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REVIEW ARTICLE

Mechanisms and functional features of polarized membrane traffic in epithelial and hepatic cells

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Epithelial cells express plasma-membrane polarity in order to meet functional requirements that are imposed by their interaction with different extracellular environments. Thus apical and basolateral membrane domains are distinguished that are separated by tight junctions in order to maintain the specific lipid and protein composition of each domain. In hepatic cells, the plasma membrane is also polarized, containing a sinusoidal (basolateral) and a bile canalicular (apical)-membrane domain. Relevant to the biogenesis of these domains are issues concerning sorting, (co-)transport and regulation of transport of domain-specific membrane components. In epithelial cells, specific proteins and lipids, destined for the apical membrane, are sorted in the *trans*-Golgi network (TGN), which involves their sequestration into cholesterol/sphingolipid 'rafts', followed by 'direct' transport to the apical membrane. In hepatic cells, a direct apical transport pathway also exists, as revealed by transport of sphingolipids from TGN to the apical membrane. This is remarkable, since in these cells numerous apical membrane proteins are 'indirectly' sorted, i.e. they are first transferred to the basolateral membrane prior to their subsequent transcytosis to the apical membrane. This raises intriguing questions as to the existence of specific lipid rafts in hepatocytes. As demonstrated in studies with HepG2 cells, it has become evident that, in hepatic cells, apical transport pathways can be regulated by protein kinase activity, which in turn modulates cell polarity. Finally, an important physiological function of hepatic cells is their involvement in intracellular transport and secretion of bile-specific lipids. Mechanisms of these transport processes, including the role of multidrug-resistant proteins in lipid translocation, will be discussed in the context of intracellular vesicular transport. Taken together, hepatic cell systems provide an important asset to studies aimed at elucidating mechanisms of sorting and trafficking of lipids (and proteins) in polarized cells in general.

INTRODUCTION

A primary function of epithelial cells is to provide a boundary between different extracellular compartments. As a consequence, these cells exhibit cell-surface polarity, which means that their plasma membrane is divided into specialized regions that are exposed to different environments and which have a characteristic protein and lipid composition. The basolateral plasma-membrane domain faces the blood circulation and adjacent cells, whereas the apical domain is in contact with the external environment, such as the bile canaliculus in hepatocytes or the lumen in renal and intestinal epithelial cells. The apical and basolateral plasma-membrane domains are separated by tight junctions, thus preventing intermixing of membrane components [1]. To generate and maintain this structural polarity, cells need mechanisms to specifically target newly synthesized proteins and lipids to the correct surface. Revealing the mechanisms and regulation of these polarized transport processes is a major challenge in current cell biology.

In contrast with our knowledge of polarized protein transport, which is fairly detailed, the understanding of lipid transport is limited. This is partly due to difficulties in methodology, as many of the techniques commonly used for studying protein transport cannot be easily applied to the study of lipid transport. These procedures include genetic, biochemical and immunological techniques that are used to identify the involvement of distinct proteins in trafficking and to study structural requirements that are involved in protein targeting, i.e., the distinct processing of apical or basolateral membrane proteins to their specific domain.

To obtain a detailed understanding of polarized membrane trafficking, it is important to reveal how transport of proteins and lipids is regulated. Obviously, vesicular transport of proteins is accompanied by lipid flow, but since apical and basolateral plasma-membrane domains differ with respect to both protein composition and lipid composition, sorting mechanisms for proteins as well as for lipids must exist. Here, the mechanisms of plasma-membrane-directed transport are discussed. We will focus on how transport of proteins and lipids is related. For simple epithelial cells, well-characterized model systems are available, and therefore most of our current knowledge is derived from this cell type. For hepatic cells, knowledge is fairly scanty. These cells excrete large amounts of lipid on their apical side and therefore must have an active lipid-transport mechanism to substitute for these lipids. Although hepatocytes share various aspects of polarized membrane traffic with epithelial cells, differences are also apparent. For example, apical protein sorting in the *trans*-Golgi network (TGN), an important sorting mechanism for epithelial cells, seems to be largely absent in hepatic cells. Hence, depending on cell type, carefully controlled molecular sorting and membrane flow occur in either a direct biosynthetic pathway

Abbreviations used: BFA, brefeldin A; C_6-NBD, 6-[*N*-(7-nitro-2-benzoxa-1,3-diazol-4-yl)amino]hexanoyl; Cer, ceramide; ER, endoplasmic reticulum; GlcCer, glucosylceramide; GPI, glycosylphosphatidylinositol; MDCK, Madin–Darby canine kidney cells; mdr/MDR, multidrug-resistant; MRP, MDR-related protein; PC, phosphatidylcholine; PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholinopropan-1-ol; PKA, protein kinase A; PKC, protein kinase C; SM, sphingomyelin; TGN, *trans*-Golgi network; PITP, phosphatidylinositol-transfer protein; VSV-G, vesicular-stomatitis-virus glycoprotein.

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between the TGN and apical membrane, or in an indirect, i.e., transcytotic pathway between basolateral and apical membrane. Several aspects of these pathways will be described and discussed in the following sections. Where appropriate to membrane biogenesis in general, reference will also be made to the trafficking of proteins and lipids in non-polarized cells.

POLARIZED CELL MODELS FOR EPITHELIAL AND HEPATIC CELLS

Morphologically, epithelial and hepatic cells differ, as shown in Figure 1. The apical domain of epithelial cells forms a continuous plane at the cell apex. By contrast, in hepatocytes *in vivo*, the apical domain, or bile canaliculus, forms a continuous network between adjacent cells that drains the apically excreted bile. In *in vitro* systems, the bile canaliculus is a closed vacuolar compartment in between two neighbouring cells. In both polarized cell types, tight junctions (TJ) prevent intermixing of apical and basolateral membrane components.

Although the morphological appearance and physiological functions of epithelial and hepatic polarized cells are different, the general organization of the apical plasma-membrane domain in both cell types is closely related. Thus common features include the apical enrichment of glycosphingolipids and glycosylphosphatidylinositol (GPI)-linked proteins. Moreover, the apical plasma membrane is abundantly covered by microvilli and is underlined by an actin microfilament meshwork [2,3]. However, although the mechanisms for polarized protein targeting are basically similar in all polarized cells, and are probably even preserved in cells that do not display cell-surface polarity [4,5], the relative importance of the targeting pathways differs strongly and depends on the cell type.

Studies on polarized membrane transport have largely relied on model systems. A widely adopted model system is the Madin–Darby canine kidney (MDCK) cell line, which, when grown on permeable filters, forms a well-polarized monolayer that can be manipulated from both the apical and the basolateral side. Caco-2 cells are used similarly as a model for polarized intestinal cells.

In hepatocytes, the canalicular membrane domain, which corresponds with the apical membrane in epithelial cells, is enclosed by adjacent cells and serves as the bile secretory pole. The sinusoidal and lateral domains are the hepatic homologues of the basolateral surface of epithelial cells and face the blood and neighbouring cells respectively. Studies on the mechanisms of polarized transport in hepatic cells have been hampered by the lack of suitable model systems. Traditionally, perfused livers have been used to study the excretion of biliary components into the bile. However, this model precludes analysis of transport of those apical membrane components that are not excreted. Transport of proteins to the apical surface has been investigated in intact liver, using pulse–chase labelling with radioactive precursors and subcellular fractionation to purify basolateral and apical membrane vesicles. Using this approach, kinetic analysis revealed that apical proteins reach the apical surface by trafficking via the basolateral surface [6–8]. However, the intact liver as a model system has limitations, as trafficking cannot be followed in real time. Neither does the model allow the analysis of intracellular transport events.

