

Different GPI-attachment signals affect the oligomerisation of GPI-anchored proteins and their apical sorting

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Summary

To understand the mechanism involved in the apical sorting of glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs) we fused to the C-terminus of GFP the GPI-attachment signal of the folate receptor (FR) or of the prion protein (PrP), two native GPI-anchored proteins that are sorted apically or basolaterally, respectively, in MDCK cells. We investigated the behaviour of the resulting fusion proteins GFP-FR and GFP-PrP by analysing three parameters: their association with DRMs, their oligomerisation and their apical sorting. Strikingly, we found that different GPI-attachment signals differently modulate the ability of the resulting GFP-fusion protein to oligomerise and to be apically sorted. This is

probably owing to differences in the GPI anchor and/or in the surrounding lipid microenvironment. Accordingly, we show that addition of cholesterol to the cells is necessary and sufficient to drive the oligomerisation and consequent apical sorting of GFP-PrP, which under control conditions does not oligomerise and is basolaterally sorted.

Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/121/24/4001/DC1>

Key words: DRMs, GPI-anchored proteins, Oligomerisation, Rafts, Sorting

Introduction

Glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs) are directly targeted to the apical domain of the plasma membrane in the majority of polarised epithelial cells. They also partition preferentially in dynamic membrane domains that are enriched in sphingolipid and cholesterol – called rafts or detergent-resistant membranes (DRMs) because of their resistance to extraction using detergent (Hancock, 2006; Simons and Ikonen, 1997; Simons and Vaz, 2004).

It has therefore been proposed that the GPI anchor acts as an apical sorting signal (Lisanti et al., 1989) by mediating the incorporation of GPI-APs into rafts (Simons and Ikonen, 1997). However, raft-association is not sufficient to determine apical sorting of GPI-APs, because DRM-associated GPI-APs are sorted both to apical and basolateral membranes of polarised epithelial cells (Benting et al., 1999a; Lipardi et al., 2000; Paladino et al., 2004; Paladino et al., 2007). By contrast, only apical GPI-APs oligomerise during their delivery to the apical membrane, and impairment of their oligomerisation leads to their basolateral missorting (Paladino et al., 2004; Paladino et al., 2007). We have previously proposed that protein oligomerisation is the key step in the apical sorting of GPI-APs (Paladino et al., 2004). However, the mechanism responsible for apical GPI-AP oligomerisation is still unknown. GPI-APs could interact with each other and with other molecules via the glycolipid anchor and/or the protein ectodomain, therefore lipid-lipid, lipid-protein and also protein-protein interactions could be involved in their oligomerisation. GPI-AP oligomers appear to be protein specific and once formed are not sensitive to cholesterol depletion, suggesting that they are maintained by protein-protein

interactions (Paladino et al., 2004). Nonetheless, several findings suggest that rafts constitute a favourable environment for their formation; indeed oligomerisation of GPI-APs begins in the medial Golgi complex – concomitantly with raft-association – and cholesterol depletion impairs the oligomer formation in the Golgi complex (Paladino et al., 2004). It is, therefore, possible that, besides the protein ectodomain, the lipid anchor also has a role in favouring clustering of apical GPI-APs. GPI anchors differ in their fatty-acid composition (Ferguson and Williams, 1988) and these differences are likely to modulate the interaction with raft lipids, which in turn might affect the ability of the proteins to oligomerise.

To understand the role of the GPI-anchor in the oligomerisation and, consequently, in the apical sorting of GPI-APs, we cloned two expression constructs in which the C-terminus of the green fluorescent protein (GFP) was fused to different GPI-attachment signals derived from either an apically sorted GPI-AP – the folate receptor (FR) (Sinn et al., 2003) (supplementary material Fig. S1), or from a basolaterally sorted one – the prion protein (PrP) (Samataro et al., 2002), yielding GFP-FR and GFP-PrP, respectively. We then analysed the properties and the sorting of these two fusion proteins in stably transfected MDCK cells. Our data show that different GPI attachment signals can influence the oligomerisation capacity differently and can determine differential sorting of the same protein ectodomain.

Results and Discussion

Expression of different GFP fusion proteins in MDCK cells

To investigate the role of the GPI anchor in the apical sorting of GPI-APs, the C-terminus of GFP was fused to the GPI attachment

signal from either the apically sorted folate receptor (FR) (Sinn et al., 2003) (supplementary material Fig. S1) or the basolaterally sorted prion protein (PrP) (Sarnataro et al., 2002), resulting in the two fusion proteins GFP-FR and GFP-PrP (Fig. 1A). Expression constructs were transfected in MDCK cells and clones that stably expressed one or the other fusion protein selected (data not shown) (Paladino et al., 2004).

