in cholesterol is required to favor oligomerization of GPI-APs in the Golgi and consequently ensure their apical sorting (Lebreton *et al.*, 2008; Paladino *et al.*, 2008).

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 than 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, 2007). Here we show that, in contrast to 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 with MDCK cells and do not incorporate uptaken cholesterol exogenously added to the culture medium. The higher cholesterol level in the Golgi of FRT cells is likely to increase membrane rigidity, resulting in a restricted diffusion of both apical and basolateral GPI-APs, which is observed in fluorescence recovery after photobleaching (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. 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 segregates 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 presents important variations in different epithelial cells, depending preferentially on either cholesterol or N-glycosylation.

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 showed that MDCK cells direct a model GPI-AP protein (GFP–prion protein [PrP]) to the basolateral domain despite its association with lipid rafts (Paladino *et al.*, 2008). Furthermore, cholesterol addition to the media reroutes 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 connecting cholesterol contents in Golgi membranes, oligomerization, and apical sorting of raft-associated GPI-APs. 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 behavior of different apical and basolateral model GPI-APs.

As an apical model protein we constructed a chimeric protein consisting of green fluorescent protein (GFP) fused to N-and O-glycosylation sequences (10 and 56 amino acids, respectively) of the low-density-lipoprotein (LDL) receptor followed by the GPI-anchor attachment signal of decay accelerating factor (DAF) protein, which we called GFP-NO-GPI (Figure 1A). This and the previously described basolateral model protein GFP-PrP (Lebreton *et al.*, 2008; Paladino *et al.*, 2008), which results from the fusion of GFP to the GPI-anchor attachment signal of the prion protein (Figure 1A), were stably transfected in FRT cells. Several clones expressing comparable levels of proteins were selected. To define the polarized distribution and detergent-resistant membrane (DRM) association of these proteins, we performed confocal microscopy (Figure 1B) and selective biotinylation assays (Figure 1C) on polarized FRT cells seeded on transwell filters as well as sucrose density gradient assays (Figure 1D). 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 1, B and C, left), whereas GFP-PrP was mainly basolateral (76 ± 2.8%; Figure 1, B and C, right). Furthermore, similar to other apical GPI-APs (Lipardi *et al.*, 2000; Paladino *et al.*, 2006), GFP-NO-GPI appeared to be directly sorted to the apical surface of FRT cells (Supplemental Figure S1). As expected from being GPI anchored, both proteins migrated to lighter

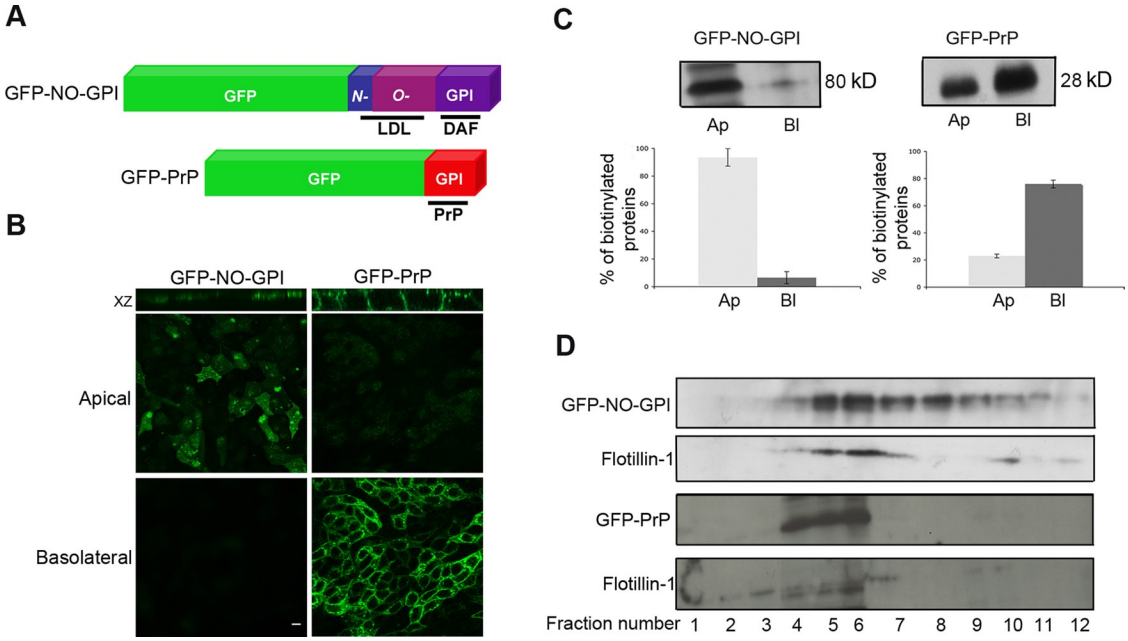

FIGURE 1: GFP-NO-GPI and GFP-PrP are respectively localized on the apical and basolateral domains in FRT cells. (A) Scheme of fluorescent model proteins, GFP-NO-GPI and GFP-PrP. In the fusion construct GFP-NO-GPI, GFP is fused to N- and O-glycosylation sequences (10 and 56 amino acids, respectively) of the LDL receptor, followed by the GPI-anchor attachment signal of DAF. In GFP-PrP, GFP is fused to the GPI-anchor attachment signal of prion protein (PrP). (B) FRT cells stably expressing GFP-NO-GPI or GFP-PrP were grown in polarized condition on transwell filters for 4 d. Cells were fixed and quenched, and serial confocal sections of 1 µm were collected from top to bottom of cell monolayers using a 488-nm laser. Scale bars, 10 µm. (C) FRT cells stably expressing GFP-NO-GPI or GFP-PrP were incubated with 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 labeled proteins was quantified by using ImageJ software by considering three independent experiments. Error bars, mean ± SD. (D) FRT cells stably expressing GFP-NO-GPI or GFP-PrP were lysed in a buffer containing 1% Triton X-100 at 4°C and separated by centrifugation at the equilibrium on 5–40% sucrose density gradients to purify Triton X-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-flotillin antibody used as a DRM marker.