Freshly isolated hepatocytes can be used as an *in vitro* cell-culture model. After isolation, the cells rapidly lose their cell-surface polarity, but they repolarize in several days when cultured between two layers of an extracellular or collagen matrix [9,10]. These repolarized cells have been used to study the endocytic and transcytotic trafficking of several membrane proteins [11]. Alternatively, hepatocytes may be isolated as pairs, or 'couplets', by limited collagenase perfusion. These couplets enclose a bile canalicular space, which reorganizes as an excretory apical pole within 3–4 h after isolation [12,13]. In hepatocyte couplets, canalicular bile secretion and apical lipid transport [14–16] have been successfully studied. Although this model system has proved to be quite useful, it suffers from some disadvantages that are inherent to the use of primary cells. These disadvantages include variations between different isolations, and the fact that couplets can only be used for short-term experiments. Also, the re-formation of the apical pole in hepatocytes that have been isolated as couplets occurs partly via targeting of apical-membrane remnants from other regions of the cell membrane to the single remaining bile canaliculus of the couplet [13,17], which could lead to an over-estimation of the importance of the transcytotic targeting route (see below).

Some cell lines have been reported to show hepatocyte-like polarity. The WIF12-1 cell line was obtained by fusion of rat hepatoma with human fibroblast cells, and forms bile canalicular structures, but is viable only at low density [18]. A derivative of this cell line, the WIF-B cell line, was selected for its ability to grow at high density. WIF-B cells polarize in culture and form enclosed bile canaliculi between adjacent cells. These bile canaliculi are sealed with tight junctions which, although more permeant than in hepatocytes *in vivo*, provide a diffusion barrier for lipids present in the outer leaflet of the basolateral and the apical domains [19,20]. Moreover, the kinetics of apical protein delivery in WIF-B cells closely resembles that of liver [21], which indicates that this cell line is a promising and useful tool for studying polarized membrane transport in hepatocytes. Interestingly, in a subclone of the WIF-B cells, WIF-B9, the establishment of hepatocyte polarity is a biphasic process. In this cell line the formation of bile canaliculi is preceded by a state of polarity similar to that of simple epithelial cells, expressing apical membrane proteins at their apex and basolateral proteins in the remaining plasma membrane [22].

The well-differentiated human hepatoma cell line HepG2 [23] is also able to form bile canalicular structures (Figure 2), to which specific apical proteins are targeted [24–28]. Although some of their functions are different from those of hepatocytes [29], this cell line has the advantage that it has been very well characterized and expresses liver-specific functions [23] such as those related to phospholipid, cholesterol, lipoprotein and bile acid metabolism [30,31]. Moreover, in contrast with other hepatoma cells, HepG2 cells can internalize bile acid via an Na^+-independent transporter [32], which, as will be discussed below,

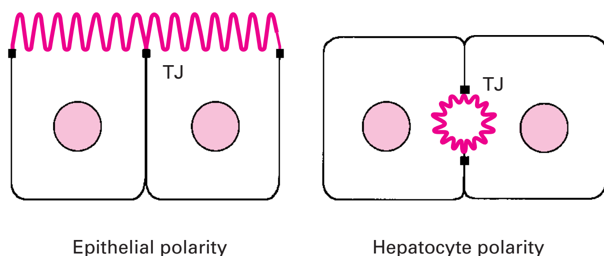


Figure 1 Transverse view of polarized epithelial and hepatic cells

The apical domains are indicated in red. Note that the apical and basolateral membrane domains are separated by tight junctions (TJ, filled black squares). The tight junction prevents lateral randomization of membrane components in the outer leaflet of the membranes only (see the text).

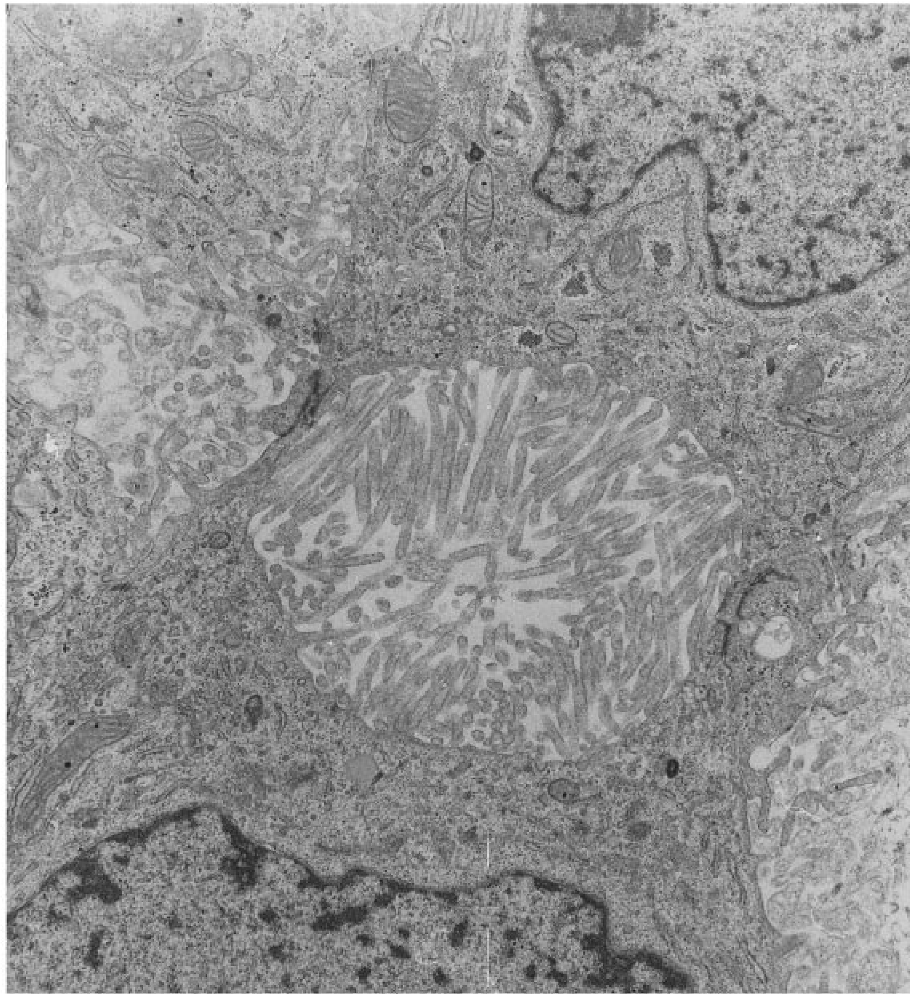


Figure 2 Bile canalicular space in HepG2 cells revealed by electron microscopy

Note the dense lining of the intercellular space with microvilli. Magnification 10 000 ×.

is probably important in intracellular hepatic lipid transport. At present, important details concerning lipid transport pathways in hepatic cells have been revealed by using the HepG2 cell line [24,27,33,34]. Before discussing these pathways in more detail, it is necessary to first describe some relevant issues related to protein trafficking.

CELL-SURFACE TRANSPORT OF PROTEINS

Pathways in polarized protein transport

In the exocytic pathway, proteins, synthesized at the endoplasmic reticulum (ER) are transported to the Golgi complex, where they are post-translationally modified. In the TGN, proteins and lipids are packaged into vesicles and further transported to the plasma membrane. Newly synthesized plasma membrane proteins can be delivered to the appropriate membrane domain by three different pathways; in all pathways, protein processing involves at least one, and often more than one, sorting step. In the direct pathway, protein sorting occurs in the TGN, where proteins are packed into vesicles that are targeted directly to the correct, i.e., basolateral or apical, domain. In the indirect pathway, all membrane proteins are first delivered to the basolateral domain. From there, apical proteins are endocytosed and transported to the apical domain by transcytosis. The evidence indicates that apical proteins reach a sub-apical compartment prior to their delivery to the apical membrane. Alternatively, proteins can reach the plasma membrane by random sorting, in which they are first transported randomly to both membrane surfaces. Those proteins that belong to the opposite surface are then either degraded, or transcytosed to their final destination.

The relative importance of each targeting pathway is dependent on the cell type [1]. MDCK cells use predominantly the direct pathway [1], whereas polarized protein targeting in Caco-2 cells also depends on indirect sorting [35,36]. In hepatocytes, prevailing evidence indicates that most apical proteins are processed along the indirect pathway [6–8]. Interestingly, the protein-sorting pathway that is used may also be related to cell development. It was shown that in a 1-day-old monolayer of epithelial Fischer-rat thyroid cells, the apical protein dipeptidylpeptidase was transported apically by random sorting, followed by transcytosis. However, in 7-day-old monolayers, apical targeting relied on direct sorting [37].