By comparing the C-terminal sequences of annotated proteins, GPI-attachment signal appears to be composed of four sequence regions: First, an unstructured linker region of about 11 residues upstream the cleavage site (ω -site); second, a region of small residues ($\omega-1$ to $\omega+2$) that include the GPI-modification site; third, a moderately polar spacer region of about 8-12 residues that contains a possibly hydrophobic island ($\omega+4$, $\omega+5$) and; fourth, a hydrophobic segment of 10-20 residues that begins at $\omega+11$ (Eisenhaber et al., 1998; Eisenhaber et al., 2003; Udenfriend and Kodukula, 1995). The C-terminal hydrophobic domain and the ω -site have been demonstrated to be essential for GPI-anchor attachment (Caras et al., 1989; Moran et al., 1991). Moreover, it has been shown that pro-proteins that contain a GPI-attachment signal without the amino acids upstream from the ω -site result in proteins being correctly modified and expressed at the cell surface (Lisanti et al., 1991). To study exclusively the role of the GPI anchor and to eliminate interference of any additional amino acids in the sequence, we used a minimal GPI-anchoring signal leaving only the two amino acids essential (ω and $\omega-1$) for the attachment of the GPI anchor in the resulting chimeric proteins. Except for the ω -site and few positions close by, the GPI modification signal has not been characterised regarding its amino-acid-type preferences but only by the physical properties of the different amino acid side chains (Eisenhaber et al., 1998; Udenfriend and Kodukula, 1995). It is unknown whether differences in the primary structure of the GPI attachment signal specify the addition of different preformed GPI-anchors. By comparing the amino acid sequences of the GPI-signals of FR and PrP, we noticed that they have the same length (24 amino acids) and that both contain a Ser residue at the ω -site, similar to 48% of all known GPI-APs (Eisenhaber et al., 1998). However, in FR the position $\omega+2$ is occupied by Ala as in 70% of all known GPI-APs, whereas in PrP this position is occupied by a Thr residue (Fig. 1A). Although the presence of a Thr residue at $\omega+2$ has been demonstrated to be less efficient than Ala or Gly residues when GPI attachment is concerned (Udenfriend and Kodukula, 1995), another GPI-protein – decay accelerating factor (DAF), which also contains a Thr residue at this position – is linked to the GPI. In addition, in contrast to PrP, DAF is efficiently sorted to the apical membrane in MDCK cells (Lisanti et al., 1991), suggesting that the presence of Thr at this position is not sufficient to redirect the protein to the basolateral surface. Another peculiarity of PrP signal are two proline residues at $\omega+8$ and $\omega+9$ (the start of the hydrophobic region; Fig. 1A), which could favour the folding of this domain and stabilise its α -helical structure (Udenfriend and Kodukula, 1995).

GFP-FR and GFP-PrP are differently sorted in MDCK cells

By treating cells with 15 μ g/ml of phosphatidylinositol-specific phospholipase C (PI-PLC) we demonstrated that both our chimeric proteins were GPI-anchored and similarly sensitive to the hydrolysis by this enzyme (Fig. 1B). We then analysed their localisation by confocal microscopy in live and in fixed cells grown under polarising conditions on polycarbonate filters (Fig. 2). At steady state, both under live (Fig. 2A) and fixed conditions (Fig. 2B), GFP-