fractions on sucrose density gradients, suggesting association to DRMs (Figure 1D). These results reproduce previous observations in MDCK cells (Paladino *et al.*, 2004, 2007), and 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 forms HMW complexes in FRT cells both in control conditions (36.1 ± 3.5% of total) and upon cholesterol addition (31 ± 5.2%; Figure 2A) and, as expected, it is apically sorted (Figure 2, B and C, left). However, whereas 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 2, B and C, right) upon cholesterol addition. Such cholesterol-insensitive behavior was not restricted to these two model proteins. Indeed, placental alkaline phosphatase (PLAP) and GFP-FR, which respectively apically and basolaterally sorted in FRT cells (Paladino *et al.*, 2007), showed similar behavior and did not change their oligomerization status and sorting upon cholesterol addition (Supplemental Figure S2). Overall these data suggest 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). Of interest, 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 to obtain information on the membrane environment of GPI-APs in the Golgi of FRT cells. We analyzed both GFP-NO-GPI and GFP-PrP, as well as two transmembrane nonraft 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, colored bars), irrespectively of their raft association and polarized sorting. This contrasted with MDCK cells, in which we found a significantly higher apparent diffusion coefficient of basolateral GFP-PrP compared with apical GPI-APs (data not shown), as previously reported (Lebreton *et al.*, 2008). Of interest, GFP-PrP exhibited a similar D of 0.05 µm^2/s at the level of the basolateral plasma membrane of both MDCK and FRT cells

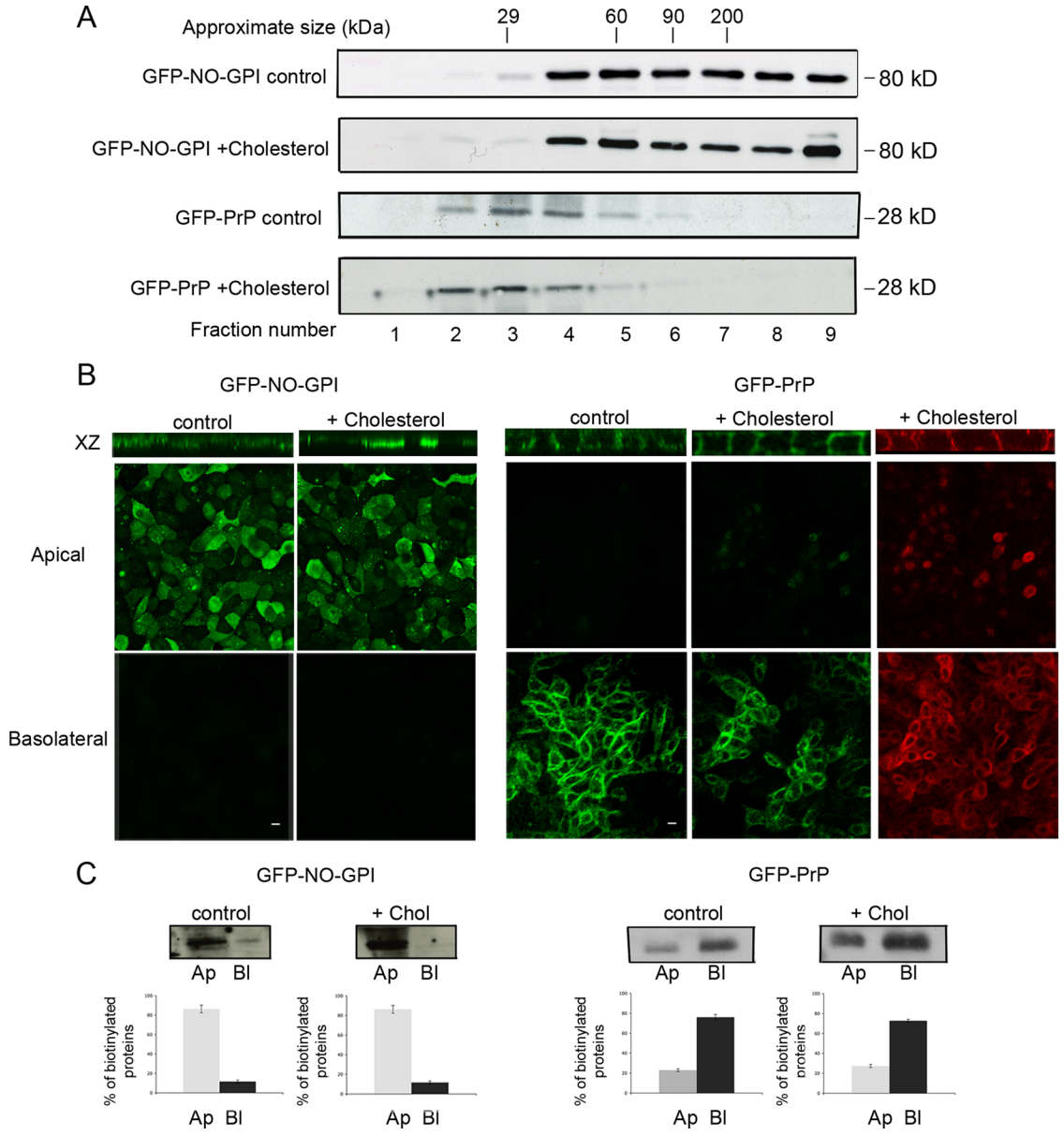

FIGURE 2: Addition of cholesterol affects neither the oligomeric state nor the polarized distribution of both apical and basolateral GPI-APs in FRT cells. (A) FRT cells stably expressing GFP-NO-GPI or GFP-PrP, grown to confluency, were incubated (+cholesterol) or not (control) with cholesterol for 45 min as described in *Materials and Methods*. Cells lysate were ran through nonlinear 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. (B, C) FRT cells stably expressing GFP-NO-GPI or GFP-PrP were grown in polarized condition on transwell filters for 4 d. (B) The plasma membrane localization of proteins was determined by analyzing the natural fluorescence of GFP with a 488-nm laser or by immunofluorescence in nonpermeabilized conditions by adding anti-GFP antibody followed by Alexa 546–conjugated secondary antibody to the apical and basolateral side of the cells in untreated (control) or cholesterol-loaded cells (+cholesterol). Serial confocal sections of 1 µm were collected from the top to bottom of the cell monolayers. Scale bars, 10 µm. (C) FRT cells stably expressing GFP-NO-GPI or GFP-PrP were incubated with 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 labeled proteins was quantified by using ImageJ software by considering three independent experiments. Error bars, mean ± SD.

