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Minireview

Sphingolipid trafficking and protein sorting in epithelial cells

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Abstract Sphingolipids represent a minor, but highly dynamic subclass of lipids in all eukaryotic cells. They are involved in functions that range from structural protection to signal transduction and protein sorting, and participate in lipid raft assembly. In polarized epithelial cells, which display an asymmetric apical and basolateral membrane surface, rafts have been proposed as a sorting principle for apical resident proteins, following their biosynthesis. However, raft-mediated trafficking is ubiquitous in cells. Also, sphingolipids per se, which are strongly enriched in the apical domain, are subject to sorting in polarity development. Next to the *trans* Golgi network, a subapical compartment called SAC or common endosome appears instrumental in regulating these sorting events. © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Sphingolipid; Subapical compartment; Cell polarity; Sorting; Trafficking

1. Introduction

To meet functional requirements, the epithelial cell surface is polarized, consisting of an apical domain, facing the lumen, and a basolateral domain, which faces adjacent cells and connective tissue. Tight junctions preclude molecular randomization between the outer, but not inner, leaflets of these membrane domains. A key issue in polarity involves the question as to how these domains are generated and, in spite of endocytic and exocytic membrane dynamics, maintained. Solving this question involves elucidation of fundamental cell biological principles of mechanisms of protein and lipid sorting, and transport to specific membrane (micro)domains. Liver cells are particularly challenging in this regard, since it has long been assumed that rather than via a direct pathway from *trans* Golgi network (TGN) to apical surface as seen in most epithelial cells, apical resident proteins in liver cells travel by transcytosis via the basolateral surface. Furthermore, microdomains, so-called 'lipid rafts', have been recognized in epithelial cells as potential sorting platform for direct apical membrane targeting. Such rafts are ubiquitously present in cellular membranes, fulfilling functions that include transmembrane signaling, thus placing their interest in a much broader context. Here, some recent progress in understanding membrane polarity development in the context of lipid and protein trafficking and sorting is discussed.

2. Lipids, microdomains and membrane polarity

Plasma membranes of all eukaryotic cells consist of a patchwork of dynamic, in terms of activity and lifetime, membrane domains (ranging from 70 nm to ~1 μm) in which raft microdomains, representing liquid-ordered sphingolipid/cholesterol-enriched phases, coexist with more fluid domains [1]. The actin-based cytoskeletal meshwork contributes to such membrane compartmentalization, creating diffusion-restricted membrane domains via anchorage of transmembrane proteins [2]. Sphingolipids and cholesterol are key players in raft microdomain formation, which are characterized by resistance to detergent extraction in the cold. A critical sphingomyelin (SM)/cholesterol ratio and cholesterol concentration per se are essential, in contrast to the presence of glucosylceramide (GlcCer) [3]. Thus, cholesterol depletion inhibits signaling events by dispersing domains, cholesterol replenishment reactivating this function [4]. In trafficking, cholesterol depletion causes missorting of apical proteins [5]. Glycosphingolipids may distribute differently over rafts [6,7], reflecting variability in individual raft composition, which in turn may determine detergent-dependent differences in solubility, as is also seen upon cholesterol depletion [8]. Protein interactions very likely contribute to domain organization, and may induce growth or coalescence of certain types of lipid domains. In this manner an efficient means would be provided of transporting preassembled signaling complexes to specific membrane areas upon stimulation of polarity development. Underlying cross-talk mechanisms remain to be clarified.

Microdomain formation and lateral assembly as an important membrane polarity organizing principle are also revealed in neutrophil front–rear plasma membrane polarity development upon directed cell migration. The process involves a reorganization of microdomains, dictated by cytoskeletal-associated transmembrane proteins like CD44/CD43 [9], as seems to also occur in domain organization at the basolateral surface of epithelial cells [10]. In migrating T-cells, GM3- and GM1-enriched raft domains segregate into leading edge and uropod, respectively, which requires an intact actin cytoskeleton [7]. Indeed, the correct redistribution appears mediated

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Abbreviations: CE, common endosome; GalCer, galactosylceramide; GlcCer, glucosylceramide; PKA, protein kinase A; SM, sphingomyelin; SAC, subapical compartment; TGN, *trans* Golgi network