Basolateral and apical protein targeting signals

Basolateral transport has long been considered to occur by default, which means that no specific signals are required to target basolateral proteins to the appropriate domain. Recently however, basolateral targeting signals have been described (for reviews, see [38,39]). The targeting signals are located in the cytoplasmic domain near the plasma membrane and, in a number of cases, they are co-linear with signals required for receptor-mediated endocytosis. Many basolateral sorting signals contain a critical tyrosine residue that is known to be essential for the formation of a β-turn. The β-turn is required for endocytosis, and probably also for basolateral sorting. Recently, a dileucine motif has also been implicated in endocytosis and basolateral sorting. Whether this motif similarly bears relevance to β-turn formation remains to be determined.

With respect to targeting of apical proteins, a unifying mechanism has not been described thus far. The best known signal involves the glycolipid GPI, which anchors proteins to the membrane. The evidence indicates that GPI-linked proteins, which are exclusively located at the apical membrane of most epithelial cells, are targeted apically by inclusion into sphingolipid microdomains. For non-GPI-linked proteins, no definite targeting signals have yet been identified. It was proposed that polarized targeting via the direct pathway is regulated by a system involving hierarchically arranged sorting signals in which the basolateral signal is dominant over the apical signal. Removal or absence of basolateral targeting signals would then reveal a cryptic apical signal that (re)directs proteins to the apical surface [40]. Apical targeting via the indirect, transcytotic route has been described in detail for the polymeric IgA receptor. After its initial targeting to the basolateral domain, mediated by its cytoplasmic sorting signal, the receptor is endocytosed. Subsequently, a serine residue in the basolateral targeting signal is phosphorylated, which stimulates the basolateral to apical transcytosis of the receptor, apparently by inactivating this signal [41]. This indicates that, also during transcytosis, inactivation of the basolateral sorting signal may reveal an apical targeting signal. At present the nature of the putative apical sorting signal(s) is not clear, but some evidence indicates that N-linked glycosylation acts as a (luminally located) apical sorting signal [42,43], possibly by interaction with lectin-like sorting receptors such as VIP36 [44]. Furthermore, it is also likely that some sorting specificity of non-GPI linked apical proteins is located in the transmembrane domain of distinct proteins [45], possibly bearing some analogy to GPI anchors in their mode of interaction with sphingolipid-enriched domains.

CELL-SURFACE TRANSPORT OF LIPIDS

Lipid diversity in cellular membranes

While proteins can be restricted to a particular organelle, lipids, except for a few species, are not. In general, differences in lipid composition are largely on the level of differences in the ratio of lipid species that are present within a membrane and of differences in the distribution of fatty acid species. As an example, although present in most membranes, the plasma membrane contains most of the cellular cholesterol and sphingolipids [46]. Another level of lipid diversity concerns the asymmetric distribution of lipids across the membrane bilayer. This asymmetry has been most clearly demonstrated in the plasma membrane, where the majority of the choline-containing lipids, phosphatidylcholine (PC) and sphingomyelin (SM), is located in the outer leaflet, whereas most of the aminophospholipids, phosphatidylserine and phosphatidylethanolamine, are located in the cytoplasmic leaflet of the membrane [47].

The spontaneous transbilayer movement of most lipids is very slow, but exceptions are diacylglycerol and probably cholesterol [48,49]. Several proteins, however, have been shown to have lipid-translocating activity. Evidence has been provided for the existence of an ATP-dependent aminophospholipid translocator that translocates aminophospholipids to the inner leaflet of the (plasma) membrane [49,50]. This protein has recently been cloned [51], but may require the complementary participation of other proteins for translocation of aminophospholipids. [52]. Furthermore, a ubiquitously expressed protein, the so-called phospholipid 'scramblase', can induce rapid transbilayer movement of phospholipids in response to increased intracellular Ca^{2+}. This protein has been recently cloned and functionally reconstituted [53,53a]. In addition, a PC translocator has been identified in canalicular membranes of rat liver, that exhibits kinetic properties similar to those of a PC translocator in the ER that was characterized previously [54]. This latter translocator is unrelated to the mdr2 protein [55], which, as will be discussed below, also acts as a translocator of PC in bile canalicular membranes [56,57]. Finally, the MDR1 P-glycoprotein and the MDR-related protein MRP1 were shown to display lipid-translocating properties [58].

Although transbilayer movement across the plasma membrane has been observed for the short-chain glucosylceramide (GlcCer) analogue C_5-DMB-GlcCer [59], as well as for its short-chain precursor Cer (e.g. C_5-*N*-[5-(5,7-dimethylBODIPY™]-1-pentanoyl-Cer or C_6-NBD {6-[*N*-(7-nitro-2-benzoxa-1,3-diazol-4-yl)amino]hexanoyl}-Cer), it has never been reported to occur for natural sphingolipids (BODIPY™ is 4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene). However, the simple glycosphingolipid GlcCer, which is synthesized at the cytosolic leaflet of the Golgi [60,61], does require translocation to the inner leaflet in order to allow for the syntheses of higher glycolipids by glycosylating enzymes that are located at the luminal side of the Golgi apparatus [61–63]. Furthermore, part of the newly synthesized GlcCer may reach the plasma membrane by monomeric transport and will require a putative GlcCer translocator for subsequent translocation across the membrane to become expressed at the cell surface ([64,65] and see below). At present, the nature of (the) putative GlcCer translocator(s) is still a matter of speculation.

Besides transbilayer heterogeneity, lipids of the plasma membrane of polarized epithelial cells are also segregated laterally into the basolateral and apical domain, as secured by tight junctions (Figure 1), each displaying a distinct lipid composition. In this regard, the most prominent difference is observed for sphingolipids, which are mainly found in the outer leaflet of the apical plasma membrane. In the inner leaflet, rapid randomization of the lipids can still occur via diffusion in the lateral plane of the membrane ([66,67] and references therein). Although it has been suggested that the lipid composition of the outer leaflet can affect that of the inner leaflet [45], it is still unknown whether the lipid compositions of the inner leaflet of the apical and basolateral plasma membrane actually differ.

Vesicular and monomeric lipid transport

As for proteins, the specific lipid composition of membranes cannot be explained by local synthesis. Moreover, despite continuous vesicular transport between the different organelles, cells are able to maintain the distinct lipid composition of the plasma membrane and that of the membranes of organelles. Therefore, as is the case for proteins, specific lipid-sorting processes must be operational within the cell. Obviously, vesicular transport of

proteins is accompanied by lipid transport, and this transport mechanism is probably the most important means for bulk transport of lipids between two membranes. On the other hand, specific lipids and proteins might co-migrate into specific microdomains ('rafts'), triggered by more-or-less specific molecular-clustering mechanisms (see [68]), thus providing a means for transport of specific lipids to distinct membrane domains.

In addition to vesicular transport, lipids can also be transported between the cytosolic leaflets of membranes as monomers. *In vitro* studies have shown that spontaneous transfer of phospholipids between membranes is very slow, and it is unlikely that this process contributes significantly to intracellular lipid transport [69]. Monomeric transfer is stimulated by phospholipid-transfer proteins, which are cytosolic proteins that carry single phospholipid molecules between membranes [70]. However, as these proteins are always found to carry a lipid molecule, they presumably do not contribute to net lipid transfer. According to recent evidence it is more likely that at least some of these proteins function in the regulation of vesicular transport and lipid synthesis. It has been demonstrated that the phosphatidylinositol-transport protein (PITP), plays a role in the formation of secretory vesicles [71,72].

Transport of glycerophospholipids and cholesterol

The majority of the enzymes that are responsible for the synthesis of glycerophospholipids and cholesterol are located at the ER [73]. Newly synthesized PC and phosphatidylethanolamine are rapidly transported to the plasma membrane with a half-time of 1–2 min. Their transport proceeds when vesicular transport is inhibited with agents such as cytochalasin B, colchicine, monensin, brefeldin A (BFA) and energy poisons, or after ATP depletion [74–76]. Neither is phosphatidylethanolamine transport inhibited in mitotic cells, i.e. in conditions under which, similarly, vesicular transport is impeded [77]. On the basis of these observations it was suggested that, rather than by a vesicular-mediated mechanism, the movement of these newly synthesized lipids is mediated by transfer of monomers, possibly via phospholipid-transfer proteins.