(Supplemental Figure S3A), 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, independent 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

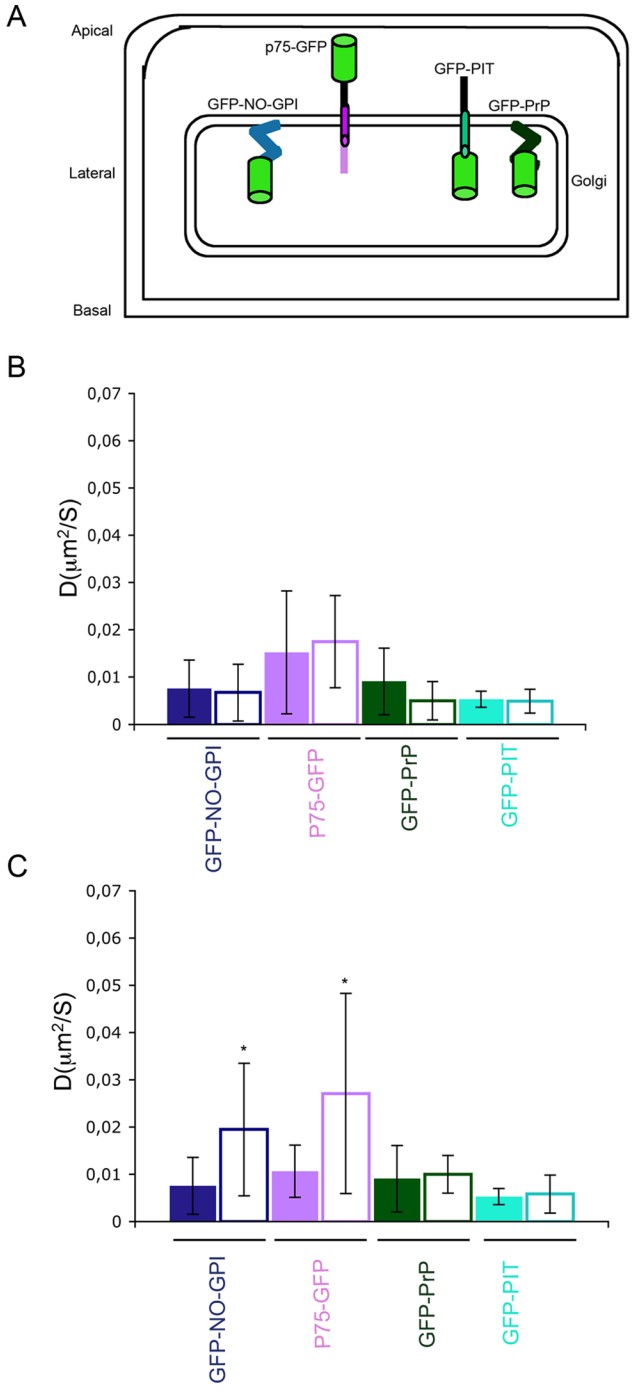

FIGURE 3: Diffusional mobilities of raft- and non–raft-associated proteins at the Golgi membranes of polarized FRT cells. (A) Scheme of fluorescent model proteins: two raft-associated proteins, GFP-NO-GPI and GFP-PrP, respectively apically and basolaterally sorted, and two transmembrane non–raft-associated 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 the 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 the Golgi complex in polarized FRT cells. Experiments were performed at least two independent times, n > 15. Error bars, means ± SD; *p < 0.05.

and basolateral GPI-APs (Figure 3B, white bars). 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 (3-hydroxy-3-methylglutaryl–coenzyme A reductase) that allows reduction by 30% the cellular cholesterol content (Lebreton *et al.*, 2008), we observed that all apical proteins (raft and not raft associated) equally exhibited an increased D ($p < 0.05$), whereas basolateral proteins (raft and not 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, independent 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, 2008; Lebreton *et al.*, 2008). Indeed, upon cholesterol depletion, both PLAP and GFP-NO-GPI were still able to oligomerize (Figure 4A) and were sorted to the apical domain (Figure 4B), reinforcing our hypothesis that the cholesterol content does not affect the oligomerization capacity of GPI-APs in FRT cells. Of interest, upon cholesterol depletion, both PLAP and GFP-NO-GPI migrated to lighter fractions of sucrose density gradient, indicating that they remain associated to DRMs (Supplemental Figure S3B). Thus 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 fumonisin B1 (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 5, A and B) but not to the basolateral GFP-PrP (Figure 5C), suggesting that missorting of GPI-APs in FRT cells is unrelated to lipid raft disruption. To support this hypothesis, we tested whether FB1 affects apical sorting of non–raft-associated transmembrane proteins such as p75-GFP and endogenous dipeptidyl peptidase-IV (DPPIV). It is striking that in FRT cells both transmembrane proteins were missorted to the basolateral domain upon FB1 treatment (Figure 5, D and E), indicating that inhibition of sphingolipid synthesis affects apical sorting of all studied proteins through some yet-unknown mechanism independent of their raft association. Consistently, we found that upon FB1 treatment both apical GPI-APs—PLAP and GFP-NO-GPI—migrated to the lighter fractions of the sucrose density gradients, suggesting that in these conditions both proteins remain associated to DRMs (Supplemental Figure S3B) while basolaterally sorted. 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 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 *Materials 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