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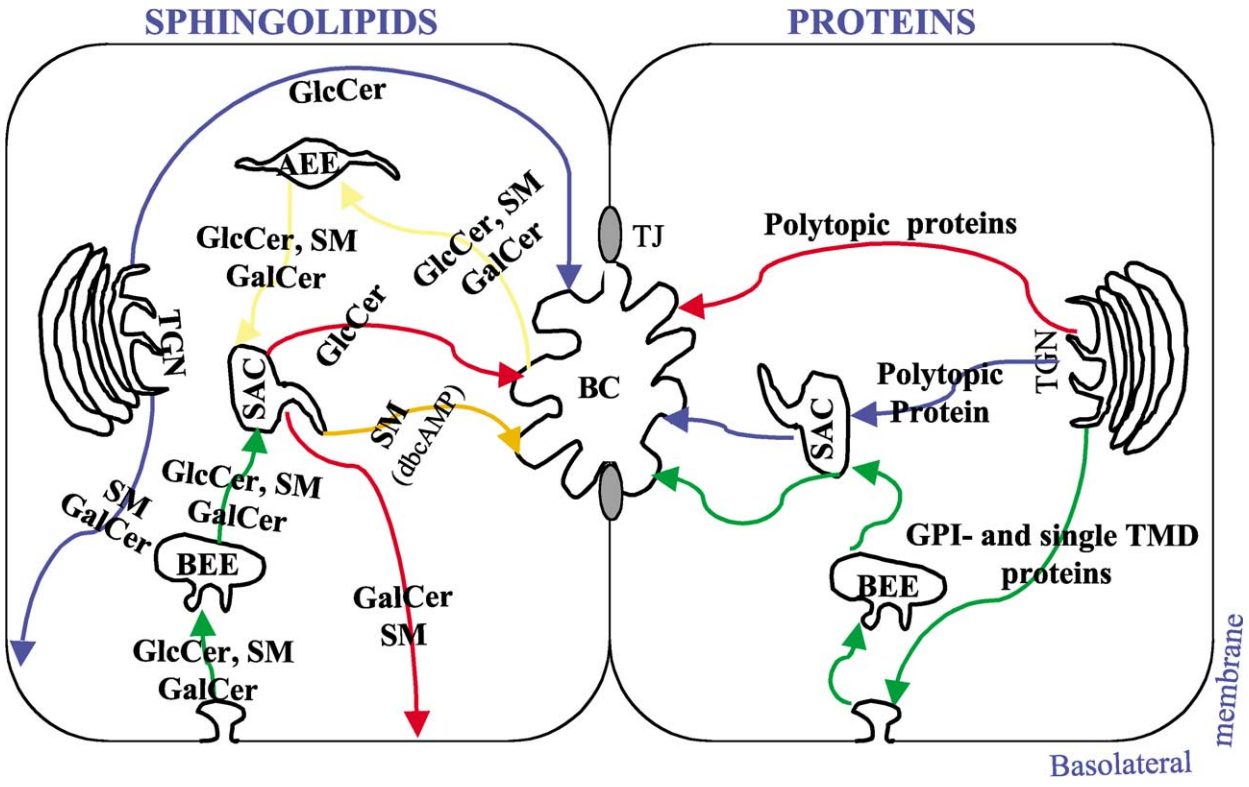

Fig. 1. Sphingolipid and protein transport pathways in polarized hepatocytes. (Left) Transport pathways for polarized trafficking of sphingolipids. Newly synthesized GlcCer, GalCer and SM are delivered directly from the TGN to the apical (bile canalicular) and the basolateral plasma membrane, respectively (blue arrows). Upon internalization, as visualized by monitoring the flow of fluorescent derivatives, sphingolipids are delivered to distinct AEE (yellow arrows) or BEE (green arrows) and transported to the SAC in which sorting and segregation of internalized sphingolipids occurs. GlcCer is delivered to the BC, whereas SM and GalCer are preferentially transported to the basolateral surface (red arrows). Note that when cells have been treated with dbcAMP, SM is redirected from the SAC to the BC (brown arrow; see text for details). (Right) Transport pathways for polarized trafficking of apical proteins. All GPI and single TMD proteins so far studied have been shown to follow the transcytotic pathway, via SAC (green arrow). Recently, a direct route followed by polytopic plasma membrane proteins (e.g. the ABC transporter, MDR1 and a copper transporter, ATP7B; red arrow) has been described. Another member of the ABC transporter family (Spgp) has been shown to accumulate in the SAC before direct delivery to the BC (blue arrow). AEE: apical early endosome, BBE: basolateral early endosome, GPI: glycosylphosphatidylinositol, SAC: subapical compartment, Spgp: sister of p glycoprotein, TJ: tight junction, TMD: transmembrane domain.