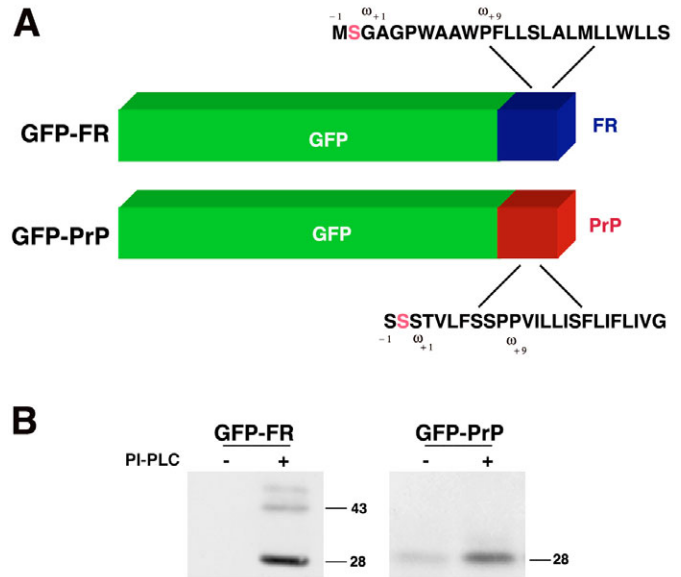


Fig. 1. GFP-fusion proteins and their treatment with PI-PLC. (A) In the fusion constructs GFP-FR and GFP-PrP, GFP is fused to the GPI-anchor attachment signal of folate receptor (FR) and prion protein (PrP), respectively. The FR or PrP sequence included in the fusion proteins is shown. The ω -site is shown in red. (B) MDCK cells stably expressing GFP-FR or GFP-PrP were extracted with Triton X-114. After phase separation, detergent phases were incubated in the presence (+) or absence (-) of PI-PLC (15 μ g/ml) as previously described (Lisanti et al., 1989). The resulting aqueous phases were precipitated with trichloroacetic acid and detected by western blotting using anti-GFP antibody. As previously shown, the band migrating at 43 kDa represents a partially denatured dimer of GFP (Paladino et al., 2004).

FR was mainly localised on the apical surface, whereas GFP-PrP was distributed on the basolateral side. The surface distribution of both proteins was quantified by selective domain biotinylation, showing ~85-90% of GFP-FR on the apical surface, whereas ~90-95% of GFP-PrP was basolaterally distributed (Fig. 2C). These results show that GFP-FR and GFP-PrP behave as native FR and PrP, indicating that different GPI-attachment signals determine the apical or basolateral sorting of the attached GFP ectodomain.

Both GFP-FR and GFP-PrP associate with DRMs, but only GFP-FR forms high-molecular-weight complexes

To investigate the mechanism of the different sorting of the two chimeric proteins we analysed their DRM-association because it has been shown to be one of the requirements for apical GPI-AP sorting (Benting et al., 1999b; Lipardi et al., 2000; Paladino et al., 2004; Paladino et al., 2007). We found that both GFP-FR and GFP-PrP migrate to the DRM fractions of sucrose-density gradients that were enriched in the ganglioside GM1 (Fig. 3A). These data confirm that the GPI anchor is sufficient to mediate the association of the protein with DRMs independently of their sorting phenotype (Benting et al., 1999b; Brown and London, 1998; Lipardi et al., 2000; Paladino et al., 2004; Paladino et al., 2007). However, it is interesting that the two proteins have a slightly different distribution profile on the gradients – apical GPI-AP peaks in fraction 5, whereas basolateral GPI-AP peaks in fraction 6 – similar to what has already been shown for native apical or basolateral GPI-APs (Paladino et al., 2004; Paladino et al., 2007; Sarnataro et al., 2002). This difference could have two explanations: either different affinities