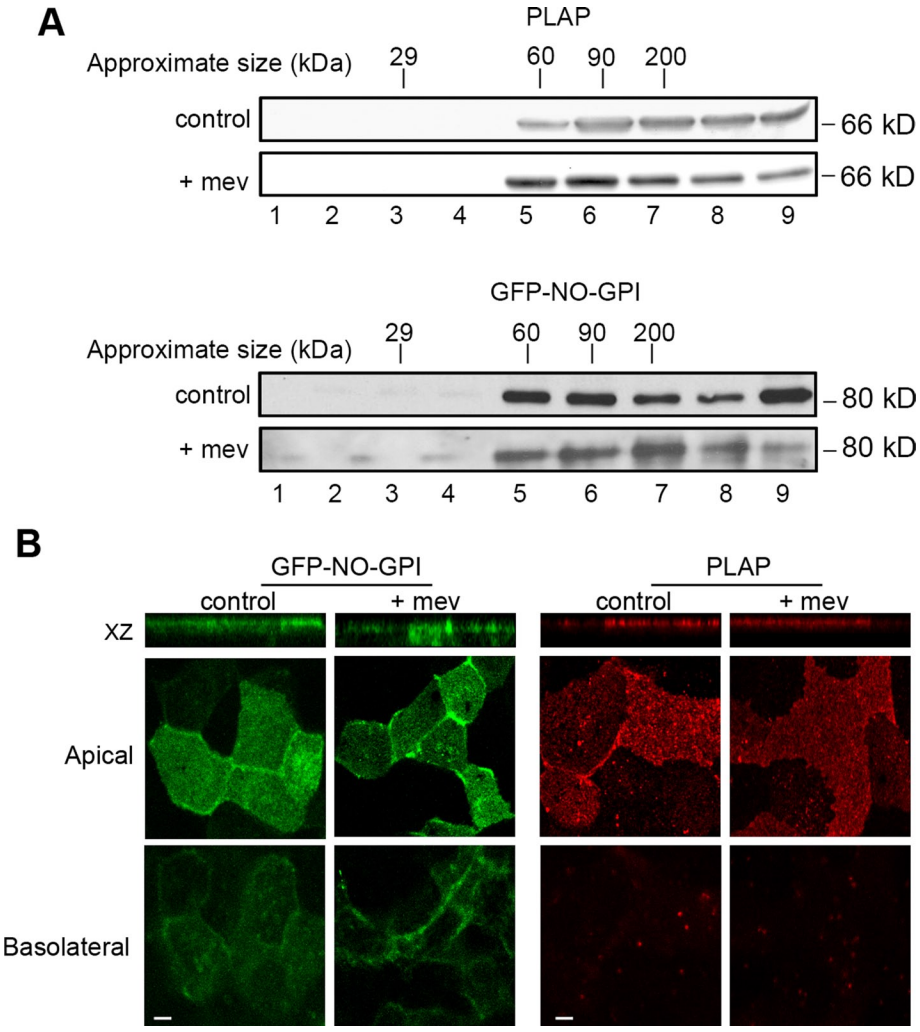

FIGURE 4: Cholesterol depletion does not affect apical sorting of GPI-APs in FRT cells. (A) FRT cells stably expressing GFP-NO-GPI or PLAP, grown to confluency, were treated or not (control) with mevinolin (+mev) as described in *Materials and Methods*. Cell lysates were run through nonlinear 5–30% sucrose 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 or anti-PLAP antibodies. The molecular weight of the monomeric form of each protein is indicated. The position on the gradients of molecular weight markers is indicated on the top. (B) Control and cholesterol-depleted FRT cells stably expressing GFP-NO-GPI or PLAP were grown on transwell filters for 4 d and fixed. Cells were analyzed by looking at the natural fluorescence of GFP or were stained with an anti-PLAP antibody followed by a TRITC-conjugated secondary antibody under nonpermeabilizing conditions. Serial confocal sections of 0.4 µm were collected from top to bottom of cell monolayers. Scale bars, 4 µm.

the cholesterol taken up 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 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 anti-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 (number of pixels) occupied by these two markers relative to the size of the cell (see *Materials 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 6, A and C, top, and E). 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 6, A and C, bottom, and E). Similar to MDCK cells, giantin occupies 4.2 ± 2.6% of the cell surface of FRT cells, whereas furin displayed a more scattered distribution (16.1 ± 4.8% of the cell surface; Figure 6, B, D, and F). It is surprising that, 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 7). 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. The role of N-glycosylation in apical sorting of GPI-APs in MDCK cells is controversial (Lisanti *et al.*, 1989; Benting *et al.*, 1999; 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 8, A–D). Similar to control conditions, we could not detect accumulation of any of the two proteins in the endoplasmic reticulum (ER; Supplemental Figure S4A), excluding that the effect of tunicamycin was indirect (e.g., due to ER stress, protein retention, or nonspecific effects). Finally, to further evaluate the effect induced by tunicamycin

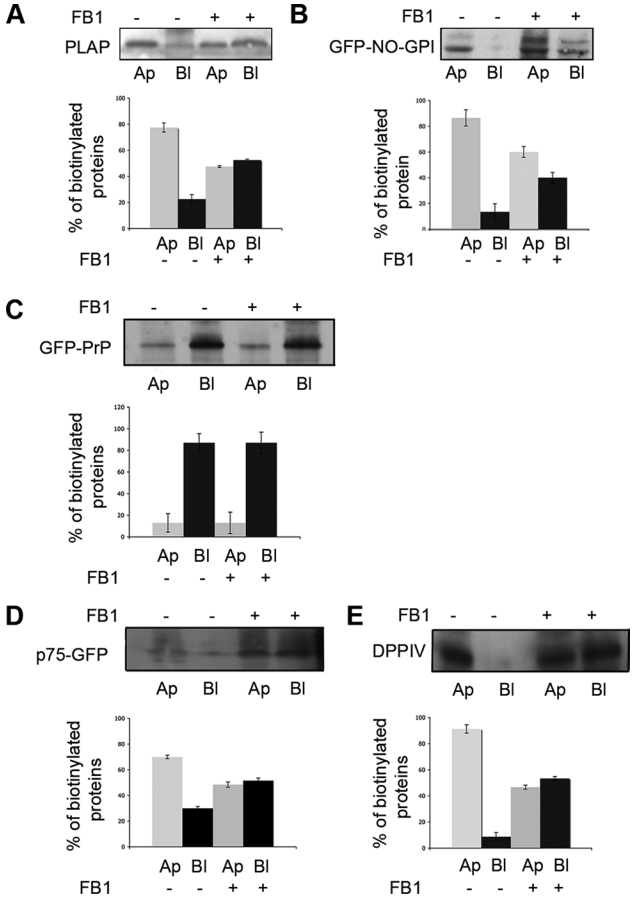

FIGURE 5: FB1 treatment induces basolateral missorting of both apical GPI-APs and transmembrane non–raft-associated proteins in polarized FRT cells. FRT cells stably expressing PLAP (A), GFP-NO-GPI (B), GFP-PrP (C), and p75-GFP (D), DPPIV (E) were grown on transwell filters in the absence (–) or presence (+) of FB1 (25 µg/ml for 3 d). 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 labeled proteins was quantified by using ImageJ software by considering three independent experiments. Error bars, mean ± SD.

treatment we investigated the surface distribution of different transmembrane proteins, p75-GFP, p75NTR, and DPPIV (Supplemental Figure S4B). As expected, tunicamycin treatment affects the sorting of DPPIV, which was previously reported to rely on N-glycans (Alfalah *et al.*, 2002). However, neither p75NTR nor p75-GFP, which are apically sorted independent of N-glycosylation (Yeaman *et al.*, 1997), were affected. These data clearly indicate that the tunicamycin treatment was affecting apical sorting of both GPI-APs through an impairment of N-glycosylation. 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). It is striking that in FRT cells this mutant was totally missorted to the basolateral domain (Figure 8, E and F) and, in agreement with our oligomerization model, it did not form HMW complexes in these cells (Figure 8G). Consistently, upon tunicamycin treatment also GFP-NO-GPI was not able to oligomerize (Figure 8H). These data disclose a previously unexpected role of N-glycosylation in the oligomerization of GPI-APs and indicate that different mechanisms may ensure this essential event for apical sorting of GPI-APs in different epithelia.