by CD43, CD44 and other adhesion receptors that favor linkage of the uropod to the cytoskeleton. Rho-GTPases, such as Cdc42, are prominently present in the leading edge, and yeast cells deficient in Cdc42 are unable to properly organize the actin cytoskeleton. Its activation appears relevant to establishing and maintaining cell polarity [11,12]. The outer leaflet of the apical membrane consists largely of glycosphingolipids and cholesterol, whereas SM is distributed equally over apical and basolateral domains. Consistently, raft-mediated trafficking constitutes a direct pathway for transfer of apical constituents from TGN to their apical destination [13]. However, from recent work in liver cells it becomes apparent that rafts reflect a specific structural organization, rather than that their biophysical properties per se imply an apical sorting signal.

3. Sphingolipid sorting and trafficking: role of microdomains

Ceramide, the sphingolipid precursor, is synthesized at the endoplasmic reticulum and reaches the Golgi by vesicular and non-vesicular mechanisms [14,15]. Here, SM and GlcCer are synthesized, the latter also serving as precursor for complex glycosphingolipids. The TGN is instrumental in their initial segregation and vesicular-mediated transport to apical and basolateral domains (Fig. 1).

Sphingolipids may display a species-dependent asymmetric distribution in the lateral plane of the bilayer [6,7,16], species-dependent sorting [17], possibly relying in part on interdigitation. Accordingly, different rafts may exist in a single membrane. A reflection of this asymmetry may be the fact that distinct sphingolipids are retrieved and/or distinctly processed (i.e. sorted) at a variety of intracellular sites, including the plasma membrane [18–20], endocytic compartments [21,22], the subapical compartment (SAC) ([23]; see below), and TGN [24]. Most of these studies were carried out with fluorescently tagged analogs, but glycolipid-bound toxins were shown to follow similar paths, although a quantitative comparison with the natural lipids remains to be determined. Whether the observed sorting relies on their assembly into particular microdomains is unclear. However, excess or depletion of cellular cholesterol redirects trafficking of plasma membrane-localized sphingolipids [25] from endo-/lysosome to Golgi, respectively. Interestingly, in polarized oligodendrocytes, overexpression of a major myelin protein PLP caused its accumulation in endo-/lysosomes and a concomitant sequestration of cholesterol [26]. In addition, fluorescently tagged lactosylceramide and galactosylceramide (GalCer), which are normally sorted to the Golgi, also accumulated at those conditions in the endo-/lysosomal system. Although

cause and consequence remain to be determined, a regulating role of cholesterol in lipid (and protein) sorting appears apparent, membrane thickness, tension and curvature being relevant parameters (cf. [16]). Differences in domain composition may cause differences in lipid–lipid interaction, clustering prompting differences in intracellular targeting. Also, relative fluidity, the principle of which also applies to cell membranes, may affect the efficiency of sorting [16,22,27]. In addition, sterol-binding proteins (e.g. oxysterol-binding protein; synaptophysin) or other, trafficking regulating proteins (e.g. caveolins, Rab proteins) may be affected upon perturbation of cholesterol homeostasis (see [16,25,28]). Finally, depletion of cholesterol, possibly interfering with membrane curvature, may frustrate exo- and endocytic vesiculation in some [29–31], but not in all cases [5,20], a feature obviously affecting trafficking.

In light of connections between cytoskeleton and polarity development as noted above, expression of dominant-active Rho-GTPase Cdc42v12 downregulates transcytosis of the poly(Ig) receptor to the apical domain in MDCK cells, while proteins normally recycling to the basolateral membrane following endocytosis appear on the apical surface [32]. In this regard, protein kinase C activation also causes membrane depolarization in liver cells [33], whereas protein kinase A (PKA) activation stimulates overall apical membrane-directed flow. This could imply a stimulation of a vesiculation event, rather than an enhanced efficiency of sorting into vesicular carriers. Canalicular membrane-directed transport may be controlled by intracellular signaling [34]. More recent work in our laboratory connects a signaling event at the basolateral membrane, mediated by cytokines of the interleukin-6 family, with apical membrane biogenesis, involving activation of a SM-marked transport pathway originating from SAC (v.d. Wouden et al., unpublished observations).