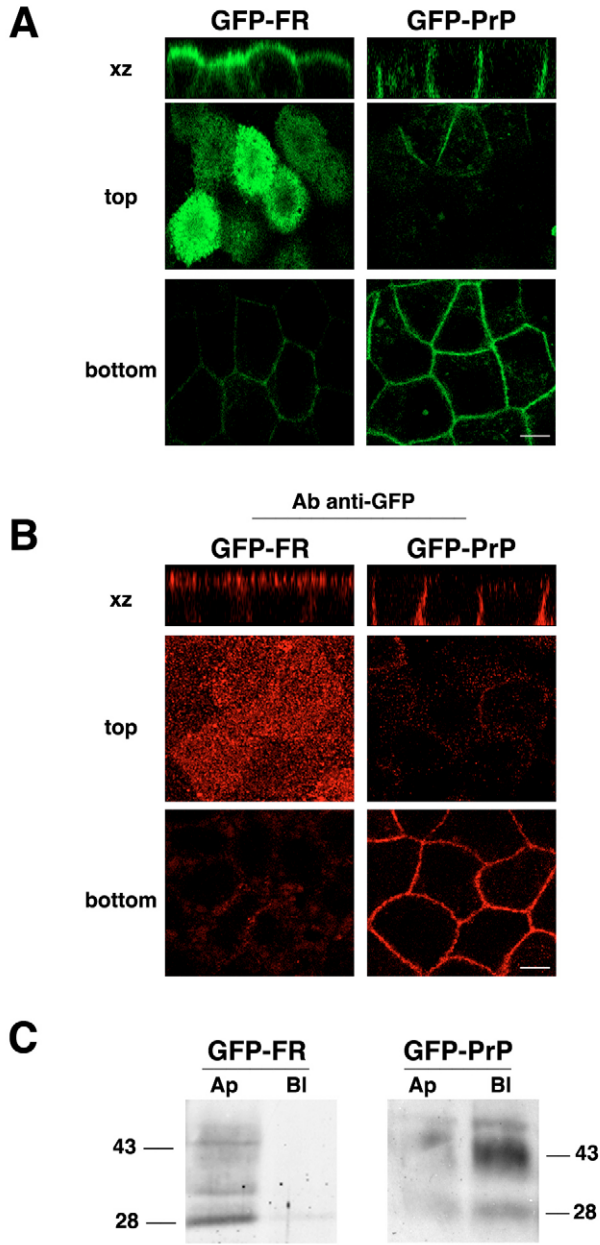


Fig. 2. GFP-FR and GFP-PrP are differently sorted in MDCK cells. MDCK cells stably expressing GFP-FR or GFP-PrP were grown on filter for 4 days. (A,B) Cells were analysed in vivo in (A) CO₂-independent medium or (B) stained with an anti-GFP antibody followed by a TRITC-conjugated secondary antibody under non-permeabilising conditions. Serial confocal sections were collected from top to bottom of cell monolayers. (C) Cells were labelled with LC-biotin, which was added to their apical (Ap) or basolateral (Bl) surface. After immunoprecipitation using anti-GFP antibody, samples were separated by SDS-PAGE and biotinylated proteins were revealed using HRP-streptavidin. Bars, 10 μ m.

of the apical and basolateral GPI-APs for the same lipid rafts or, a different lipid environment surrounding the differently sorted proteins.

GPI anchors can have differences in their fatty acid composition and/or the modifications of their sugars (Ferguson and Williams, 1988; McConville and Ferguson, 1993). In particular, the presence

