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REVIEW ARTICLE

# Clustering in the Golgi apparatus governs sorting and function of GPI-APs in polarized epithelial cells

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**Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are lipid APs attached to the extracellular leaflet of the plasma membrane (PM) *via* a glycolipid anchor. GPI-APs are commonly associated with cholesterol- and sphingolipid-enriched membrane microdomains. These microdomains help regulating various biological activities, by segregating different proteins and lipids in (nanoscale) membrane compartments. In fibroblasts, GPI-APs form actin- and cholesterol-dependent nanoclusters directly at the PM. In contrast, in polarized epithelial cells GPI-APs cluster in the Golgi apparatus, the major protein-sorting hub for the secretory pathway. Golgi clustering is required for the selective sorting of GPI-APs to the apical PM domain, but also regulates their organization and biological activities at the cell surface. In this review, we discuss recent advances in our understanding of the mechanism of GPI-AP sorting to the apical membrane. We focus on the roles of the protein moiety and lipids in the regulation of the clustering of GPI-APs in the Golgi apparatus.**

**Keywords:** calcium; cholesterol; clustering; Golgi complex; GPI-anchored proteins; lipid microdomains; sorting

The acquisition of cell polarity is crucial for the physiology and homeostasis of epithelial tissues, which line the outside of the body (skin) and the inside cavities of organs (such as lung, kidney, liver and gastrointestinal tract) and constitute a protective and selective barrier regulating the exchanges with the extracellular space.

Polarization of epithelial cells require the activation of specific processes that are directed by external clues (such as cell–cell and cell–extracellular matrix adhesion), integrated in time and in space and devoted to the establishment of a unique cytoarchitecture [including specific organization of the cytoskeleton, organelles distribution and plasma membrane (PM) specialization] and to the acquisition of distinct trafficking pathways [1–3].

To achieve the unique vectorial functions of epithelia, the PM of epithelial cells is divided into structurally and functionally distinct domains (apical and basolateral), which display different protein and lipid composition. The acquisition and maintenance of this asymmetric distribution is ensured by the establishment of tight junctions, physically separating the two domains, and by the continuous sorting of newly synthesized lipids and proteins and their regulated internalization [2,4,5]. Apical and basolateral proteins must be correctly delivered along the secretory pathway to their final destination, *via* the sequential action of several sorting signals and multiple sorting events. The Golgi complex is the major protein-sorting station. Apical and basolateral proteins have been shown to be completely segregated

**Abbreviations**
GPI, glycosylphosphatidylinositol; GPI-APs, GPI-anchored proteins; PM, plasma membrane; SPLs, sphingolipids.

at the level of the trans-Golgi network (TGN) [6–10]. Nevertheless, endosomal compartments have also been implicated in the biosynthetic trafficking of proteins in polarized epithelial cells [2,4,5,11].

Glycosylphosphatidylinositol-anchored proteins (GPI-APs), a family of lipid APs expressed from lower eukaryotes to humans, are selectively localized at the apical surface of the major part of epithelia, where they must exert their physiological functions. They represent roughly 0.5% of total proteins in eukaryotes and so far more than 150 GPI-APs have been identified in mammalian cells exhibiting a wide range of functions including surface antigens, receptors, enzymes and cell adhesion molecules [11,12]. GPI-APs are confined to the outer leaflet of the cell surface *via* the lipid portion of GPI anchor that is synthesized in the endoplasmic reticulum (ER) through about 20 sequential reactions and transferred *en bloc* by the multienzymatic complex, GPI-transamidase, to the C-terminal part of the protein after recognition of the GPI attachment signal sequence [11,13–16].

The presence of both lipid anchor and protein portion confers unique trafficking features to these proteins. Besides post-translational protein modifications, recent studies have highlighted that GPI anchor remodelling has profound effects on their traffic along the secretory pathway. Moreover, GPI-APs partition with cholesterol and sphingolipids (SPLs) enriched membrane microdomains that could influence both their trafficking and their surface organization [11,17–22].

Protein oligomerization is a key step to determine the apical sorting of GPI-APs in epithelial cells of different origin [23,24]. Specifically, it has been shown that clusters of single GPI-AP species (named homoclusters) form in the Golgi complex of fully polarized cells. Importantly, homocluster formation in the Golgi requires cholesterol and leads to the segregation of GPI-APs in specific membrane microdomains and their subsequent apical sorting [23,25,26]. Conversely, once formed, GPI-AP homoclusters become insensitive to cholesterol depletion. Current data suggest that both protein–lipid and protein–protein interactions are involved in the formation and stabilization of GPI-AP clusters [23,27]. Although the biochemical nature of the interactions that determine apical GPI-AP clustering prior to their apical sorting are not totally clear, the bulk of the evidence point out on the integrated action of the protein ectodomain and the GPI anchor together with the requirement of a favourable lipid environment (i.e., need of threshold levels of cholesterol in the Golgi membranes). Overall, these data indicate that the composition of Golgi membranes modulate the protein and lipid interactions leading to GPI-AP clustering and apical sorting. How these two cooperate and whether there is a reciprocal regulation is still to be determined.

Golgi GPI-AP homoclusters are required for the subsequent organization of these proteins in larger cholesterol-dependent clusters formed by multiple GPI-AP species (heteroclusters) at the apical PM, of polarized epithelial cells [27]. Thus, this clustered organization is achieved only in fully polarized cells when the sorting mechanism in the Golgi is active [27]. Strikingly, this clustered organization appears to regulate also the activity of GPI-APs at the PM, so that only when the GPI-APs are correctly sorted they are organized in functional clusters at the surface.