4. The SAC: a traffic center in polarized cells

A tubulovesicular compartment, visualized by electron and fluorescence microscopy, defined as common endosome (CE, [35]) or SAC ([23]) appears important in the intracellular pathway and mechanism of membrane and lipid flow in polarized cells. In this compartment, which functionally mimics the recycling endosome in non-epithelia [23,36], endocytic transport originating from apical and basolateral membrane surface merges. Via this compartment GlcCer recycles to the apical membrane in HepG2 cells [37] (Fig. 1) and, in a Rab17-dependent and microtubule-independent manner [38,39], the transferrin receptor recycles via SAC to the basolateral membrane [40,41]. Also, SAC appears instrumental in transcytotic vesicular transport of phosphatidylcholine for biliary secretion and recycling [42].

For simple sphingolipids like GlcCer, GalCer and SM, sorting occurs in SAC in the transcytotic pathway [37], rather than at the plasma membrane [24,36]. Thus, within the luminal leaflet of this compartment, SM and GalCer on the one hand, and GlcCer on the other are sorted into transport vesicles, at steady state being directed towards the basolateral and apical membrane surface, respectively (Fig. 1). Also in enterocytes, transcytosis of GalCer from the apical to basolateral membrane occurs, where HIV to travel across the intestinal epithelium exploits the lipid [43]. Intriguingly, during HepG2 cell polarity development, a microtubule-dependent apical-directed pathway is triggered, which is marked by SM transport, implying a reversed transport direction of this lipid compared to steady state. This apical pathway differs from the vesicle-mediated apical recycling pathway of GlcCer [44], and appears regulated by a transient activation of intrinsic PKA (Fig. 1).

It is tempting to suggest that in SAC GlcCer partitions in cholesterol-poor domains, which could favor its recycling to the apical membrane [25]. In developing neurons, an upregulation of SM synthesis directs random trafficking of GM1 to an axonal (apical) localization, concomitant with raft formation [45]. Whether rerouting of SM in HepG2 to the apical domain underlies a similar raft-recruiting principle remains to be established. It has been noted that cholesterol is highly enriched in the CE [46]. Also, in small intestine enterocytes, the apical membrane protein aminopeptidase N becomes raft-associated according to kinetics, following pulse labeling, that would be consistent with recruitment in a SAC [47]. As noted, to potentially regulate these pathways, a set of Rab proteins has been identified, including Rab11, 17 and 25. Our preliminary data suggest that Rab11 is operating distally in SAC, reflecting functional sub-compartmentalization [23,39,42] via which SM and the pIgR/dIgA complex travel specifically to the apical membrane.

Finally, next to transcytosis, and like in other epithelial cells, also in liver cells a direct sphingolipid transport pathway exists between TGN and apical membrane [33,34].

5. Sorting and transport of proteins to apical and basolateral membrane domains

5.1. Basolateral sorting

Basolateral targeting of proteins is mediated by short amino acid sequences located in their cytoplasmic tails. Sequences involve a critical tyrosine residue within a consensus sequence NPXY or YXXΦ, where Φ is a bulky hydrophobic residue and X is any amino acid. Most of these tyrosine-based signals are colinear with rapid endocytosis signals [48]. Less frequent signals include Leu-Leu and di-hydrophobic motifs [49,50]. Also, basolateral signals with no apparent consensus sequence have been described [51,52].