DISCUSSION

The mechanism(s) of GPI-APs sorting in polarized epithelial cells remain unclear and include some controversial aspects regarding the roles of rafts and glycosylation. We previously showed 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, 2007). Of interest, the requirement of clustering in the Golgi complex for GPI-AP 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, 2007). However, whereas 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 an 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 toward 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, 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 behaviors. 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-AP sorting (Zurzolo *et al.*, 1993; Paladino *et al.*, 2004, 2007).

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, in contrast 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 take up similar amounts of exogenously added cholesterol (see *Materials and Methods*). Nevertheless, in FRT cells addition of cholesterol does not affect basolateral sorting of GFP-PrP (Figure 2), as it does in MDCK cells (Paladino *et al.*, 2008). Of interest, FRAP experiments revealed that in the Golgi of FRT cells, all studied proteins

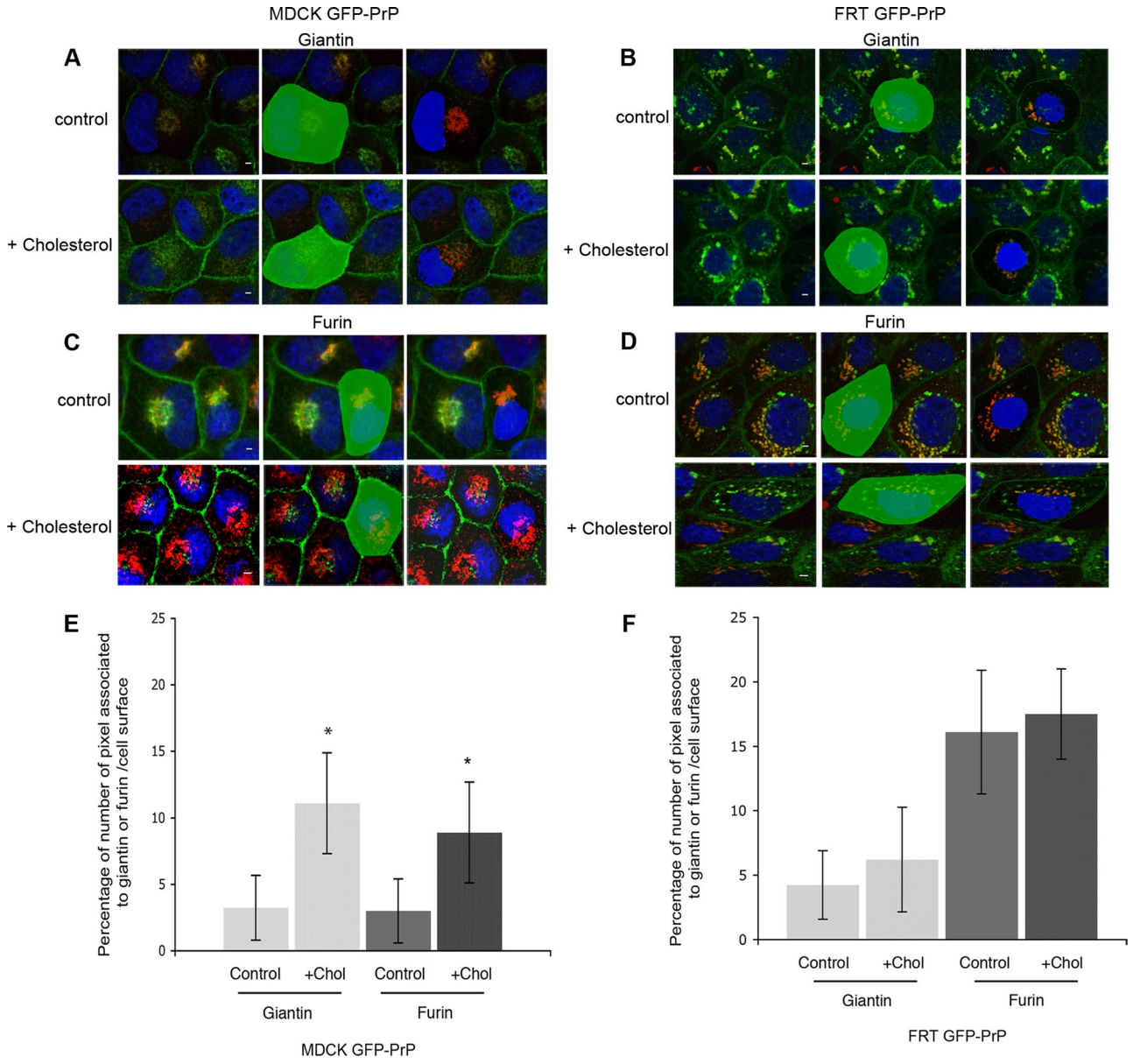

FIGURE 6: Addition of cholesterol does not affect Golgi morphology in polarized FRT cells. Equal number of MDCK (A, C) and FRT (B, D) cells stably expressing GFP-PrP were plated on the coverslips and grown until they reach high confluency. Untreated (control) or cholesterol-loaded (+cholesterol) cells were fixed, permeabilized, and 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 analyzed by using Quia software. Middle panels (in A–D) show the green mask of the cell used to measure the total number of pixels of cell surface. DAPI staining is used to evaluate the number of pixels of the nucleus. (E, F) Number of pixels associated to the Golgi marker (giantin and furin) normalized to cell surface and expressed as percentage in MDCK (E) and FRT (F) cells, in both conditions. Experiments were performed at least two independent times ($n \geq 60$ cells). Error bars, means ± SD; *$p < 0.0001$.

(apical, basolateral, raft and not raft associated) exhibit restricted diffusion, which is not affected by cholesterol addition (Figure 3B). Furthermore, the TGN of FRT cells appears already scattered, and, in contrast to other cells, the Golgi apparatus does not vesiculate following cholesterol addition (Stuven *et al.*, 2003; Ying *et al.*, 2003; Figure 6, B, D, and F). Overall our data indicate that the Golgi membranes of FRT cells are saturated in cholesterol (Figure 7), resulting in the inability to incorporate uptaken cholesterol and in a rigidified membrane environment, which restricts protein diffusion (Figure 3B).