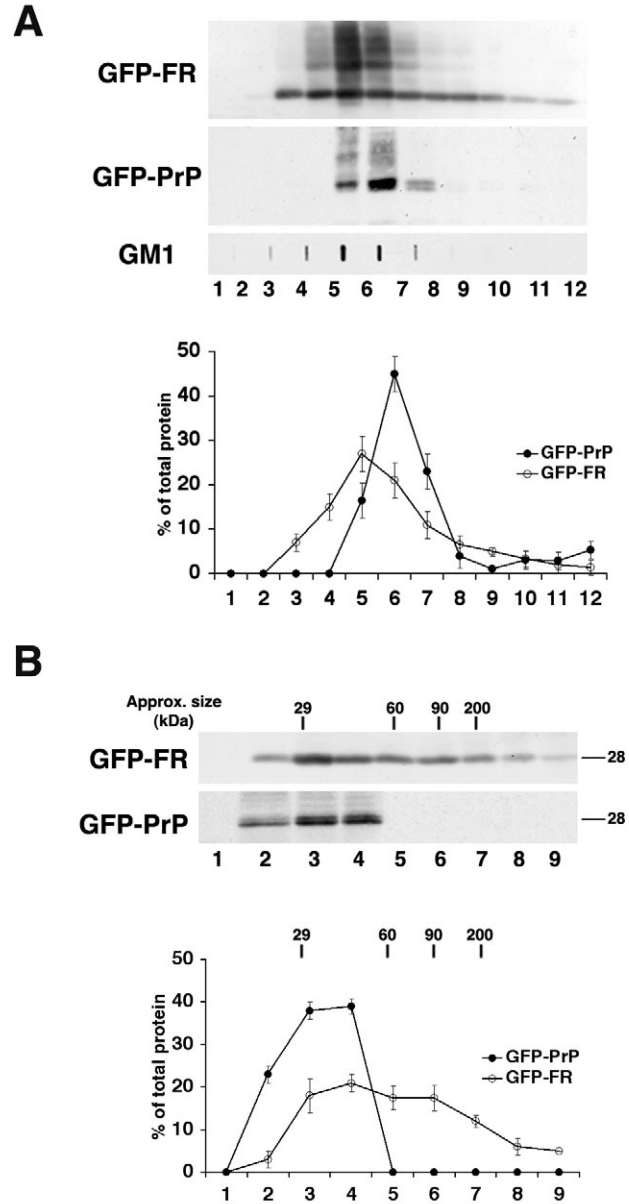
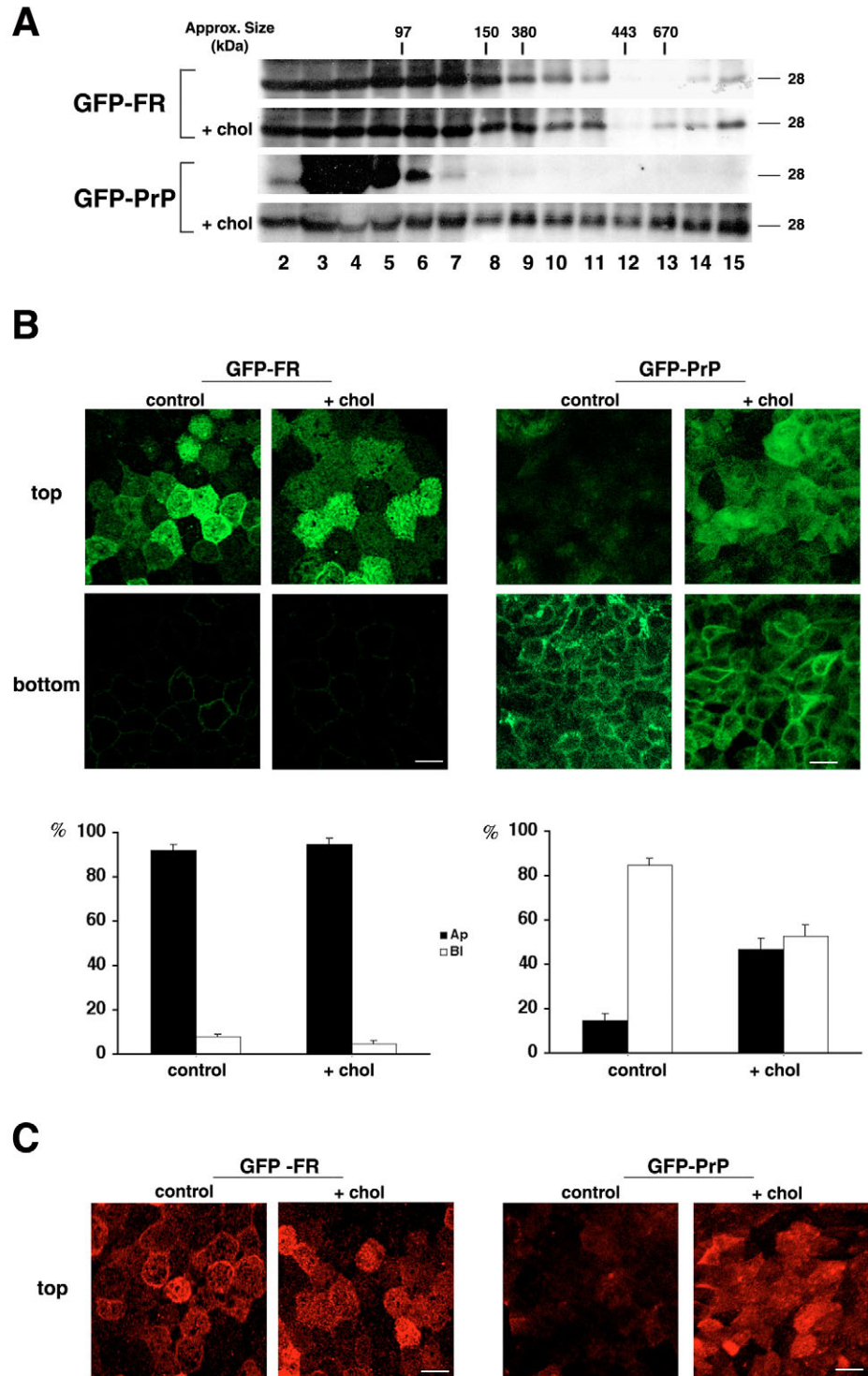