The functional diversity of tyrosine-based and dileucine-based sorting signals suggests that a family of receptors is able to recognize and interact with these signals in order to specify protein sorting to the basolateral membrane. The similarity between some basolateral and endocytic signals suggests that basolateral sorting may use a similar clathrin-based mechanism involving adaptor protein (AP) complexes. Four adaptor complexes, AP-1, AP-2, AP-3 and AP-4, have been identified [53,54]. AP-2 is used for endocytosis at the plasma membrane, while AP-1, AP-3 and AP-4 mediate sorting from the TGN to endosomes. Tyrosine-based motifs bind specifically to the medium (μ) chains of adaptor complexes [55,56]. At least in vitro, dileucine signals interact with β rather than μ subunits [57]. Involvement of the AP-1 adaptor complex in basolateral sorting of the pIgR, prior to transcytosis, is supported by coimmunoprecipitation experiments [58]. Furthermore, a novel isoform of μ1 (AP-1), μ1B, has been identified as an epithelial cell-specific AP required for basolateral sorting [59,60]. Polarized LLC-PK1, lacking μ1B, missorts many basolateral proteins (LDL and TfR) to the apical surface [60,61]. The correct basolateral sorting is restored following

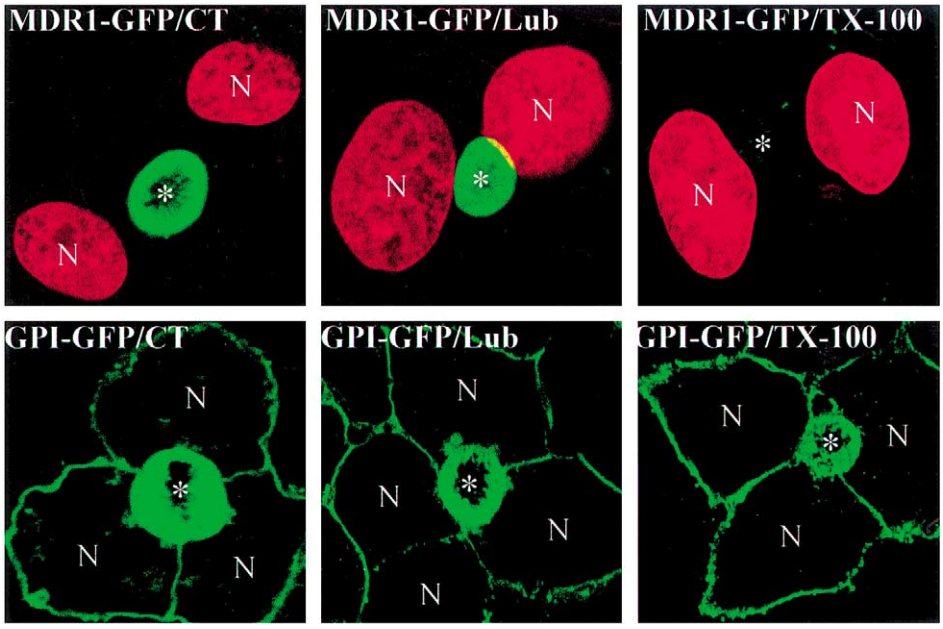

Fig. 2. Confocal microscopic examination of the expression of MDR1-GFP and GPI-GFP constructs in stably transfected polarized HepG2 cells. Note that MDR1-GFP, which uses the direct pathway from the TGN to the BC (asterisk), is restricted to the apical membrane, whereas GPI-GFP which travels along the transcytotic pathway is detected at both the BC and basolateral domains. Analysis of microdomain association of these constructs by in situ extraction of HepG2 cells with detergents shows that MDR1-GFP is resistant to Lubrol WX extraction, but is efficiently removed from the BC after TX-100 extraction. In contrast, GPI-GFP is resistant to both treatments, indicating its association with both lubrol WX and TX-100 microdomains. CT: control; N: nuclei stained with propidium iodide.

transfection of μ1B into LLC-PK1 cells. Interestingly, μ1A and μ1B localize to different subdomains of the TGN, where μ1B acts to sort basolateral proteins from those selected by μ1A for transport to endosomes and lysosomes [62]. Whether the AP-1 complex is able to bind all types of basolateral signals, and the uniqueness of this mechanism for basolateral sorting, remains to be determined. For instance, in LLC-PK1 cells, μ1B is not required for the sorting of two basolateral membrane proteins which contain a di-leucine type targeting signal ([61]; V. Bello and M. Maurice, personal communication). This leaves open the question of how basolateral proteins with di-leucine motifs are sorted in the TGN. Moreover, the fact that both hepatocytes and neurons, which do not express μ1B [59], can transport proteins to their sinusoidal and somatodendritic surfaces, respectively, strongly suggests that other μ1B-independent mechanisms for basolateral sorting exist. Yet, in liver cells, it is not clear whether sorting of basolateral proteins is signal-mediated or occurs by default, nor whether hepatocytes use the same signals as in other epithelial cells. Recently, it was reported that a basolateral signal is required for basolateral targeting of the LDL receptor, a mutant receptor being mistargeted to the bile canalicular surface [63].