In contrast 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 irrespective of their raft association. The increased diffusion of a nonraft 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

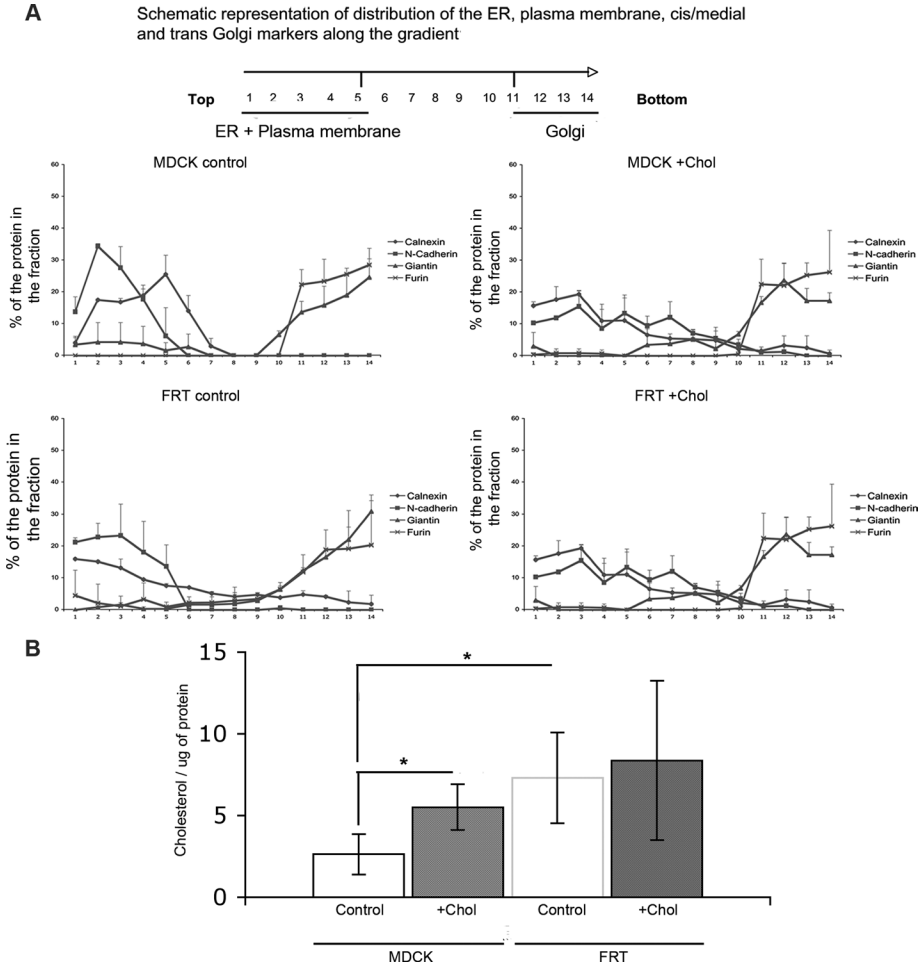

FIGURE 7: Cholesterol quantification after subcellular fractionation of MDCK and FRT cells. MDCK and FRT cells stably transfected with GFP-PrP were subjected to cell fractionation in control condition (control) or after addition of cholesterol (+Chol). The distribution of ER, plasma membrane, and *cis*/medial and *trans*-Golgi was analyzed along the gradient. (A) Schematic representation of the distribution of ER, plasma membrane, and *cis*/medial and *trans*-Golgi along the 14 fractions of the gradient. The following proteins were quantified in each fraction and expressed 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 microgram of protein in control condition (white bars) and upon addition of cholesterol (black bars). This experiment was performed two independent times. Error bars, means ± SD; *p < 0.05.

depletion does not result in their basolateral missorting (Figure 4, A and B), as 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, which was shown to contribute to apical protein sorting at the level of the Golgi (Lazaro-Dieguez *et al.*, 2007; Goswami *et al.*, 2008; Lebreton *et al.*, 2008), as we observed a similar increased diffusion under latrunculin A treatment (results not shown). Indeed, a putative relationship between cholesterol and actin cytoskeleton has been shown in fibroblasts, where actin would be the driving element of the cholesterol-dependent nanocluster organization of GPI-APs at the plasma membrane (Goswami *et al.*, 2008). It would be therefore interesting to explore further whether a similar relationship exists in the Golgi and has a role in apical protein sorting.

Of interest, in contrast with MDCK cells (Paladino *et al.*, 2004), in FRT cells cholesterol depletion affects neither oligomerization and apical sorting of GPI-APs (Figure 4, A and B) nor their association to lipid rafts (Supplemental Figure S3B). All of this evidence suggest that in FRT cells apical and basolateral GPI-APs are segregated at the level of the Golgi complex independent 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 (Supplemental Figure S3B) but induces missorting of all apical proteins (raft and not raft associated) (Lipardi *et al.*, 2000) (Figure 5). These results are in apparent contrast with previous reports on FRT cells (Lipardi *et al.*, 2000), where only apical GPI-APs were missorted to the basolateral side of the cell upon sphingolipid depletion. However, whereas here we are considering the steady-state distribution, in the previous report the effect of FB1 was evaluated on a pool of newly synthesized proteins. Therefore these different experimental approaches could explain the observed differences. Nonetheless it is interesting to note that whereas for GPI-APs the effect of FB1 is the same if we consider newly synthesized proteins or steady-state distribution, FB1 does not have the same effect on the newly synthesized or the stable pool of p75NTR, suggesting a general but differential effect of sphingolipids on apical sorting. Nonetheless, 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 independent 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.*, 1999). 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;