Fig. 3. GFP-FR and GFP-PrP both associate with DRMs, but only GFP-FR forms HMM complexes. (A) MDCK cells stably expressing GFP-FR or GFP-PrP were lysed at 4°C in buffer containing 1% Triton X-100 and separated by centrifugation until equilibrium on 5–40% sucrose-density gradients to purify Triton-X-100 insoluble microdomains. Fractions of 1 ml were collected from top (fraction 1) to bottom (fraction 12) and, after TCA-precipitation, run on SDS-PAGE and detected using anti-GFP antibody. One aliquot of each fraction was transferred onto nitrocellulose membrane, and GM1 (a typical raft-marker) was revealed by using cholera toxin conjugated to HRP. (B) Cells were lysed in buffer containing 0.4% SDS and 0.2% Triton X-100 and run through 5–30% sucrose gradients. Fractions of 500 μ l were collected from the top (fraction 1) to the bottom (fraction 9) of the gradients. Proteins were TCA-precipitated and detected by western blotting using a specific GFP antibody. The position on the gradients of molecular mass markers is indicated. The graphs show the mean values of protein distribution on the gradients from three different experiments \pm s.d.

of an additional acyl substitution of the inositol ring and/or carbohydrate and phospho-ethanolamine substitution of mannose residues has been found (Ferguson, 1999; Kinoshita and Inoue, 2000). This in turn could mediate a different affinity for lipid rafts.



Acyl- and alkyl-chain length of GPI-anchors has been demonstrated to be crucial for raft association *in vitro* (Benting et al., 1999a). Several enzymes, such as GUP1 and PER1, have been shown to be involved in remodelling the GPI-anchor in *Saccharomyces cerevisiae* and in *Trypanosoma brucei* (Bosson et al., 2006; Fujita et al., 2006; Jaquenoud et al., 2008). Recently, by using post-GPI-attachment to protein 2 (PGAP2)-deficient cells, it has been shown that fatty-acid remodelling of GPI-AP is crucial for their raft

association (Maeda et al., 2007), thus supporting the hypothesis that anchor remodelling affects the affinity for lipid rafts. Moreover, a recent study has demonstrated that two different recombinant GPI-APs [GFP-DAF and GFP-conjugated tumor-necrosis-factor-related apoptosis-inducing ligand receptor 3 (GFP-TRAIL-R3)] partition in lipid rafts at different extents, both after their transfection into HEK 293 cells or after insertion in primary lymphocytes by cell-surface painting (Leglar et al., 2005), suggesting that the two fusion

proteins have a different affinity for these lipid microdomains (Legler et al., 2005). However, it has been shown that two other GPI-APs, PrP and thymocyte differentiation antigen1, have a different subcellular localisation in neurons and are surrounded by a different lipid environment (Brugger et al., 2004), thus suggesting the existence of more than one type of lipid microdomain. On the same line, we found that DRMs associated with an apical or a basolateral protein contain the same lipid species, but in different ratios (Tivodar et al., 2006). Therefore, it is also possible that a different GPI anchor leads to the partitioning of GPI-APs in different lipid microdomains. Interestingly, it has been reported that the GPI-anchor of PrP has one or two more hexose units in addition to the three basic ones and contains sialic acid, a sugar not previously found to be a component of GPI anchors (Baldwin, 2005; Stahl and Prusiner, 1991).

Whereas DRM-association is required but is not sufficient for the apical sorting of GPI-APs (Benting et al., 1999b; Paladino et al., 2004) a distinct feature of the apically sorted GPI-APs is their ability to form high molecular mass (HMM) complexes (Paladino et al., 2004; Paladino et al., 2007). Thus, we analysed whether the different sorting of the two chimeric proteins correlates with a difference in their ability to oligomerise. To this aim, cell lysates were purified on velocity gradients according to their molecular mass (Fig. 3B). We found that, similar to the native GPI-APs (FR and PrP) at steady state, ~20-25% of GFP-FR occurred in HMM complexes, whereas GFP-PrP was purified exclusively as monomer (Fig. 3B). These findings confirm that oligomerisation is required for apical sorting of GPI-APs (Paladino et al., 2004; Paladino et al., 2007) and suggest that the basolateral sorting of GFP-PrP is a consequence of its inability to oligomerise. Furthermore, they show that differences in the GPI attachment signal to the same ectodomain result in fusion proteins with different oligomerisation capacity.