By contrast, evidence has also been provided for a particular class of lipid-rich vesicles that carry newly synthesized cholesterol to the plasma membrane [78,79]. This process is dependent on ATP, but is not sensitive to drugs that affect the cytoskeleton (cytochalasin and colchicine) or the Golgi apparatus (BFA and monensin), a finding that is consistent with a vesicular transport process independent of protein secretion [78,79]. Recently, it was suggested that caveolin, a protein which, as will be discussed below, resides in cholesterol/sphingolipid-rich microdomains, is involved in transport of newly synthesized cholesterol to the plasma membrane [80]. Finally, extensive evidence supports the notion that bulk internalization of most (phospho- and sphingo-)lipids from the outer leaflet of the plasma membrane occurs via a vesicle-mediated recycling pathway [48,62,69].

Transport of sphingolipids

Cer, the precursor of sphingolipids, is synthesized at the ER, but, unlike phospholipids and cholesterol, the synthesis of SM and most glycosphingolipids occurs in the Golgi. An exception to this rule is galactosylceramide (GalCer), which, at least in some cell types, appears to be synthesized at the ER [81]. Sphingolipids are largely located at the outer leaflet of the plasma membrane. This topology is presumably correlated to their functions in cell adhesion, protection, recognition and signal transduction [82,83].

In numerous studies, plasma-membrane transport of newly synthesized GlcCer and SM has been investigated by using fluorescent C_6-NBD-Cer as a precursor [84]. Other methods, mainly relying on radioactive precursors, have also been used. Kinetic experiments have shown that endogenous glycosphingolipids move to the cell surface at a rate which is consistent with estimates for bulk protein flow [85], suggesting a vesicular mechanism of transport. Indeed, the mechanism of transport of SM and GlcCer is thought to be vesicular, because it is sensitive to monensin [84,86], BFA [64] and microtubule inhibitors [86,87]. Some studies have reported an inhibition of transport of these lipids during mitosis [77,88]. Moreover, putative transport vesicles carrying these sphingolipids between the TGN and plasma membrane have been isolated from permeabilized cells [89,90], while reconstitution experiments revealed that transport is dependent on ATP and cytosol [89]. However, when interpreting such data it is pertinent to take into account the properties of the lipid analogues that are used. Distinct treatments such as those with BFA can give rise to 'spontaneous translocation' of C_6-NBD lipids. Given the relative water-solubility of these analogues, they might then readily move as free monomers through the cytoplasm and reach the inner leaflet of the plasma membrane. Cell-surface exposure is subsequently accomplished by MDR P-glycoprotein-mediated translocation [91].

For GlcCer, alternative, non-vesicular transport mechanisms have also been proposed. This was based on observations that under some conditions, such as those obtaining after BFA treatment and at 15 °C, transport of SM and the glycolipid GM3 is blocked, while GlcCer transport proceeds [64,65,86]. Also, GlcCer reaches the plasma membrane with a shorter lag time than SM [65]. This would be consistent with the notion that different transport vesicles may exist, varying in relative enrichment of either SM or GlcCer [90]. Alternatively, part of the mechanism of transport of GlcCer might indeed involve monomeric flow, mediated by the action of a specific glycolipid-transfer protein [92] and the action of the MDR1 P-glycoprotein [91] in order to accomplish translocation across the plasma membrane. A mechanism of monomeric transfer through the aqueous phase would be consistent with the occurrence of the synthesis of GlcCer at the cytosolic leaflet of Golgi membranes, which allows direct access of this lipid to a transfer protein in the cytosol or vice versa. Also, the ability of GlcCer to use an alternative mechanism of transport compared with other simple sphingolipids such as GalCer or SM might explain the observations that this lipid is sorted from these sphingolipids during endocytosis [93], transcytosis [33] and in the biosynthetic pathway [94].

The role that MDR and MRP P-glycoproteins may play in the ultimate localization at the plasma-membrane surface of the fraction of sphingolipids that reaches the inner leaflet by monomeric transport is intriguing. Indeed, it has been proposed that the lipid translocase activity of MDR and MRP proteins at the plasma membrane plays a role in the generation of the distinct sphingolipid composition of the apical and basolateral plasma membrane [58]. If so, it is evident that specific (sphingo)lipids, except for newly synthesized GlcCer, first have to translocate from the Golgi lumen to the cytoplasmic surface. Subsequently, specific mechanisms, including sorting, must then exist that mediate GlcCer transfer to the inner leaflet of the apical and, for example, SM or GalCer, to that of the basolateral membrane. Next, P-glycoprotein-mediated translocation should take place, which, in the case of SM and, presumably, PC [56], should display some selectivity with regard to the species which are translocated (i.e. PC, for subsequent excretion into bile in the case of hepatocytes). Selectivity should also be expressed in terms of the fraction of the lipid which is translocated, since, for example, endogenous SM is distributed in both leaflets. Several

of these steps have yet to be demonstrated, and it should also be noted that part of the scenario described above is based on work done with short-chain analogues of the lipids. Hence it will also be pertinent to evaluate the fate of the endogenous, natural (sphingo)lipids.

SPHINGOLIPID TRANSPORT IN POLARIZED CELLS

In polarized epithelial cells, the apical membrane is enriched in glycosphingolipids, whereas SM is distributed equally over the apical and basolateral domains (see [95,96]). Using fluorescently tagged sphingolipid analogues, it has been shown that in MDCK and Caco-2 cells, C_6-NBD-GlcCer, in contrast with C_6-NBD-SM, is preferentially delivered to the apical domain. This localization is entirely consistent with the enrichment of endogenous GlcCer in the apical membrane. Evidence was provided that the polarized delivery of GlcCer is accomplished via direct sorting, i.e., without prior delivery of the lipid to the basolateral plasma membrane followed by transcytosis [94,95]. In fact, it was shown that, in both MDCK and Caco-2 cells, lipid sorting is not taking place during transcytosis, but does occur in the direct transport pathway [95,97]. Interestingly, in hepatic cells, the same lipid analogues are subject to sorting in the retrograde apical-to-basolateral transcytotic pathway, as was shown in HepG2 cells [33]. Moreover, these sorting processes were taking place in vesicle-mediated transport events and, as revealed by the use of specific inhibitors, were independent of P-glycoproteins [98]. As for MDCK and Caco-2 cells, a direct sphingolipid transport route from the Golgi to the apical domain was also demonstrated in HepG2 cells [24,27] and in (rat) hepatocyte couplets [15,27]. Most interestingly, as opposed to the indirect transport pathway of apical proteins in hepatocytes [6–8], apical sphingolipid transport in hepatic cells does not depend on indirect transport. Given the likelihood, therefore, of the functioning of a direct apical pathway in these cells, which is in contrast with previous suggestions (see, e.g. [45,99]), the intriguing questions remain as to whether and which proteins participate in this pathway, and why GPI-linked proteins examined thus far are first delivered to the basolateral membrane. The question also arises as to whether sphingolipid rafts are actually formed in hepatic cells and, if so, why distinct apical proteins are excluded. In this context it should be noted, though, that transport of a GPI-linked protein in a direct apical pathway in hepatic cells, has been suggested [100].