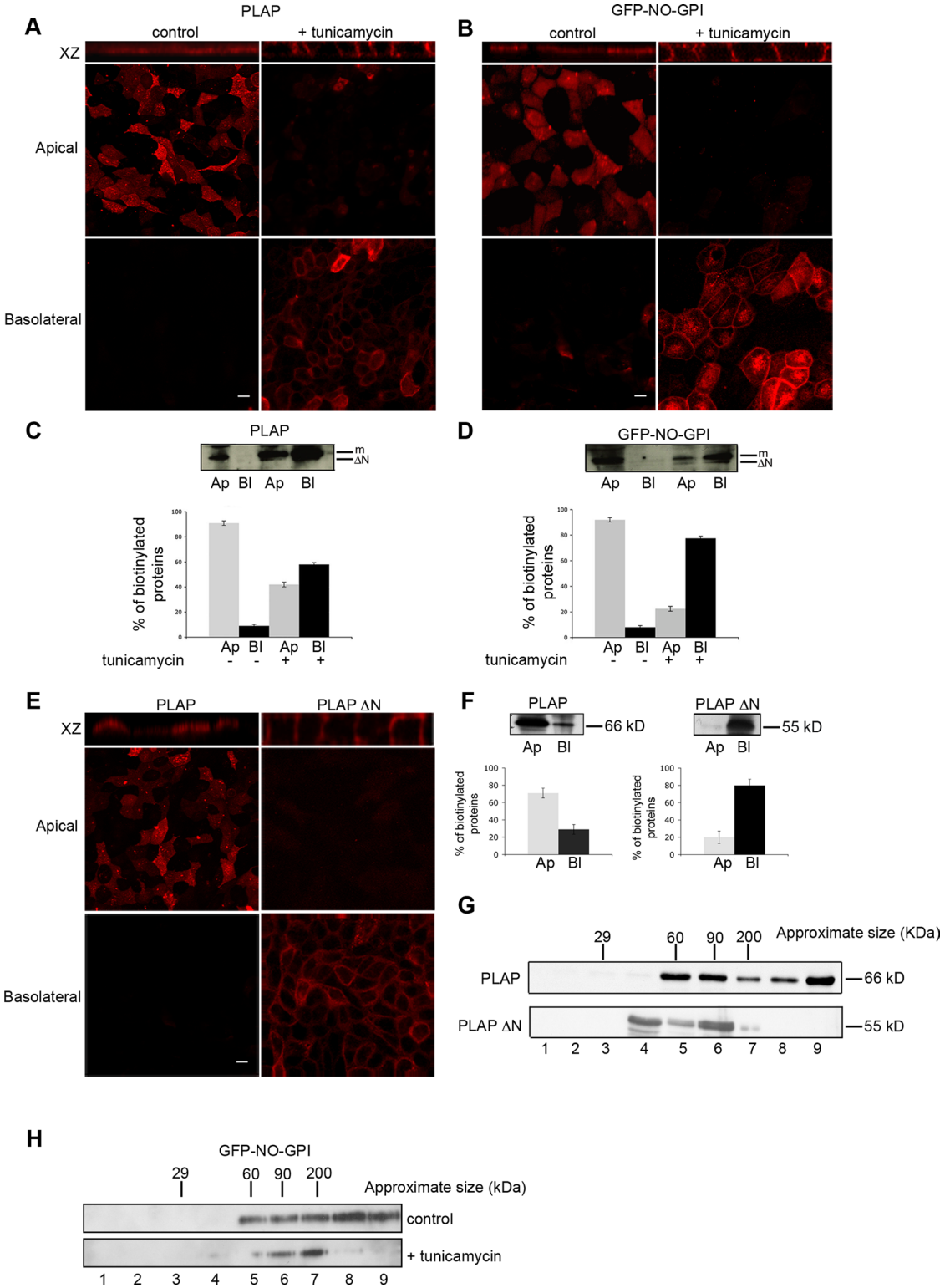

FIGURE 8: N-Glycosylation is essential for apical sorting of GPI-APs in polarized FRT cells. FRT cells stably expressing PLAP (A, C) or GFP-NO-GPI (B, D) were plated on filters, and after 48 h tunicamycin (10 μg/ml) was added to the cell culture medium for 20 h. (A, B) Cells were fixed, permeabilized, and labeled with specific primary antibodies (anti-PLAP and anti-GFP), followed by Alexa 546–conjugated secondary antibodies. Serial confocal sections of 1 μm were collected from the top to bottom of cell monolayers. Scale bar, 10 μm. (C, D) 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. Here m and ΔN indicate respectively the mature (fully glycosylated) and deglycosylated forms of the proteins. Amount of labeled proteins was quantified by using ImageJ software by considering three independent experiments. Error bars, mean ± SD. (E) FRT cells stably expressing PLAP or PLAPΔN were fixed, permeabilized, and labeled with specific primary antibody (anti-PLAP) and

Catino *et al.*, 2008). Strikingly, here we found that in FRT cells PLAPΔN is missorted to the basolateral membrane (Figure 8, E and F) and does not form oligomers (Figure 8G). Furthermore, inhibition of N-glycosylation with tunicamycin leads to basolateral missorting of both PLAP and GFP-NO-GPI, which remains O-glycosylated (Figure 8, A–D). However, both the nonglycosylated and fully glycosylated forms of both proteins are present at the basolateral surface (Figure 8, A–D). This could be due to the fact that the effect of tunicamycin on the newly synthesized proteins is partial. It is possible that the amount of fully glycosylated proteins (which enable the oligomerization process) might be the limiting step in the formation of the oligomers and hence not be sufficient to ensure their apical sorting. Alternatively, the fully glycosylated form present on the basolateral surface could derive from the basolateral recycling of the mature protein present on the apical plasma membrane prior to tunicamycin treatment. Although it will be interesting to discriminate between these two hypotheses, taken together our findings indicate that in FRT cells N-glycosylation is the critical event for oligomer formation and apical sorting of GPI-APs, independent of the cholesterol content of the surrounding membrane environment in the Golgi.

Alternatively, there could be a hierarchy with regard to the different mechanisms of apical GPI-AP oligomerization, which could become predominant in different cell lines. Indeed, considering our previous data in MDCK cells, we showed that addition of cholesterol is sufficient to drive the oligomerization and consequent apical sorting of GFP-PrP, but it is not sufficient to reroute to the apical domain the Cys mutant of GFP-FR (Paladino *et al.*, 2008), which is unable to oligomerize because it does not possess the two Cys residues in the ectodomain that allow its oligomerization (Paladino *et al.*, 2004). Thus we previously proposed that both the membrane environment and a "permissive" protein ectodomain are required (Paladino *et al.*, 2008). Consistent with this scenario, in FRT cells where the Golgi membranes are saturated in cholesterol and extremely rigid such as to restrict protein diffusion, the protein ectodomain becomes the main actor in this process.

Nonetheless, our results show a fundamental difference in the role of N-glycans in FRT and MDCK cells, the mechanistic elucidation of which 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 nonraft 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 they 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 not 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, 2007), suggesting that they are segregated at this level. Live-cell imaging experiments, currently underway in our laboratory, will test this hypothesis.