This regulation is specific for GPI-APs in polarized epithelial cells and appears to be very different from fibroblasts. In these cells, GPI-APs are monomeric in the Golgi and clustering occurs exclusively at the PM in response to surface cues (e.g., cholesterol, cortical actin) [19,28–33]. The different regulation of GPI-AP clustering in the diverse cell types might be linked to the functions that each cell type exerts. The strict interdependence of GPI-AP clustering, sorting and polarized phenotype of GPI-APs in epithelial cells might ensure functional GPI-AP clusters in functional epithelial tissues. Thus, understanding the molecular mechanisms regulating Golgi clustering is crucial for understanding how these processes might be altered upon pathological conditions.

In this review, we discuss the mechanism of Golgi clustering of GPI-APs and more specifically the role of (a) protein itself; (b) the lipids and (c) actin in the Golgi.

## Clustering of GPI-APs in the Golgi regulates apical sorting

Like all PM proteins, the journey of the GPI-APs begins with their synthesis in the ER thanks to a cleavable, hydrophobic amino-terminal signal sequence that targets the newly polypeptide chain to this organelle. The attachment of the GPI anchor occurs post-translationally in the ER. The multienzymatic complex, GPI-transamidase, recognizes a hydrophobic signal sequence at the C terminus (named GPI attachment signal) of the protein, cleaves it and transfer the preassembled GPI moiety to the protein [13–16,34].

Once entered the secretory pathway, as for other PM proteins, GPI-APs are delivered to their correct destination *via* the sequential action of several sorting signals and multiple sorting events. Proper sorting and trafficking is crucial for exerting their activity and this is particularly relevant in the case of epithelial tissues in which the major part of GPI-APs is selectively

localized on the apical surface. Both the protein portion and the GPI anchor are involved in the regulation of GPI-AP trafficking. This implies that pathways involved in lipid and protein modifications may influence the intracellular trafficking of these proteins, conferring them unique trafficking features.

While in yeast the ER is the site where GPI-APs are sorted from the other secretory proteins [35,36], in mammals the Golgi complex, and in particular the TGN, is the major sorting station [18,22,36–38]. In polarized epithelial cells, apical and basolateral proteins are sorted and incorporated into distinct vesicles at the TGN level upon recognition of specific apical or basolateral signals [2,5]. Time-lapse experiments showed that GPI-APs travel together with other cargo molecules in the early Golgi cisternae of both polarized and nonpolarized cells and segregate completely in the TGN where they exit in distinct vesicles [6,8,9]. Moreover, it was ascertained that the rate-limiting step for GPI-AP transport was the exit from the Golgi complex [39], supporting that GPI-AP sorting occurs at the TGN. Whether GPI-AP and transmembrane proteins destined to the same domain travel in the same or different carriers is an open question. So far, there is one study showing that two apical transmembrane proteins, one associated with lipid microdomains and one not, are transported in distinct carriers in non-polarized Cos-7 cells [40], suggesting that proteins enriched in lipid platforms use different carriers to reach the PM. Moreover, the fact that the molecular determinants for the transport of GPI-APs and transmembrane proteins are different [2,6,7,9,10,41–49] further supports this hypothesis.

On the other hand, while it has been well established that transmembrane and secreted, both apical and basolateral, proteins transit through endocytic compartments en route to the cell surface [50–55], few studies have reported an involvement of these compartments for GPI-APs [56]. Most likely, the use of this pathway could be dependent on the protein and/or on the cell type; therefore, further studies are necessary to elucidate this.

A peculiarity of GPI-APs is that they are associated with cholesterol and SPL-enriched membrane microdomains (also called lipid rafts), which could influence their trafficking. Lipid rafts help regulating various biological functions by segregating different proteins and lipids in micro-/nanomembrane domains and at the beginning they have been considered to act as apical sorting platforms for GPI-APs [57]. However, both apical and basolateral GPI-AP partition into lipid rafts proving that their association with these lipid domains is not sufficient for GPI-AP apical sorting [23,24]. Protein oligomerization is a key step to drive the apical sorting of GPI-APs in epithelial cells of different origin [23,24]. Indeed, only apical GPI-APs are able to oligomerize into high molecular weight complexes and the impairment of oligomerization leads to protein missorting [23,24]. As shown by pulse-chase experiments GPI-APs homoclusters (containing a single GPI-AP species) form in the Golgi complex of fully polarized cells, in particular when the protein traverse the medial Golgi, concomitantly with their association with lipid microdomains [23,24]. Importantly, homocluster formation in the Golgi requires cholesterol and allows segregation of GPI-APs in specific membrane microdomains and their subsequent apical sorting [23,25,26]. Hence, clustering and partition in specific lipid domains drive the apical sorting of GPI-APs (Fig. 1). Clustering stabilizes GPI-APs into lipid domains possibly increasing their affinity for certain lipids as shown for some receptors and viral proteins [58–61]. This facilitates GPI-AP segregation from other secretory cargoes and their incorporation into apical vesicles, similarly to what has been previously proposed for sorting of GPI-APs in early endosomes [60,62].