Addition of cholesterol results in GFP-PrP oligomerisation and apical sorting, but does not affect the behaviour of GFP-FR

Our previous results might be explained by the fact that different GPI attachment signals mediate a different affinity for lipid rafts or the association of the two different GPI-APs to different lipid rafts, which in turn influences the oligomerisation state of the protein and, therefore, its sorting behaviour. To test these hypotheses we decided to alter the amount of cholesterol in the MDCK cell membrane, because it appears to be a crucial player in the apical sorting of GPI-APs. Indeed cholesterol depletion affects both the delivery of apically sorted GPI-APs and their ability to oligomerise in the Golgi complex (Ehehalt et al., 2008; Paladino et al., 2004; Paladino et al., 2007). By contrast, the basolateral sorting of PrP is not affected by cholesterol depletion and the protein remains in its monomeric form during its delivery to the basolateral domain of the plasma membrane (Paladino et al., 2004; Sarnataro et al., 2002). We therefore decided to analyse the effect the addition of cholesterol has on oligomerisation and sorting of our two chimeric GPI-APs (Fig. 4). As previously shown (Patel et al., 2002), the pre-treatment of MDCK cells with cholesterol (2 mM) in a complex with methyl- β -cyclodextrin (β CD) results in an increase of ~50% in cellular cholesterol levels (see Materials and Methods), without detectable cytotoxicity or morphological and functional changes in the cell monolayer (supplementary material Fig. S2). Whereas the addition of cholesterol does not affect oligomerisation (Fig. 4A) and/or apical sorting of GFP-FR (Fig. 4B), we – surprisingly – purified ~20% of GFP-PrP as HMM complexes that contained more than a trimer upon cholesterol loading (Fig. 4A). In agreement with these data,

GFP-PrP was re-directed to the apical surface of the cells, as assessed by confocal microscopy in (living or fixed) polarised cells (Fig. 4B,C). Quantification of the fluorescence intensity showed that after cholesterol addition ~45 \pm 3% of GFP-PrP reached the apical surface compared with ~20 \pm 5% in control cells (Fig. 4B).

These data, therefore, support the hypothesis that oligomerisation is a necessary event for the apical sorting of GPI-APs, and show that the lipid environment (specifically the levels of cholesterol in the MDCK cell membrane) may drive oligomerisation and apical sorting of GPI-APs. However, because a double Cys to Ser mutation (referred to as S49/71) in the GFP ectodomain of GFP-FR impairs both oligomerisation and apical sorting of this protein (Paladino et al., 2004), it appears that the protein ectodomain is also required to permit or stabilise the occurrence of oligomers in rafts. We postulate that, although the S49/71 mutant possesses a GPI-anchor that can promote partition in a favourable environment to form HMM complexes, it does not oligomerise because it does not possess the two Cys residues in the ectodomain that enable its oligomerisation. In agreement with this hypothesis we found that cholesterol addition does not affect the sorting of the S49/71 mutant (supplementary material Fig. S3). Indeed, although this mutant already partitions in a favourable membrane environment, it does not have the permissive ectodomain that is needed to allow or stabilise the HMM oligomers. By contrast, the addition of cholesterol favours oligomerisation and apical sorting of the basolateral GFP-PrP (which has a different GPI-attachment signal compared with apical GFP-FR, but contains the two Cys residues in the ectodomain, Fig. 4).

In conclusion, we show that different GPI-anchor-signal attachments result in both different sorting and different oligomerisation capacity of the same anchored ectodomain GFP. This is probably owing to the attachment of a structurally different GPI anchor. Interestingly, by increasing the cholesterol content we were able to ‘convert’ a non-oligomerising basolateral protein into an oligomerising apical one. This clearly indicates that a specific lipid environment is required to enable a permissive ectodomain to oligomerise. These data can be explained in at least two different ways: Either, the addition of cholesterol stabilises the interaction between the basolateral GPI-AP and lipid rafts (i.e. by rigidifying the raft membranes or interfering with the free diffusion of proteins) (Lebreton et al., 2008), thus allowing the stabilisation of the protein in rafts and consequently its oligomerisation and apical sorting; or, cholesterol addition may change the characteristics of the surrounding lipid environment (i.e. conversion of basolateral rafts to apical rafts), thus enabling the protein originally associated with the basolateral raft to oligomerise and to be delivered apically. However, it could also be envisaged that a cholesterol-enriched environment recruits into the raft domains other proteins, which can stabilise GFP-PrP oligomers through interaction with the ectodomain of the protein. Possible candidates involved in the stabilization of GPI-AP oligomers are proteins belonging to the annexin family (e.g. annexin II and annexin XIIIb), which have been found to be enriched in rafts and involved in the apical transport of raft transmembrane proteins (Fiedler et al., 1995; Jacob et al., 2004; Lafont et al., 1998). Alternatively, galectin 4, which interacts with glycosphingolipids and the depletion of which induces the intracellular accumulation of apical raft-associated proteins (Delacour et al., 2005) might be another candidate.