Sphingolipid transport to the basolateral and apical domains is differentially regulated in both epithelial and hepatic cells. In MDCK cells, the microtubule-inhibitor nocodazole inhibits the apical, but not the basolateral, targeting of SM and GlcCer. Also, apical transport of both lipids in these cells is inhibited by monensin, which, in addition, inhibits basolateral transport of SM, but not of GlcCer [86]. Likewise, in HepG2 cells, Golgi-to-apical sphingolipid transport is dependent on microtubules, whereas transport to the basolateral plasma membrane is only slightly affected by a disruption of microtubules [87]. Furthermore, in these cells the apical targeting of sphingolipids, via both the direct and the transcytotic pathway, is inhibited by protein kinase C (PKC) activity and stimulated by activation of protein kinase A (PKA) [24,33]. Their basolateral targeting, however, is not regulated by the activity of these kinases [24]. At present it is unknown how the above-described effects of PKA and PKC on sphingolipid transport are related to their role in other polarized membrane-transport processes. In hepatocytes and MDCK cells, apical targeting of proteins is stimulated by cAMP or PKA [101–105], a process that may be regulated at the level of the Golgi apparatus [106]. Indeed, recent studies have shown that PKA activity stimulates the biogenesis of vesicular carriers that mediate transport from the Golgi to the plasma membrane, although the exact role of the phosphorylating activity of the kinase, and the exclusiveness of the effect on vesicle budding, remains to be determined [107]. Interestingly, cAMP and PKA activity induce an enlargement of the total apical membrane surface in HepG2 cells and hepatocytes [17,24], which indicates that PKA does not merely stimulate apical trafficking of specific proteins, e.g., by their phosphorylation-induced recruitment into specific vesicles, but rather stimulates overall membrane flow to the apical membrane.

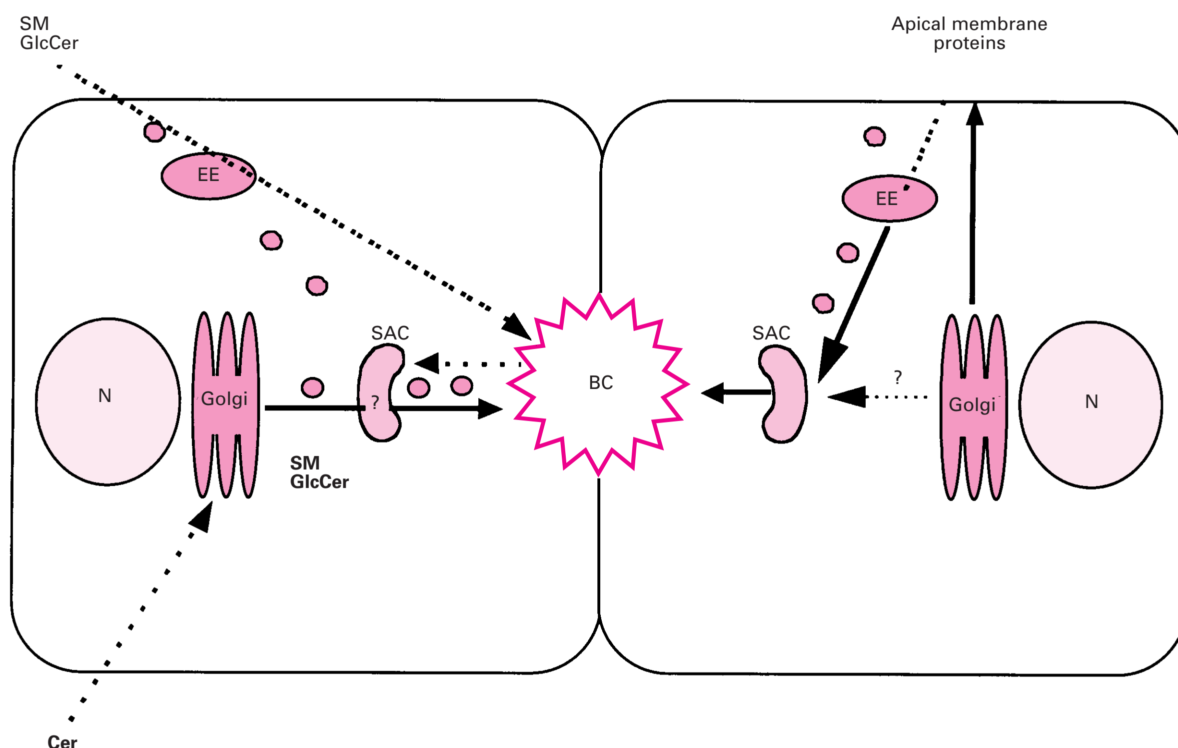
Similar observations have been reported upon PKC activation. In MDCK cells, activation of PKC by phorbol esters stimulates apical delivery of proteins that are endocytosed from either the basolateral or the apical surface [108]. In addition, PKC stimulates direct protein transport from the TGN to the apical surface in these cells [103]. Although phosphorylation of membrane proteins has often been implicated as a determinant for protein trafficking (see [109]), evidence suggests that the effects of PKC on protein transport are not caused by a PKC-induced phosphorylation of these proteins [108,109]. Thus, by analogy to PKA activation, recent evidence indicates that PKC activity can also stimulate vesicle budding from the Golgi apparatus, independently of its phosphorylating activity [110,111]. It has been proposed that PKC, or a PKC-related protein, in concert with PITP, can activate phospholipase D, which hydrolyses PC and, accordingly, generates phosphatidic acid. After translocation to the luminal face of the membrane by a putative flippase, a high local concentration of the negatively charged phosphatidic acid may then induce/facilitate membrane fusion at the neck of budding vesicles, thus promoting the release of vesicles from the Golgi membrane [111,112]. Alternatively, the production of phosphatidic acid may promote vesicle budding by regulating the binding of coat proteins to Golgi membranes [113]. Indeed, it has been shown that treatment of Golgi membranes with phospholipase D stimulates coatomer binding [114], which may be part of the mechanism which regulates budding of vesicles from the Golgi. The extent to which PKC-mediated phosphorylation of these coat proteins is also of relevance to these binding processes remains to be determined. In light of the foregoing, it is furthermore important to note that the presence of acidic phospholipids in a membrane can be a determining factor for the budding of vesicles. This was shown in recent work in which the formation of COPII-coated vesicles was reconstituted in a system containing artificial liposomes and yeast coat proteins. In this system it was demonstrated that binding of the coat proteins, which allows the subsequent formation of COPII-coated vesicles, depends on the presence of acidic phospholipids like phosphatidic acid or phosphatidylinositol lipids [115].

In spite of the general stimulation that PKC seems to exert on apically directed membrane traffic, activation of PKC inhibits apical sphingolipid transport in intact HepG2 cells [24]. This inhibition is probably not due to an overall inhibiting effect on plasma-membrane-directed transport, since PKC does not affect basolateral sphingolipid transport [24]. Moreover, PKC stimulates the formation of constitutive, secretory protein-carrying vesicles from HepG2 Golgi membranes [110]. Assuming that PKC stimulates the formation of these vesicles via the mechanism described above [111,112], the final vesicle scission is mediated by a reorganization of the lipid environment. This reorganization may perturb (the formation of) specific sphingolipid domains destined for apical delivery. Possibly such a perturbation may result in an inhibition of sorting, vesiculation or transport of this domain. Alternatively, PKC may inhibit direct apical sphingolipid transport at a later step, after con-

verging with the transcytotic transport route [24]. However, irrespective of the mechanism that is responsible for the inhibition of apical sphingolipid transport by PKC, the results indicate that, in HepG2 cells, sphingolipids can be excluded from bulk membrane flow. It is also apparent that the role of the different protein kinase activities in intracellular transport needs to be further defined, in particular their regulation and potential cross-talk, given their similiarity in vesiculation capacity. However, the data also indicate that these regulatory activities may not be exclusively restricted to an interference with membrane flow at the level of vesicle budding only. Identification of these additional mechanisms will be a future challenge.