Protein–protein crowding has been proposed as a new mechanism enabling membrane deformation [63–66]. Interestingly, it has been shown that the effect of protein crowding on membrane bending increases with asymmetric distribution of protein portions across the bilayer. This mechanism could be mostly suitable for

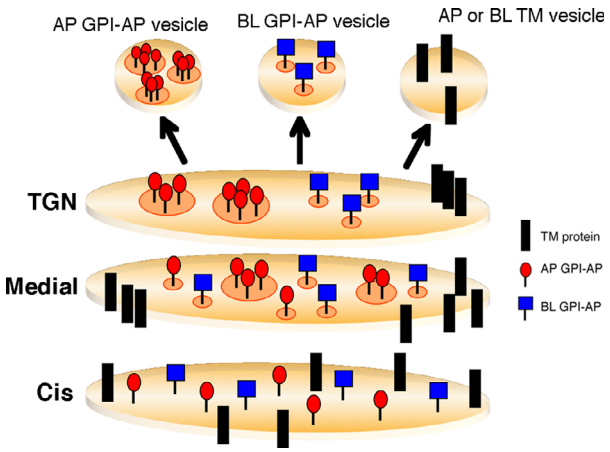


**Fig. 1.** Model of TGN sorting of GPI-APs in polarized epithelial cells. Both apical (red) and basolateral (blue) GPI-APs partition into lipid rafts at the medial Golgi and segregate from nonraft transmembrane proteins (black). At the medial Golgi, only apical-destined GPI-APs start to oligomerize. Clustering stabilizes GPI-APs into lipid domains allowing the segregation from basolateral GPI-APs and the inclusion into the apical vesicle. Cholesterol is required for GPI-AP homoclustering.

GPI-APs because of their asymmetric structure. Therefore, at the TGN GPI-AP oligomers might promote the bending of Golgi membranes with consequent formation of a GPI-AP–enriched vesicle, similar to what has been proposed in the case of steric-dependent sorting of synthetic GPI-AP analogues that are excluded from endocytic vesicles containing transferrin receptors [67]. Moreover, membrane curvature can be also modulated by changes in lipid composition, which supports a role of the lipid driving force in vesicle budding [68–71]. Considering the mutual cooperation between GPI-AP clustering and association with lipid microdomains and the role of cholesterol in driving GPI-AP oligomerization, it appears plausible that a more complex scenario should be envisaged where GPI-AP crowding contributes to the curvature-driven lipid sorting. In turn, it is envisaged that proximal homoclusters would function as nucleation points allowing the coalescence of different lipid phases and thus enabling the membrane curvature necessary to drive vesicle formation. It would be interesting to directly test this hypothesis in model membranes *in vitro* using different GPI-AP mutants. On the other hand, in the case of the ER exit of GPI-APs it was reported that Sec13p is required for vesicle formation in yeast [63]. Copic and colleagues have proposed that Sec13p would keep the rigidity of COPII coat balancing the opposite forces generated by the local crowding of asymmetrically distributed cargoes [63]. Similarly, we can speculate that tether and coat factors might help the GPI-AP vesicle/tubule formation at the TGN, thus helping the membrane deformation caused by protein crowding. So far, the nature of these factors acting at the TGN on GPI-AP carriers is not known. Galectin-4, which binds glycosphingolipids [44], could be a good candidate as it could crosslink raft-associated proteins into lipid platforms from which vesicle could bud. Furthermore, the new findings showing the involvement of clathrin and AP1 in the Golgi-to-PM transport of GPI-APs [72] point out a putative role of these proteins in promoting GPI-AP vesicle formation.

Interestingly, Golgi homoclustering not only mediates apical sorting of GPI-APs but it is also required for their subsequent organization at the apical PM [27]. Specifically, it has been shown that newly arrived homoclusters [27,73] coalesce in heteroclusters (containing at least two different GPI-AP species) that are sensitive to cholesterol depletion (Fig. 2). It is worth noting that in nonpolarized cells, in the absence of homocluster formation in the Golgi, GPI-APs remain unclustered at the cell surface. These data indicate that in epithelial cells the membrane organization of GPI-APs is strictly related to the presence of an active mechanism of sorting and to the acquisition of the polarized phenotype. Furthermore, the clustered organization is crucial for the biological activities of GPI-APs at the cell surface [11,27], so that only when GPI-APs are correctly sorted to the apical membrane in fully polarized epithelial cells, they are organized in functional clusters at the surface. This interdependence of the sorting of GPI-APs with their surface organization ensures that GPI-APs are fully active at the proper domain of residency, but rapidly inactive upon pathological conditions that harm epithelial polarization (such as infection, inflammation, cancer). This is very different from fibroblasts where GPI-APs arrive from the Golgi complex in the form of monomers and cluster at the cells surface and here their dynamics are modulated by both cholesterol and actin cytoskeleton [19,28–30,74]. It would be interesting to test this hypothesis by forcing GPI-AP clusters in the Golgi of fibroblasts or in nonpolarized epithelial cells (e.g., by crosslinking) and look at the organization and activity of the proteins at the PM.