MATERIALS AND METHODS

Reagents and antibodies

Cell culture media were purchased from Sigma-Aldrich (St. Louis, MO) and EuroClone (Pero, Italy). Antibodies were purchased as follows: polyclonal α-GFP and monoclonal α-GFP from Invitrogen (Eugene, OR), α-PLAP from Rockland (Gilbertsville, PA), monoclonal anti–flotillin-1 antibody from BD Transduction Laboratories (Lexington, KY), polyclonal giantin from Covance (Emeryville, CA; 1:5000 for WB and 1:500 for IF), polyclonal furin from Thermo Scientific (Waltham, MA; 1:1000 for WB and 1:100 for IF), polyclonal calnexin from Stressgen (Ann Arbor, MI; 1:1000) and monoclonal N-cadherin from BD Transduction Laboratories (1:1000). Biotin and horseradish peroxidase (HRP)-linked streptavidin were from Pierce (Rockford, IL). FB1, tunicamycin, and all other reagents were purchased from Sigma-Aldrich.

Cell culture and transfections

MDCK and FRT cells were grown in DMEM and F12 (F12 Coon's modification medium), respectively, containing 5% fetal bovine serum. MDCK cells stably expressing GFP-PrP and FRT cells stably

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 PLAPΔN polarized for 4 d 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 labeled proteins was quantified by using ImageJ software by considering three independent experiments. Error bars, mean ± SD. The white and black bars indicate the percentage of protein labeled at the apical and basolateral domains, respectively. (G) FRT cells stably expressing PLAP or PLAPΔN were lysed and run through nonlinear 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 an anti-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. (H) FRT cells stably expressing GFP-NO-GPI grown to confluency were treated or not with tunicamycin as described. Cells were lysed and run through nonlinear 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 an anti-GFP antibody. The position on the gradients of molecular weight markers is indicated on top.

expressing PLAP, GFP-PIT, and p75-GFP were 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 [10 amino acids] and O-glycosylation [56 amino acids] sequences of LDL receptor followed by the GPI signal attachment of the DAF 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 d were selectively biotinylated from the apical or the basolateral side using sulfo-*N*-hydroxysulfosuccinimide-long chain (LC)–biotin. Lysates were immunoprecipitated using a monoclonal anti-GFP antibody (Molecular Probes, Invitrogen, Carlsbad, CA) for GFP-PrP and GFP-NO-GPI and with a polyclonal anti-PLAP antibody for PLAP and PLAPΔN and analyzed by Western blotting using 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 24 h after plating, mevinolin (30 μM) and mevalonate (200 μM) were added to the cells in F12 medium supplemented with 5% delipidated calf serum for 48 h.

To load the cells with cholesterol, we used a previously published protocol (Lebreton *et al.*, 2008). Cells were plated on filters for 4 d, and water-soluble cholesterol (10 mM in methyl-β-cyclodextrin was added to warm medium containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (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, La Jolla, CA) according to the manufacturer's instructions. Following cholesterol addition, both FRT and MDCK cells uptake similar amounts 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 (PBS) 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, and 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 (5–30%) 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; Beckman Coulter, Brea, CA), fractions of 500 μl were harvested from the top of the gradient and trichloroacetic acid (TCA) precipitated. The proteins were revealed by Western blot using specific antibodies.

Sucrose density gradients

Sucrose density gradient analysis of Triton X-100–insoluble material was performed using previously published protocols (Paladino *et al.*, 2008). Cells ([20–25] × 10^6) were grown on 150-mm dishes to confluence, were scraped from dishes in PBS containing $CaCl_2$ and $MgCl_2$, and resuspended in 1 ml of lysis buffer containing 1% Triton X-100, 10 mM Tris buffer (pH 7.5), 150 mM NaCl, and 5 mM EDTA and sheared through a 23-gauge needle. The lysate was mixed with an equal amount of 85% sucrose and run on discontinuous sucrose gradients (5–40%) for 16 h at 4°C. Twelve 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-flotillin antibody (a typical raft marker).

Cell fractionation

FRT cells (12 plates of 150 mm) were homogenized by 10 strokes in an Isobiotec (Heidelberg, Germany) cell homogenizer with a tungsten carbide ball in 500 μl of 20 mM HEPES/KOH, pH 7.3, and 120 mM sucrose. A postnuclear supernatant fraction was obtained by centrifugation at 600 × g for 5 min in an Eppendorf tube. The postnuclear supernatant was loaded on the top of a discontinuous sucrose gradient (0.6 ml of each fraction of 15, 20, 25, 30, 35, 40, and 45% sucrose with 0.5 ml of sucrose 60% on the bottom) made up in the same buffer. The gradient was spun in an SW 50.1 rotor for 1 h at 45,000 rpm in a Beckman centrifuge, 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, and cis/medial and *trans*-Golgi markers.

FRAP measurements and analysis

FRAP analysis was performed as described previously (Lebreton *et al.*, 2008) on a confocal LSM 510 META from Zeiss (Thornwood, NY) using the Plan Apo 63× oil immersion (numerical aperture [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 of interest (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 a second one that is the level of fluorescence background. The raw data were fitted with the IGOR Pro software (WaveMetrics, Portland, OR) and an application developed in EMBL (The European Molecular Biology Laboratory) with the Soumpasis equation. We grew the cells on filters upside down in polarized conditions as previously reported and performed all our analysis at 37°C in order to reproduce physiological conditions as closely as possible (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 and MDCK cells, grown either on coverslips or on transwell filters, were washed with phosphate-buffered saline containing $CaCl_2$ and $MgCl_2$, fixed with 4% paraformaldehyde, quenched with 50 mM NH_4Cl, and, depending on the experiment, stained with specific antibodies in nonpermeabilized or permeabilized (0.2% Triton X-100) conditions. Primary antibodies were detected with Alexa 546– or tetramethylrhodamineisothiocyanate (TRITC)–conjugate secondary antibodies. The images were acquired using a laser scanning confocal microscope (LSM 510; Zeiss) equipped with a Plan Apo 63× oil immersion (NA 1.4) objective lens.

Morphometric analyses of Golgi apparatus were carried out by using Quia-ICY software (www.bioimageanalysis.org). A robust image analysis was applied on the immunofluorescence pictures by considering a Z-stack of all acquired images from top to bottom of the cell layer. On the Z-stack the plasma membrane staining of GFP-PrP is used to define the whole cell surface area (100%). We then evaluated the number of pixels associated with Golgi subcompartments that

is normalized to the whole cell surface. In addition, 4′,6-diamidino-2-phenylindole (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.

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