CO-TRANSPORT OF SPHINGOLIPIDS AND PROTEINS

Current evidence indicates that glycoproteins and sphingolipids can be co-transported in the same vesicles from the Golgi to the plasma membrane. In reconstitution experiments and in studies with intact and semi-intact cells, transport of both proteins and sphingolipids through the Golgi and to the plasma membrane has been shown to be mechanistically very similar. Both processes are dependent on temperature, ATP and cytosol, and can be inhibited with *N*-ethylmaleimide and guanosine 5′-[γ-thio]triphosphate [85,89,116,117]. Indeed, transport of SM was found to be associated with a membrane fraction carrying a secretory protein [118]. Moreover, detergent-insoluble complexes of distinct proteins and sphingolipids can be isolated that are thought to be derived from microdomains, i.e. so-called ‘rafts’. It is claimed that in epithelial cells these rafts play a prominent role in the sorting and trafficking of newly synthesized apical membrane compounds, such as distinct sphingolipids and GPI-linked proteins. The central concept in this model is that GPI-linked proteins and sphingolipids are sorted in the TGN by clustering in a distinct microdomain, or raft, which is formed by self-aggregation of sphingolipids and which requires the presence of cholesterol for raft-stabilization [45,96]. Upon membrane vesiculation, this microdomain is subsequently transported to the apical domain. Experimental support for such a domain has been derived from observations that GPI-linked proteins that are targeted to the apical surface, are associated with a low-density sphingolipid/cholesterol enriched membrane complex, which is resistant to detergent solubilization at low temperature [119]. Although these complexes are thought to be formed in TGN, their intracellular localization can only be determined with certainty upon isolation of the organelles of interest, prior to detergent extraction. Apart from involvement in the sorting of apical GPI-linked membrane proteins, other apical transmembrane proteins which favour partitioning into a



Scheme 1 Direct and indirect transport pathways in hepatic cells

Sphingolipids are transported to the bile canaliculus (BC) of hepatic cells by a direct route and by transcytosis (left panel; see [24] for details). The direct Golgi-to-apical membrane-transport pathway can be revealed by incubating cells with a cell-permeable fluorescent Cer analogue, which enters the cell at low temperature (bottom). After warming to 37 °C, Cer accumulates in the Golgi, where it is metabolized to GlcCer and SM. These products are subsequently transported to both the basolateral and the apical plasma membrane. As the presence of BSA, which extracts basolaterally arriving sphingolipids from the cell, does not prevent sphingolipid transport to the apical membrane, a direct transcytosis-independent sphingolipid transport pathway must exist. In addition, sphingolipids can be transported to the bile canaliculus by basolateral-to-apical transcytosis. This transport route (top, left panel) is revealed by incubating cells with fluorescent analogues of either GlcCer or SM. These sphingolipids remain at the basolateral plasma membrane at low temperature, but are efficiently transported to the bile canaliculus after warming the cells to 37 °C. The involvement of sub-apical compartments (SAC) in lipid transport in either pathway requires further investigation, although it is evident that the compartment plays a role in sorting of apically derived GlcCer and SM to the apical and basolateral membrane respectively (see [33,98]). In the processing of newly synthesized apical membrane proteins (right panel), SAC plays a role in their sorting from non-apical proteins [21]. Prior to delivery, apical proteins are first transferred to the basolateral membrane. By transcytosis, the proteins reach the apical membrane via SAC. The extent to which apical proteins are also transferred via a direct transport pathway in hepatic cells, as in epithelial cells, is at present unclear (see the text). Abbreviation: EE, early endosome.

sphingolipid/cholesterol-enriched membrane domain [120], might also be sorted along the same raft mechanism.

Sphingolipid rafts are structurally related to caveolae, which form flask-shaped invaginations at the plasma membrane. The integrity and function of caveolae is dependent on the presence of cholesterol [121–123] and, in all likelihood, sphingolipids [124]. The presence of caveolin, a major caveolar protein, correlates with caveolae formation [125,126]. Caveolae are ubiquitously present on cell surfaces and play a role in endocytic-like events and, according to recent studies, in cholesterol transport [80,127,128]. However, in polarized epithelial cells such as MDCK, caveolae are exclusively present at the basolateral membrane surface, where they are involved in transcytosis.

The above results indicate that sphingolipid rafts and caveolae, the function of which seems to be dependent on lipid composition in the first place, are of major importance with respect to intracellular transport processes. However, although the raft model is highly attractive in explaining apical sorting, it is far from settled (cf. [129]), not least because it has been based mostly on circumstantial evidence. In particular, the molecular mechanism underlying raft formation is entirely obscure. Speculations include the notion that clustering is strongly facilitated by interdigitation, which implies that the long fatty acid chain of glycosphingolipids may cross the membrane midplane and protrude among acyl chains of the opposing monolayer, as previously described [62]. Other factors, such as fatty acid chain saturation [45], including those contained in the GPI anchor of apical proteins [62] and cholesterol [45,62,130,131], may also play a role in triggering/mediating the clustering of specific molecules in rafts. However, clarification of these issues will require detailed studies in which model membranes (liposomes) may prove to be an indispensable tool.

Also, involvement of caveolin in raft-mediated apical transport is at present unclear, although a role for this protein in the transport of cholesterol from the ER to the plasma membrane was demonstrated [128]. In addition, caveolin was shown to be involved in the formation of caveolae [125,126]. The question as to whether this protein also plays a direct role in the apical targeting of GPI-linked proteins was recently addressed using the epithelial Fisher-rat thyroid cell line. These cells neither express caveolin, nor do they form caveolae; they deliver GPI-linked proteins and fluorescent glycolipids to the basolateral surface. Upon expression of caveolin-1 in these cells, the formation of caveolae was observed. However, the expression of this protein does not redirect GPI-linked proteins to the apical surface, nor does it promote their inclusion into a cholesterol/sphingolipid-enriched domain. The data suggest, therefore, that caveolin-1 might not be directly involved, or its expression alone does not suffice in the apical targeting of GPI-linked proteins [126].

Obviously, as molecules can easily redistribute upon treatment with detergent, caution is needed to draw conclusions about the exact composition of these lipid domains on the basis of detergent-extraction experiments. The use of photoreactive sphingolipids, which, as reported in some recent studies [34,64,132], can label raft-associated protein components *in situ* prior to detergent extraction, may serve as an attractive alternative to identify proteins that are located in, and functionally relevant to the biogenesis and ensuing processing of, sphingolipid rafts.

Although the mechanisms remain to be clarified, the above discussion emphasizes that sphingolipids can be co-transported with both secretory and membrane proteins. However, the results of several studies also indicate that sphingolipid transport routes exist which operate independently of protein transport. A striking example of sorting of SM and glycoproteins in the exocytic pathway has been shown in HeLa cells, infected with the intracellular pathogen *Chlamydia trachiomatis*, which forms an intracellular vacuole termed an inclusion. In infected cells, the vesicular transport of endogenously synthesized SM from the Golgi to the plasma membrane was disrupted and specifically redirected to the inclusion [133,134]. By contrast, under these conditions, the trafficking of newly synthesized plasma-membrane marker glycoproteins like the vesicular-stomatitis-virus glycoprotein (VSV-G) and the transferrin receptor was not significantly impaired [135]. Although the trafficking of typical apical proteins was not examined in this system, no host proteins, including the GPI-linked protein 5′-nucleotidase, were as yet identified in the inclusion membrane [134]. Since the inclusion does not communicate with the endosomal pathway, it was concluded that sorting of SM and glycoproteins probably takes place in the TGN.

Other studies, too, suggest that sphingolipids can be transported independently of plasma-membrane proteins. In Caco-2 cells, sphingolipids are directly sorted, i.e., from the TGN to the appropriate membrane domain [95], whereas polarized protein transport relies partly on indirect sorting, i.e., involves a transcytotic step. Similarly, in hepatocytes, most apical proteins are sorted indirectly, whereas a substantial part of the sphingolipids is transported directly to the apical domain [27]. Moreover, it has also been shown that, after BFA treatment, SM transport proceeds, whereas albumin secretion is arrested, in hepatocytes [136]. Taken together, and although co-transport may occur, the data indicate that transport of sphingolipids and membrane proteins is not necessarily tightly coupled. This raises intriguing questions as to the potential involvement of molecular factors regulating either mode of transport.

Scheme 1 summarizes the various sphingolipid-transport pathways involved in polarized trafficking in hepatic cells. Note that very recent evidence indicates that subapical compartments, analogous to those previously identified as apical recycling compartments in MDCK cells [137,138], also appear to operate in sorting and polarized transport in hepatic cells [21,33,98].

(SPHINGO)LIPIDS AS REGULATORS OF VESICULAR TRANSPORT

In recent years, evidence has been mounting that lipids do not only accompany protein transport routes but may also be involved in the regulation of intracellular membrane-transport processes. An example is the involvement of acidic phospholipids in the formation of COPII-coated vesicles and the regulation of the secretory pathway by PITP and phosphatidylinositol lipids, as indicated in the previous section. In addition, phosphatidylinositol lipids are involved in a number of other membrane-trafficking events, and their role is now only beginning to emerge [71,72,139–141].