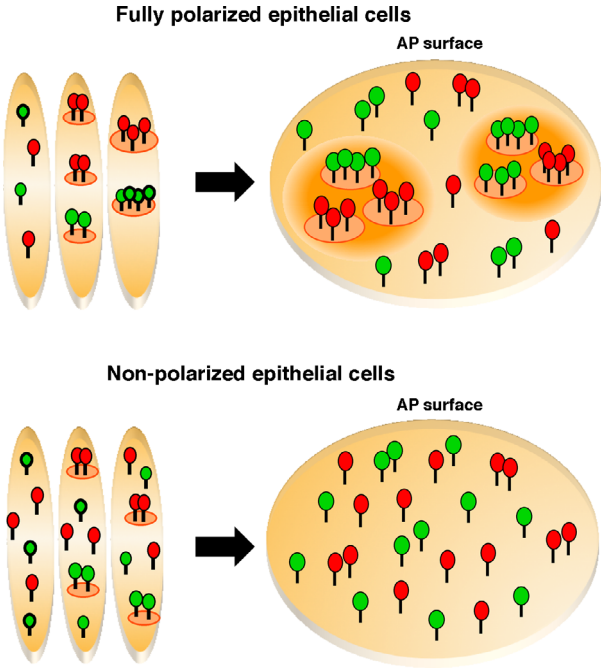


**Fig. 2.** Membrane organization of GPI-APs at the apical surface of epithelial cells. GPI-APs are organized into cholesterol-independent homoclusters containing a single GPI-AP (red and green aggregates). Homoclusters of different species can coalesce in cholesterol-dependent heteroclusters (depicted here as pale orange domain). Golgi homoclustering is required for the subsequent formation of GPI-AP heteroclusters at the PM. Differently from fully polarized epithelial cells, in nonpolarized epithelial cells, GPI-APs do not form clusters in the Golgi complex and remain in monomeric form at the apical surface.

## Role of glycolipid anchor and protein moiety in the clustering of GPI-APs

Cholesterol is crucial for the formation of GPI-AP clusters in the Golgi [23,25,26]; yet, once formed GPI-AP homoclusters become insensitive to cholesterol depletion. This suggests that both protein–lipid and protein–protein interactions are involved in the formation and stabilization of GPI-AP clusters [23,27]. Below we discuss the data supporting this hypothesis.

### Role of glycolipid anchor

Nonprotein-linked GPI anchors are distributed in a nonpolarized manner on both domains of the PM in MDCK cells [75], indicating that GPI per se cannot act as a sorting signal. However, the GPI anchor appears to have a role in favouring GPI-AP clustering. Compelling evidence, in support of this, is that different GPI attachment signals (derived from an apical and basolateral native GPI-AP, respectively, Folate Receptor and PrP) attached to secretory GFP affect the behaviour of the resulting GFP fusion proteins [26]. While GFP-FR was able to oligomerize and was apically sorted, GFP-PrP did not oligomerize and was sorted basolaterally [26]. Most likely, different GPI attachment signals result in the attachment of a structurally different GPI anchor. How this occurs is not yet understood; however, it has been shown that, beside a common core, GPI anchors can have different fatty acid compositions and/or sugar modifications [14,76]. Whether and how different GPI attachment signals affect the subsequent remodelling of the anchor is unknown and requires further investigations. Nevertheless, research in recent years has revealed that GPI anchor remodelling might influence the traffic of GPI-APs along the secretory pathway, at different steps (e.g. ER, Golgi, PM), and their properties. In particular, Maeda and colleagues have shown that replacement of an unsaturated chain in the sn2 position with a long saturated one, a process occurring in the Golgi complex thanks to the sequential actions of PGAP3 and PGAP2 enzymes, is required for raft association of GPI-APs [77]. Other studies also supported that differences in the GPI anchor (in terms of either length of acyl and alkyl chains or remodelling of glycan portion) mediate a different affinity for lipid microdomains [78–81]. Thus, GPI anchor remodelling could contribute to apical sorting of GPI-APs modulating their affinity with different lipids and membrane domains. On the other hand, fatty acid remodelling was found to have an impact on the oligomerization of GPI-APs [82]. Specifically, the oligomerization of different GPI-APs was reduced in PGAP3-deficient cells [82], indicating that unremodelled GPI-APs are inefficient to cluster. Understanding how the presence of an unsaturated chain, and generally how lipid and glycan remodelling can affect the GPI-AP oligomerization ability are important issues to elucidate.

Overall, all these findings suggest that GPI anchor might contribute to the apical sorting of GPI-APs strengthening their partition into lipid microdomains, from one side, and favouring oligomerization from the other side.

### Role of post-translational modifications of the protein moiety

Once formed GPI-AP oligomers are insensitive to cholesterol depletion and are resistant to the extraction with different detergents, while they appear to be sensitive to urea treatment, indicating that GPI-AP oligomers are maintained by protein–protein interactions. In agreement with this, we have shown that oligomerization of the reporter GPI-AP GFP-FR is stabilized by disulphide bonds between protein ectodomains [23].

It is reasonable to speculate that in native GPI-APs, noncovalent interactions, possibly mediated by post-translational modifications, could be responsible of GPI-AP clustering. Glycosylation, which mediates weak interactions, might be a good candidate. While *N*- and *O*-glycans have been shown to act as apical determinants for transmembrane proteins [83–86], there are contrasting data regarding the role of glycosylation in the sorting of GPI-APs. *N*-glycans mediate the apical targeting of the native GPI-anchored membrane dipeptidase and CD59 in epithelial cell lines of different origin [79,87]. Consistently, the addition of N-linked glycans to the GPI-anchored form of rat growth hormone confers its apical targeting [88]. In contrast to these data, it has been shown that *N*-glycosylation is not required for the sorting of the GPI-anchored form of endolyn [89]. In addition, mutagenesis of *N*-glycosylation sites does not affect the oligomerization and apical sorting of the native PLAP and the chimeric protein NTR-PLAP bearing the ectodomain of $p75^{NTR}$ in MDCK cells [90]. Instead, the same mutant of PLAP does not oligomerize and is missorted in FRT cells [91].