Further studies are required to better characterise the structural composition of GPI anchors of differently sorted proteins and the chemical-physical properties of lipid microdomains that surrounding GPI-APs at the level of the Golgi complex, and also to investigate

further the existence of possible interactors or motifs present on the protein ectodomain that allows oligomerisation to occur.

Materials and Methods

Cell culture and transfections

MDCK cells were grown in DMEM (Dulbecco's modified Eagle's medium) containing 5% FBS. The GFP-FR construct (referred to before as GFP-GPI) (Paladino et al., 2004) was a kind gift of Stephen Lacey (Southwestern University, Georgetown, TX) and MDCK cells stably expressing this construct had been obtained previously (Paladino et al., 2004). In GFP-PrP, the signal peptide sequence and the GPI-attachment-signal sequence of mouse PrP were fused 5' and 3' of GFP, respectively, into a pEGFP vector (Clontech Laboratories). Sequences were amplified using PCR and specific primers containing unique restriction sites of the vector: the signal peptide was inserted 5' *NheI* 3' *AgeI*, the GPI attachment signal 5' *BglII* 3' *EcoRI*. MDCK cells were transfected with cDNA coding for GFP-PrP using lipofectin (Invitrogen). Stable clones were selected by resistance to neomycin.

Biotinylation assay

Cells grown on polycarbonate filters for 4 days, were selectively biotinylated from the apical or the basolateral side. Lysates were immunoprecipitated using a monoclonal anti-GFP antibody (Molecular Probes, Invitrogen) and analysed by western blotting using horseradish peroxidase (HRP)-streptavidin (Pierce).

Fluorescence microscopy

MDCK cells, grown on transwell filters for 4 days, were fixed using 4% PFA and stained using an anti-GFP antibody that can be detected with TRITC-conjugated secondary antibody. For live imaging, cells were grown upside down on filters that had been then mounted on a optical glass within a Petri dish containing CO₂-independent medium (150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES pH 7.4) as previously described (Paladino et al., 2006). Images were collected using an LSM 510 confocal microscope (Carl Zeiss).

As previously described (Nichols et al., 2001), quantification of mean fluorescence intensities in selected regions of interest were performed using the LSM software (Carl Zeiss). In particular, the fluorescence intensities of same-sized areas within a single z-plane through the cell monolayer (ranging from 1-3 μm to 8-12 μm, starting from the top of the cell for apical and basolateral signals) were measured and corrected for background.

Sucrose-density gradients

Sucrose-density-gradient analysis of Triton-X-100-insoluble material was performed according to previously published protocols (Brown and Rose, 1992; Paladino et al., 2004). Cells grown to confluency in 150-mm dishes were lysed for 20 minutes in TNE buffer (Tris, NaCl, EDTA) with 1% Triton X-100 on ice. Lysates were scraped off the dishes, brought to 40% sucrose and then placed at the bottom of a centrifuge tube. A discontinuous sucrose gradient (5-35% in TNE) was layered on the top of the lysates and the samples were ultracentrifuged at 39,000 rpm (SW41 rotor; Beckman) for 17 hours. From the top of the gradient, 1-ml fractions were harvested.

Velocity gradients

Cells grown to confluency in 100-mm dishes, were lysed for 30 minutes in 20 mM Tris pH 7.4, 100 mM NaCl, 0.4% SDS, 0.2% Triton X-100 on ice. Lysates were scraped off the dishes, sheared through a 26-g needle and nuclei were pelleted. A sucrose gradient (30-5%) was layered into a centrifuge tube and the lysate, added on the 5% part of the gradient, was ultracentrifuged at 45,000 rpm (SW50 rotor; Beckman) for 16 hours. Fractions of 500 μl were harvested from the top of the gradient (Paladino et al., 2004; Scheiffèle et al., 1998). In the case of longer-velocity gradients, lysates were layered on the top of a 40-20% glycerol gradient and, after ultracentrifugation [45,000 rpm (SW50 rotor; Beckman) for 16 hours], fractions of 300 μl were collected (Meunier et al., 2002).

Cholesterol addition

Cholesterol was added to cells by using cholesterol-saturated βCD (2 mM cholesterol in 10 mM βCD), which was purchased from Sigma as water-soluble cholesterol already balanced with βCD (ratio 1:6). Cholesterol-saturated βCD was added to cells in medium (containing 20 mM HEPES pH 7.5 and 0.2% bovine albumin) at 37°C for 30 minutes. A colorimetric assay (Merck Chemicals) was used to measure cholesterol levels.

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