Recently, sphingolipids were also found to play a role as regulators in vesicular transport in both the biosynthetic and endocytic pathway [142–144]. When cells are treated with the sphingolipid synthesis inhibitor D-*threo*-1-phenyl-2-decanoyl-amino-3-morpholinopropan-1-ol (PDMP), plasma-membrane-directed transport of both newly synthesized sphingolipids and the VSV-G protein was delayed [143], while ER-to-Golgi transport of the M protein of infectious-bronchitis virus was similarly interrupted [144]. It was suggested that the inhibitory effects of PDMP were related to an increase of the sphingolipid precursor Cer, which accumulates when the biosynthesis of sphingolipids is inhibited by PDMP. Indeed, addition of a cell-permeable Cer analogue also inhibited VSV-G transport in the Golgi [142]. However, exogenous Cer addition did not display the same effect as PDMP on ER–Golgi transport, while the transport process as

such did not appear to include an inhibition of ER–Golgi transport of Golgi-resident proteins, suggesting a specific interference with M-protein cycling between ER and Golgi [144]. Therefore the exact role of Cer in this particular process remains to be identified. However, in this context it is noteworthy that PDMP is able to interfere with BFA-induced retrograde Golgi-to-ER membrane transport [145]. Interestingly, rather than being related to an effect on sphingolipid metabolism, this interference was related to an effect of PDMP on calcium homoeostasis, its perturbation giving rise to an inhibition of BFA-induced retrograde transport from the Golgi to ER [146].

An increase of the intracellular Cer concentration, as accomplished either by PDMP treatment or by addition of Cer analogues, was also found to inhibit fluid-phase endocytosis of horseradish peroxidase and, to a lesser extent, receptor-mediated endocytosis of low-density lipoprotein [147]. Taken together, prevailing evidence supports the view that Cer can inhibit membrane transport. As discussed above, its effect may be exerted at the level of vesicular trafficking and, interestingly, possibly at the level of protein sorting, as exemplified by interference of Cer with corona M-protein transport. Finally, in addition to these effects, a perturbation of sphingolipid metabolism can also interfere with polarized protein sorting. In MDCK cells, the apical sorting of the GPI-linked protein GP-2 and the basolateral sorting of Na^+/K^+-ATPase was abolished in cells that had been treated with the sphingolipid-synthesis inhibitor fumonisin B_1. This inhibitor blocks the synthesis of Cer and, accordingly, that of sphingolipids. By addition of a cell-permeable Cer analogue that circumvents the fumonisin B_1 block, the polarized sorting was re-established [148]. Whether in that study Cer addition inhibited the rate of transport of these proteins to the plasma membrane, as was found by Pagano and co-workers [147], remains to be established. Nevertheless, these data support the view that the polarized sorting of GP-2 is coupled to sphingolipid synthesis.

APICALLY DIRECTED LIPID TRANSPORT AND BILIARY LIPID SECRETION IN HEPATOCYTES

At the bile canaliculus, i.e. the apical domain of hepatocytes, considerable amounts of lipids are secreted into the bile. According to a commonly accepted model, biliary lipids are released from the bile canalicular membrane by bile-acid-mediated solubilization. Thus lipids destined for biliary secretion are first incorporated into the bile canalicular membrane before they are excreted into the bile. Most studies concerning apically directed lipid transport in hepatocytes have focused on characterizing lipid excretion into the bile, which is in fact the net result of two different processes: (i) the intracellular transport of lipids to the apical membrane, and (ii) their subsequent secretion into the bile. The latter process presumably involves (specific) translocation and sorting steps, since solubilization and secretion of biliary lipids does not occur randomly. This follows from the notion that the lipid composition of bile, which consists mainly of cholesterol and PC, is completely different from that of the bile canalicular membrane (Table 1). Moreover, the secreted PC has a specific fatty acid composition and contains predominantly palmitate ($C_{16:0}$) in the *sn*-1 position and oleate ($C_{18:1}$) or linoleate ($C_{18:2}$) in the *sn*-2 position.

Table 1 Specific phospholipids are secreted into bile

The phospholipid compositions of bile canalicular and sinusoidal membranes (rat) are given. A comparison of the compositions illustrates the specificity of the phospholipid fraction secreted into bile. The Table is compiled from data presented in [150,179].

	Composition (% of total phospholipid)		
Phospholipid	Whole bile	Canalicular membrane	Sinusoidal membrane
Phosphatidylcholine	94.8	35.5	44.6
Lysophosphatidylcholine	0.5	1.6	0.8
Sphingomyelin	0.1	22.1	11.0
Phosphatidylethanolamine	4.5	23.8	28.4
Phosphatidylinositol	–	4.4	6.4
Phosphatidylserine	–	11.2	7.6

Lipid transport to the canalicular membrane

In bile canalicular membranes, SM is most likely located at the luminal leaflet and is therefore in direct contact with the bile. However, the amounts of SM that are found in the bile are very low. Since sphingolipid-enriched membrane domains are resistant to detergents, including the bile acid deoxycholate [119], SM very likely participates in protecting the bile canalicular membrane from solubilization by bile acids. As only small fractions of biliary PC and cholesterol (5 and 20% respectively) are newly synthesized, most lipids that are secreted into the bile originate from a pre-existing intracellular pool. Apart from the bile canalicular membrane, lipids derived from the ER, Golgi and endosomal or lysosomal membranes might also contribute to the pool, secreted as biliary lipid [149,150].

At present it is unclear whether lipids destined for biliary secretion are co-transported in membrane vesicles that also contain apical membrane components that are not secreted. However, experiments with microtubule inhibitors have shown that biliary lipid secretion depends on vesicular transport [151–153], but neither the nature nor the intracellular transport route of these vesicles have been elucidated with certainty [150]. Also, an inhibitory effect of monensin on biliary lipid secretion has been reported [154], but this was not confirmed by others [155]. Some evidence points towards the Golgi apparatus as an assembly point for lipids and bile acids that are destined for biliary secretion. Most likely, biliary lipids are part of the membrane of the carrier vesicle and are not complexed within the interior of the vesicles, as is the case for lipoproteins [149]. As biliary-secreted PC species display acyl-chain specificity, an intracellular phospholipid sorting mechanism exists that selects phospholipids according to their fatty acyl chain length. This hypothesis is supported by studies of Cassagne and co-workers, who showed that, in leek (*Allium porrum*) seedlings, C_{20}–C_{24} phospholipids are transported to the plasma membrane via the ER–Golgi pathway, and that this transport is blocked by monensin and low temperature. The transport of C_{16}–C_{18} phospholipids, however, was not arrested under these conditions [50,156]. The latter observation merits further investigation, since it could imply that biliary-specific PC species, at least in part, can be transported via a non-vesicular mechanism.

Since both bile-specific PC and sphingolipids have to be transported to the apical surface, it is tempting to suggest that bile-specific PCs may reside in the cytoplasmic leaflet of sphingolipid rafts. However, whether this is the case remains to be established, as the fatty acid composition of PC found in (potential) hepatocyte rafts has never been analysed. By subcellular fractionation, Ahmed et al. [157] have isolated a vesicle fraction that may be involved in biliary lipid transport. These vesicles were enriched in microsomal and apical proteins and contained PC that had a fatty acid pattern typical of biliary PC.

Also, the PC/cholesterol ratio in this fraction could be correlated with that in bile [157]. These data would therefore suggest that apical proteins and bile-specific lipids can be co-transported, as both components were recovered in this vesicle fraction.

Unfortunately, lipids other than PC were not analysed in that study, and it is therefore unclear whether this fraction was enriched in sphingolipids and/or related to sphingolipid rafts.

In couplets or perfused rat liver, biliary lipid secretion is not influenced by treatments that block the transcytotic delivery of horseradish (*Armoracia rusticana*) peroxidase (BFA, monensin), indicating that the transcytotic pathway has no major physiological relevance in the process of lipid secretion into the bile [155,158]. Thus these results would argue against co-transport of lipids (destined for biliary secretion) and apical proteins because, as noted above, in hepatocyes transport of proteins to the apical membrane domain largely depends on transcytosis for apical delivery. Also, in HepG2 cells and hepatocyte couplets, fluorescent analogues of sphingolipids are efficiently transported to the bile canaliculus via a transcytotic pathway [24,27,33], which may suggest that, during transcytosis, these lipids, which are enriched in the bile canalicular membrane but not in bile, follow a pathway different from the one used for lipids that are secreted into bile.