The scenario is even more complicated by the finding that the partial remodelled form of CD59 (lysoGPI-anchored CD59), bearing only one long saturated acyl chain, is apically sorted independent of association with lipid rafts, but dependent on *N*-glycosylation in MDCK cells [79]. Most likely, lysoGPI-APs do not cluster and use a different apical route with respect to the fully remodelled counterpart, similar to what is shown for

**Table 1.** Role of *N*- and *O*- glycosylation in apical sorting of GPI-APs. N.D., not determined; n.a., not applicable because protein does not have *O*-glycosylation sites.

| GPI-AP | Cell type | *N*-glycans | *O*-Glycans | References |
|---|---|---|---|---|
| CEA | Colon (HT-29) | N.D. | Yes | [85] |
| CD59, lysoCD59 | Kidney (MDCK) | yes | N.D. | [79] |
| Dipetidase | Kidney (MDCK) | Yes | n.a. | [87] |
| | Colon (CaCo-2) | | | |
| Endolyn | Kidney (MDCK) | No | No | [89] |
| gGH (GPI anchored) | Kidney (MDCK) | Yes | n.a. | [88] |
| NTR-PLAP | Kidney (MDCK) | No | No | |
| PLAP | Kidney (MDCK) | No | n.a. | [90] |
| | Thyroid (FRT) | Yes | n.a. | [91] |

soluble form of PLAP and NTR-PLAP [90,92]. However, this has not been tested.

Based on these different data, we can infer that *N*-glycosylation might play a role in apical sorting of GPI-APs in dependence on the protein and/or on the cell type. Moreover, the findings that (a) the treatment with tunicamycin impairs PLAP oligomerization; and (b) in Con A-resistant MDCK cells, which are defective in high-mannose residues synthesis, newly synthesized GPI-APs arrive at the surface unclustered [73] are consistent with a general role of *N*-glycosylation in promoting GPI-AP clustering, possible through the involvement of a putative *N*-glycosylated receptor/factor.

Finally, there are few and opposite data on the role of *O*-glycosylation in the sorting of GPI-APs, whereby we cannot make any conclusions regarding this pathway: carcinoembryonic antigen (CEA) has been shown to accumulate intracellularly after pharmacological inhibition of *O*-glycosylation [85], while *O*-glycosylation mutant of NTR-PLAP oligomerizes and is apically sorted [90].

In conclusion, all data pointed out that the integrated action of protein moiety and glycolipid anchor mediate GPI-AP clustering in epithelial cells; however, this seems to be dependent on both the protein and cell type (Table 1); thus possibly linked to the presence/absence of other determinants.

## Role of additional factors

Besides the role of glycolipid anchor and protein moiety in oligomerization, additional factors could also promote and modulate, in spatial and temporal terms, the clustering of GPI-APs (Table 2). It is also unknown whether cytosolic proteins are required for the budding and formation of the apical GPI-AP–enriched vesicle.

Several proteins have been implicated in regulating the apical transport of raft-associated transmembrane and GPI-linked proteins (such as VIP17/MAL, caveolins, annexins, galectins), but they do not seem to be specific for GPI-APs and their mechanistic role is not completely clear [18,22,42,47,48,93]. Among them, galectin-4 could favour the segregation of apical rafts-associated proteins from not associated apical and basolateral cargoes by binding sulfatides with long chain-hydroxylated fatty acids and clustering glycosphingolipid platforms [44]. Although galectin-4 is not a specific factor for GPI-APs, it could help the coalescence of GPI-AP homoclusters and their subsequent incorporation in the apical GPI-AP–enriched vesicle providing the local energy at the boundary between order and disordered lipid phases. Belonging to the galectin family is an interesting candidate galectin-9, which binds to the Forsmann glycolipid, the more abundant lipid in fully polarized MDCK cells [94]. It has been shown that galectin-9 is implicated in the maintenance of apical-basal axis of MDCK cells [95,96]. Moreover, the knockdown of galectin-9 results in the intracellular accumulation of E-cadherin and mislocalization to the basolateral surface of the GPI-AP CEA [96], suggesting a possible specific role in GPI-AP clustering and apical transport.

Interestingly a recent study has shown that clathrin and AP1 are required for apical sorting of GPI-APs [72]. The knockdown of either clathrin or AP1 leads to the basolateral missorting of the GPI-AP CD59 in MDCK cells, while it does not affect the

**Table 2.** Role of cytosolic factors in apical sorting of GPI-APs.

| Cytosolic factor | CELL TYPE | GPI-AP | References |
|---|---|---|---|
| Galectin-4 | Colon (HT-29) | CEA | [44] |
| Galectin-9 | Kidney (MDCK) | CEA | |
| AP-1 | Kidney (MDCK) | CD59 | [72] |
| | | lysoCD59 | |
| Clathrin | Kidney (MDCK) | CD59 | [72] |
| | | lysoCD59 | |

apical and basolateral transport of transmembrane proteins [72]. However, whether the reduced expression of these two proteins affects the GPI-AP clustering remains to be checked. This finding opens many questions: how AP1 is specifically recruited in the GPI-AP–enriched domains? Is there a putative receptor for GPI-AP cargoes? Further studies will be necessary to address these questions.