Transport vesicles carry sorted proteins to the basolateral surface and the involvement of p200/myosin II in the budding of basolateral transport vesicles has been suggested [64]. However, there appears to be no consensus since p200 is not required for formation of TGN-derived vesicles containing the basolateral marker, VSV-G [65]. Recent reports have implicated the small Rho-GTPase Cdc42 in the generation of basolateral transport vesicles [12]. The mechanism of how these proteins mediate vesicle formation remains to be elucidated.

5.2. Apical sorting signals

Apical sorting signals have been identified throughout the sequence of apical resident proteins. In contrast to basolateral sorting, apical sorting is likely mediated by more than one mechanism. In the majority of epithelial cells studied, it has been shown that all GPI-anchored proteins are sorted to the apical surface [3]. Furthermore, the transmembrane domains of viral [66] and polytopic membrane proteins [67] also mediate apical targeting, although apical sorting information has also been identified in the cytoplasmic tail of two polytopic proteins [68,69]. N- and O-glycosylation of the ectodomain also appears to play a role in apical sorting [70]. However, glycosylation does not constitute a universal apical sorting signal, as several non-glycosylated proteins are also targeted to the apical domain [71,72]. Models have been proposed [70] in which N- and O-glycans, by changing the conformation of proteins, would allow a functional proteinaceous signal to become accessible. A mechanism involving recognition of glycans by sorting lectins has also been postulated, but the evidence is scanty.

5.3. Apical sorting and lipid microdomains

Apical sorting has been proposed to involve partitioning of apical proteins into lipid microdomains that form in the inner leaflet of the Golgi [73]. There have been several reports that the presence of GPI-anchored and some transmembrane proteins in these microdomains correlates with their direct delivery to the apical surface. However, such a mechanism poses a potential exception to the hepatocytes, which are distinguished from other epithelial cells by the fact that they transport both GPI-anchored and single transmembrane proteins via the transcytotic pathway [74]. This raises the question whether and if so when and where lipid rafts are involved

in apical sorting in these cells, particularly since in polarized HepG2 cells newly synthesized sphingolipids can be delivered directly from the TGN to the apical surface [33] (Fig. 1). Indeed, a direct route has recently been described for polytopic plasma membrane proteins ([75,76], our unpublished results) (Fig. 1). More interestingly, we have obtained evidence that lipid rafts are implicated in apical sorting in HepG2 cells, and they are operating in both the direct and indirect routes (unpublished results). These results emphasize the ubiquitous presence and functioning of rafts in cells, the need to differentiate between distinct lipid microdomains [77,78], and the role of raft 'cargo' as co-determinant(s) in sorting. Indeed, following extraction of HepG2 cells with either Triton X-100 (TX-100) or lubrol WX, we found that polytopic proteins which travel along the direct pathway are preferentially incorporated into lubrol-insoluble but TX-100-soluble microdomains, whereas GPI-anchored and single transmembrane proteins which traffic along the transcytotic pathway were retained into microdomains, resistant to both TX-100 and lubrol WX (Fig. 2). Based on their common detergent-resistance solubilization properties (lubrol WX-resistance) ([78,79], our unpublished results), it is tempting to speculate that polytopic plasma membrane proteins may prefer a similar structural environment.

Several proteins have been proposed to function in the apical sorting machinery, including cholesterol-binding caveolin-1. Moreover, large caveolin-1 homooligomers are targeted to the apical surface and play a role in apical transport of HA in MDCK cells [80]. MAL/VIP17 has also been implicated in apical transport and downregulation of MAL expression specifically inhibited transport to the apical surface [81,82]. Furthermore, antibodies specific for annexin XIIIb significantly inhibited the transport of apical proteins without affecting basolateral transport [83]. Finally, also microtubule motors that transport TGN-derived vesicles to the apical surface may well be involved [84,85]. We submit that the complexity of these various pathways and their distinct regulation in liver cells, compared to other epithelial cells, will reveal novel mechanisms involved in the generation and maintenance of membrane polarity.

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