## Role of the Golgi environment in clustering of GPI-APs

Besides the cooperation between the glycolipid anchor and the protein moiety in regulating the Golgi clustering of GPI-APs and their apical sorting, compelling evidence shows that the Golgi environment is also instrumental in regulating GPI-AP clustering. In this section, we will describe the contribution of lipids and actin.

### Role of lipids

The Golgi complex is enriched in a mix of lipids containing glycerol phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol), SPLs (e.g. ceramide, sphingomyelin, and glycosphingolipids) and cholesterol. Mammalian cells produce their own cholesterol whose cellular levels are finely regulated by three different routes: biosynthesis ex novo, cell efflux and influx. Because mammalian cells are devoid of the enzymes allowing breakdown of cholesterol, the efflux is critical for cell homeostasis [97]. Cholesterol efflux occurs continuously by desorption or by binding of lipoproteins to their receptor at the cell surface. Regarding the exogenous influx, cholesterol is carried in the bloodstream as lipoproteins that can be taken up through binding with specific receptors [e.g. low-density lipoprotein (LDL) receptor] *via* clathrin-mediated endocytosis. Regarding cholesterol biosynthesis, this lipid is synthesized in the ER and can be transported throughout cell *via* vesicular or nonvesicular pathways. The vesicular pathway is also involved in the retrograde transfer of cholesterol between the PM and the ER [98,99] and relies on protein transporters (named stereogenic acute regulatory-related lipid transfer protein) as STARD4, while the nonvesicular pathway between ER–Golgi would require proteins such as oxysterol-binding protein, VAPA and VAPB, ACAT, enriched at the ER–Golgi membrane contact sites [100–102]. Additionally, esterification and storing esterified cholesterol as lipid droplets concur to cellular cholesterol homeostasis [103,104]. The proper balance between free and esterified cholesterol is critical for the cells to buffer excess or lack of free cholesterol [103]. Interestingly, recent findings showing the plasticity of lipid droplets and their ability to interact with most organelles through membrane contact sites have transformed the static view of lipid droplets as simple lipid storage organelles to an essential hub of cellular metabolism [104].

Among cell compartments, the maintenance of proper cholesterol levels in the Golgi complex is critical considering its role in the Golgi export (Fig. 3). In polarized epithelial cells, cholesterol has an impact on the entire traffic exiting from the Golgi complex: upon cholesterol depletion apical transport is reduced in polarized MDCK cells [105,106], while exogenous addition of cholesterol blocked the VSV-G exit from the TGN in COS-1 cells [107]. Furthermore, cholesterol is required for the formation of both regulated and constitutive secretory vesicles in neuroendocrine cells [108], revealing the crucial role of this lipid in protein exocytosis. Regarding GPI-AP trafficking, it has been reported that in PGAP2/PGAP3 defective CHO cells, the unremodelled form of the GPI protein CD59, which does not associate with lipid domains, is transported to the cell surface with comparable kinetic to the remodelled protein in wild type CHO cells [80]. Interestingly, in the same study the authors showed that cholesterol depletion slows down Golgi to cell surface transport of both unremodelled and remodelled GPI-APs revealing an essential role of cholesterol in the Golgi exit of GPI-APs independently of their remodelling and association to membrane lipid microdomains [80]. This finding implies a possible role of cholesterol *per se* in promoting the formation of GPI-AP vesicle. On this line, cholesterol affects various physical properties of cellular membranes such as permeability, fluidity and thickness [109]. Interestingly, a recent study showed that cholesterol induces changes in the shape of liposomes from irregular, when composed only of dipalmitoylphosphatidylcholine (DPCC), to regular and spherical vesicles proportionally to the increase of cholesterol concentration [110]. Similarly, shape fluctuations of large unilamellar DPCC vesicles were observed after cholesterol addition [111]. All together these findings support a role for cholesterol in modulating the properties of lipid bilayer, beyond its possible role in promoting lipid segregation and membrane domains formation [112]. Thus, one can hypothesize that cholesterol might actively contribute by itself or together with other lipids, in different ways to the membrane curvature and formation of carriers.

In polarized epithelial cells, studies based on the manipulation of the cellular content of cholesterol

have revealed that it is instrumental for clustering of GPI-APs in the Golgi complex and their subsequent apical sorting [23,26]. Cholesterol depletion impairs the Golgi clustering of apical GPI-APs leading to their basolateral missorting in MDCK cells [23]. Similarly, reduction of cholesterol levels with lovastatin or methyl-β-cyclodextrin led to a significant increase of basolateral delivery of CEA in Caco-2 cells [113]. Conversely, exogenous addition of cholesterol is sufficient to induce clustering in the Golgi of the basolateral GFP-PrP, which is then redirected apically [26]. Cholesterol addition might modify the properties of Golgi lipid environment thus enabling clustering of GFP-PrP. Alternatively, the high levels of cholesterol could stabilize the association of the basolateral protein with lipid domains, therefore, allowing its clustering and apical sorting. According to its oligomeric state, GFP-PrP exhibited a lower diffusion coefficient, measured by FRAP, upon cholesterol addition with respect to control conditions [25]. Importantly, cholesterol depletion or addition does not modify the diffusional properties and the organization state of apical and basolateral transmembrane proteins indicating that cholesterol modulates specifically the membrane organization of GPI-APs [25].

There are still many open questions as to whether the Golgi content of cholesterol would lead to different remodelling of the GPI anchor, or could be critical for the biological activity of Golgi resident or recruited proteins, which in turn could regulate the GPI-AP clustering. Moreover, whether the proteins regulating the cholesterol content of Golgi membranes (e.g. OSBP, VAPA/VAPB etc.) might modulate GPI-AP clustering remains undetermined. Golgi vesiculation and dispersion of cis/medial and trans-Golgi markers upon exogenous cholesterol addition, respectively in HeLa and FRT cells was observed [91,114]. Similarly, removal of cholesterol led to partial fragmentation of Golgi complex in enterocytes [115]. Whether there is a correlation between cholesterol levels, Golgi structure and the clustering capacity of GPI-APs is unknown.

Besides cholesterol, the Golgi membranes are enriched in SPLs. Interestingly, SPL content varies largely between pre- and post-Golgi compartments with the former being poor and the latter more enriched [116]. Among these lipids, sphingomyelin is the more abundant species in the Golgi complex, where it is synthesized from the ceramide. Through the vesicular pathway ceramide is transported to cis-Golgi where it is converted in glucosylceramide, a substrate for the production of gangliosides. Alternatively ceramide can also be transported *via* a nonvesicular pathway to TGN relying on CERT (Cer-transfer protein) leading to the production of sphingomyelin and diacylglycerol [116]. Moreover, also glucosylceramide can use a nonvesicular pathway, *via* the glucosylceramide transfer protein FAPP2, to be delivered to the TGN, where it can be utilized for the formation globosphingolipids [117]. This complex scenario implies that the SPL metabolism and the proteins involved in its regulation might have an impact on the organization of Golgi membranes and for the dynamics of proteins associated with lipids. So far, several studies have highlighted the role of SPL in protein trafficking. The inhibition of glycosphingolipid synthesis by using a toxin derived from the fungus Fusarium moniliforme (FB1) leads to the basolateral missorting of the GPI-AP GP-2 in MDCK cells, while it does not alter the apical secretion of gp84 and the basolateral transport of E-cadherin [118]. The same treatment affects the apical delivery of PLAP and the chimeric GFP-NO-GPI in FRT cells [91]. Interestingly, two apical transmembrane proteins, $p75^{NTR}$ and DPPIV, were also basolaterally missorted upon FB1 treatment [91], indicating that in FRT cells SPLs might be required for the apical transport of both GPI-APs and transmembrane proteins.

Based on these findings, together with cholesterol, SPLs could concur to create a favourable environment that might promote clustering, segregation and apical sorting of GPI-APs. Moreover, changes in the local concentration of different SPL species could also influence the membrane curvature facilitating the budding of apical GPI-AP–enriched vesicle.

A recent study pointed out a novel role by which SPLs might control the trafficking Golgi-PM [71]. Deng and colleagues have shown that the inhibition of sphingomyelin synthesis (by acute depletion of sphingomyelin synthase 1 and 2 or incubation of the cells by the specific inhibitor D609) impairs the secretion of a subset of proteins whose exit from the Golgi complex is dependent on the Golgi resident calcium-binding protein Cab45 [71]. The authors proposed that sphingomyelin content might modulate the activity of the Golgi $Ca^{2+}/Mn^{2+}$ pump SPCA1 such that increasing the Ca-influx SPCA1 induces the activation of Cab45, and therefore their concerted action drives the export of the secretory proteins [71].

Considering that GPI-APs abound in cholesterol- and sphingolipid-enriched membrane domains, it will be interesting to explore whether SPCA1 and calcium might promote clustering and apical sorting of GPI-APs. Moreover, studying whether sphingomyelin and/or other sphingolipids could regulate the activity of this pump in polarized epithelial cells would be intriguing.

## Role of actin

Actin, one of the three cytoskeletal components, is involved in the regulation of different steps of protein trafficking. At PM, actin is crucial for the formation of endocytic vesicles as well as for docking and fusion of secretory vesicles. Several findings indicate that actin is important for the maintenance of the Golgi structure [119,120]. Indeed, it has been observed that the treatment with actin-depolymerizing (such as cytochalasin D, latunculin B) and actin-stabilizing (jasplakinolide) drugs leads to either swelling or vesiculation of the Golgi, respectively [121]. It seems that actin might help to establish the membrane tension along cisternae stacks that is essential for the maintenance of Golgi structure as its function. Moreover, actin might also modulate the membrane curvature of TGN membranes facilitating the vesicle budding. Recent findings suggest that contractility of actomyosin can also help the protein segregation [120,122], implying a role of protein regulating the actin dynamics in controlling protein sorting and trafficking. Interestingly, it has been shown that Rab6, myosin II and KIF20A are critical for the fission of the Rab6-positive transport carriers from the TGN membranes, highlighting an involvement of actin and microtubules in this process [123]. Miserey-Lenkei and colleagues propose that the coordinated action of actin and microtubules ensures the spatial organization of the fission events, which occur in limited regions of Golgi (Golgi hotspots), and exit along microtubules [123]. However, this mechanism seems to be not active for GPI-APs because Rab6 vesicle carriers are devoid of GPI-APs [122].

Several actin-binding proteins like ankyrin, spectrin, myosin, cortactin, cofilin, cdc42 have been localized at the Golgi complex. The small GTPase cdc42 has been shown to regulate the exit of both apical and basolateral transmembrane proteins in MDCK cells [49,124]. While the overexpression of a dominant negative cdc42 mutant accelerated the exit from the TGN of the apical transmembrane p75-GFP, the exit of the basolateral LDL receptor is drastically delayed [49,124]. These defects in protein trafficking are correlated with the disappearance of actin perinuclear filaments, indicating that the Golgi pool of actin might be involved in the polarized trafficking [49,124]. Nonetheless, it remains understood whether actin filaments play a role in cargo selection or in membrane budding/fission in polarized cells.

Cortactin, which is recruited to the Golgi *via* the actin regulator ADP-ribosylation factor ARF1 and interacts with dynamin-2, also seems to be involved in the regulation of basolateral transport. Specifically, the inhibition of cortactin–dynamin2 interaction leads to a significant accumulation of VSV-G in the TGN of BHK-21 cells [125], implying a role of this complex in the exit from the Golgi. However, whether this complex regulates actin dynamics at the Golgi complex remains to be checked. On the other hand, VSV-G export from the Golgi is also promoted by Dynamin2/syndapin II complex in Hepg2 cells [126]. Based on these data, it is plausible that the two different complexes would regulate protein sorting from the Golgi differently depending on the cell type and cargoes.

So far, some actin-binding proteins have been found to be involved in the regulation of apical trafficking. LIM Kinase1 and cofilin mediate specifically the delivery of apical transmembrane proteins (e.g. p75-GFP), but not that of apical GPI-APs or basolateral proteins in MDCK cells [127]. The apical transport of the transmembrane raft-associated haemagglutinin (HA) relies on the activity of the phosphatidylinositol 5-kinase (PI5K) that promotes the formation of Arp2/3-dependent actin comets [128].

All these studies clearly indicate that actin cytoskeleton plays a critical role for basolateral and apical transport of transmebrane proteins, but not for GPI-APs. This is also consistent with a pioneering study showing that actin-stabilizing/depolymerizing agents inhibit the exit of apical and basolateral proteins (p75 and VSV-G) without affecting the exit of GPI-APs in Cos-7 cells [129]. Interestingly, FRAP experiments have shown that in unpolarized MDCK cells latrunculin A decreases the apparent diffusion coefficient of transmembrane $p75^{NTR}$, but not that of GPI-AP GFP-FR [25]. The scenario is completely different in fully polarized MDCK cells where latrunculin A affects the mobility of all apical proteins, but does not have any effect for basolateral proteins [25]. These findings provide important evidence that the sorting machinery at the Golgi level changes during establishment of cell polarity and that in the Golgi complex of polarized cells apical and basolateral proteins do not share a common membrane environment. Of interest, treatment with latrunculin A does not affect the homoclustering of GPI-APs at the Golgi nor their apical sorting (S. Lebreton, S. Paladino & C. Zurzolo, unpublished data). The apparent contrasting data indicate that the formation of GPI-AP homoclusters is independent on actin, but actin might modulate their local diffusion.

Overall, these findings highlight that a specialized population of actin filaments at the Golgi complex controls the export from this organelle. Actin-binding proteins that can change, in time and in space, the

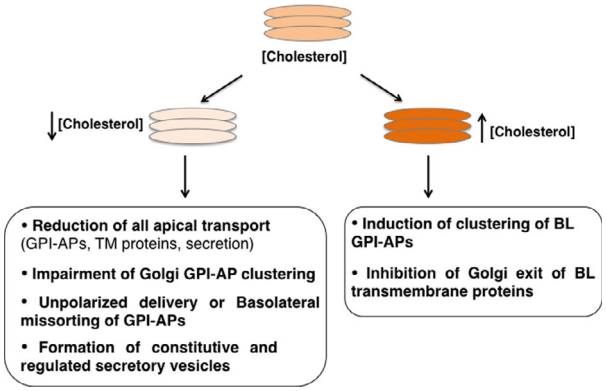


**Fig. 3.** Cholesterol content of the Golgi complex regulates multiples pathways of protein trafficking. The effects caused by cholesterol depletion or addition are summarized in the scheme.

organization of these filaments, will play a key role. It is clear that the involvement of one or other actin regulators might depend on the cargo and on the cell type, but their mechanistic role remains to be understood.

## Conclusions and perspectives

The data discussed in this review highlight that:

- Glycosylphosphatidylinositol-anchored proteins are a family of proteins very peculiar in terms of sorting, trafficking and organization due to the presence of both a protein moiety and a glycolipid anchor. Both portions undergo different modifications when the proteins travel along the secretory pathway and therefore protein and lipid modifiers might contribute to the regulation of their trafficking.
- The mechanism of GPI-AP clustering in the Golgi appears to be highly complex supporting a role for cholesterol.
- The PM organization of GPI-APs is dependent on their Golgi homoclustering that occurs only when cells are fully polarized. This clustered organization is crucial for the biological activities of GPI-APs and this is physiologically relevant in case of loss of polarity.

Although we have now many piece of the puzzle, further studies are required to elucidate how the composition of Golgi membranes might modulate the protein and lipid interactions leading to GPI-AP clustering and apical sorting as, if any, the relationship between cholesterol and SPLs in the formation of GPI-AP enriched vesicle. Moreover, if Golgi luminal factors, as for example the content of calcium ions similarly to secretory cargoes, might regulate GPI-AP sorting and trafficking in epithelial cells remains to be determined. Finally, understanding how the molecular machinery regulating Golgi GPI-AP clustering is activated during the polarization of epithelia remains an important and unresolved challenge.

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