Involvement of intracellular signaling in hepatic apical lipid transport

Canalicular-membrane-directed lipid transport may be controlled by intracellular signalling. It has been proposed that, e.g., papaverine (which inhibits lipid secretion) and some bile acids (which stimulate lipid secretion) exert their effects via a second-messenger mechanism [159]. It has been shown that dibutyryl cAMP, a cell-permeable analogue of cAMP, stimulates transcytosis of horseradish peroxidase and bile-acid-induced secretion of phospholipids in perfused rat liver [160]. In addition, the apical targeting of the Cl^-/HCO_3^- exchanger protein was stimulated by cAMP [101]. These results suggest that cAMP has an overall stimulating effect on apically directed membrane traffic. Indeed, in hepatocyte couplets, dibutyryl cAMP treatment significantly increases the canalicular circumference and bile acid secretion, which are both partly microtubule-dependent processes [17]. Moreover, as noted above, cAMP stimulates transport of sphingolipids to the bile canalicular membrane, which similarly is accompanied by an enlargement of the apical membrane surface [24]. Finally, an elevation of intracellular cAMP enhances the formation of canalicular vacuoles in single hepatocytes that do no longer maintain a polarized apical membrane [161]. Also this observation is probably related to an enhanced membrane trafficking to the canalicular membrane (see Scheme 1). To date, the mechanism that is responsible for the stimulation of apically directed transport in hepatocytes is unknown, but it might be similar to the mechanism(s) that stimulate(s) apical transport in MDCK cells. In both cell types the involvement of cAMP-dependent PKA has been demonstrated [24,102,104,105]. In MDCK cells, PKA activity has been shown to stimulate basolateral-to-apical transcytosis of ricin and the polymeric IgA receptor [102,105], which may be regulated at the endocytic level [105], or at an intracellular level, after endocytic uptake [102]. In addition, PKA was shown to stimulate protein transport from the TGN to the apical, but not to the basolateral, membrane [102,104]. However, it remains to be determined whether these (protein-) transport pathways are also stimulated in hepatic cells and whether they are responsible for the enhanced apical membrane targeting that is observed in this cell type.

Bile acid and intracellular hepatic lipid transport

Biliary lipid secretion is stimulated by, and tightly linked to, the secretion of bile acids, which are secreted independently, and probably prior to lipid secretion [149,162]. The mechanism of bile-acid-induced lipid secretion is not fully understood yet. As noted above, micellar bile acids in the bile act as a detergent and solubilize phospholipids from the canalicular membrane. Within the canalicular membrane, the bile-type phospholipids may be phase-separated into more fluid microdomains, thus allowing specific secretion of bile-specific PC species [149,162]. Apart from bile-acid-mediated lipid secretion at the level of the bile canalicular membrane, bile acids may also affect lipid secretion at an intracellular level. Several studies indicate that bile acids can have a general stimulating effect on apically directed vesicular transport (see [149]). It was demonstrated that bile acids stimulate transcytosis of BSA, horseradish peroxidase and plasma-membrane proteins, while the secretion of lysosomal protein appears to be enhanced as well. In addition, bile acids have been shown to increase the number, and change the morphology, of vesicles and vesicular structures near the bile canaliculus [163–166]. Moreover, bile acids can stimulate the transport of Golgi-derived sphingolipids to the bile canaliculus in hepatocyte couplets [15].

As mentioned above, bile acids possibly exert their effects on canalicular-membrane-directed traffic via intracellular signalling. Indeed, tauroursodeoxycholic acid can activate PKC in isolated rat hepatocytes [167], and it was shown that PKC inhibits both bile secretion [168] and cAMP-induced stimulation of vesicular targeting to the apical domain [101]. Moreover, sphingolipid delivery to the canalicular domain of HepG2 cells is also inhibited by this kinase [24]. Some studies, however, have reported a stimulating effect of PKC activity on bile secretion [167]. Interestingly, evidence has been provided showing that, in hepatocytes, fluorescent derivatives of lithocholate and ursodeoxycholate associate with intracellular membranes and induce transcytosis and a relocation of annexin II toward the apical membrane [169]. Annexin II has been implicated in membrane-fusion events leading to exocytosis [170]. A translocation of annexin II to the apical membrane might therefore play a role in fusion of vesicles with the apical membrane and, as a consequence, stimulate bile secretion. In this respect it is relevant to note that, in adrenal chromaffin cells, the participation of annexin II in exocytosis requires its phosphorylation by PKC [171], although others have proposed that phosphorylation of annexin II by this kinase could be a mechanism for its inactivation [170]. However, whether annexin II is (also) a target by which PKC can regulate apical directed transport or secretion remains to be established.

The role of mdr2 in biliary lipid transport and its implications for intracellular lipid transport

Recently it was demonstrated that the mdr2 P-glycoprotein, which is found in the bile canalicular membrane, is essential for the excretion of phospholipids into the bile. It was shown that, in bile of transgenic mice in which the *mdr2* gene was disrupted, phospholipid was completely absent, while cholesterol secretion was strongly decreased [56]. From these and other studies it was concluded that biliary phospholipid secretion entirely depends on the mdr2 protein, while cholesterol is, at least partly, secreted by an mdr2-independent mechanism [172,173]. By analogy with other mdr P-glycoproteins, which function as ATP-dependent drug efflux pumps, it was hypothesized that mdr2 may function as a lipid translocator. Indeed, *in vitro* studies in which the mdr2 protein was expressed in a yeast secretion mutant revealed an

ATP-dependent PC translocase activity of the mdr2 protein [57]. In addition to mdr2, another, structurally unrelated ATP-independent PC translocator has been demonstrated in canalicular membranes [54,55].

Mdr2 glycoprotein probably translocates PC, destined for biliary secretion, from the cytosolic to the luminal leaflet of this membrane. This hypothesis is supported by the fact that the mdr2 protein and its human homologue MDR3 are exclusively located at the bile canalicular membrane [174]. Also, since disruption of the *mdr2* gene does not affect the intracellular trafficking of cholesterol [172,173], transcytosis of horseradish peroxidase and IgA, or the secretion of lysosomal enzymes [175], it is unlikely that the mdr2 protein has a function in the regulation of membrane transport at the intracellular level. However, the finding that a functional mdr2 protein is an absolute requirement for lipid excretion may have important implications for the mechanism of intracellular transport of bile-destined lipids. The fact that PC secretion is completely dependent on the presence of the mdr2 protein suggests that the total pool of PC that is excreted into bile is initially delivered to the cytosolic surface of the bile canalicular membrane. Only after subsequent translocation to the luminal leaflet do they become accessible for solubilization by bile acids. Delivery of bile-specific PC to the cytosolic leaflet of the canalicular membrane implies that the vesicular carriers must have an asymmetric lipid distribution. Therefore, these putative vesicles should carry the bile-specific PC in the cytosolic leaflet, which, after fusion, results in a cytosolic location at the canalicular membrane. Such a localization of PC in these vesicles is consistent with the topology of PC synthesis, which occurs at the cytosolic leaflet of the ER [73]. Evidently, the lipids that reside at the luminal leaflet of these carrier vesicles, are, after fusion, delivered to the outer leaflet of the canalicular membrane. Thus, conceivably, lipids in the outer leaflet of the transport vesicles consist of lipids that are enriched in the outer leaflet of the canalicular membrane. Possibly the luminal side of these asymmetric vesicles is enriched in detergent-resistant sphingolipids [176], which would be consistent with the topology of the site of synthesis of most of these lipids, i.e., at the luminal leaflet of the Golgi apparatus [177]. By means of a fusion mechanism the sphingolipids would thus be directly inserted into the outer leaflet of the canalicular membrane. It is therefore tempting to postulate that bile-specific phospholipids are recruited at the cytosolic side of sphingolipid rafts. In this regard it is noteworthy that sphingolipids usually have long fatty acids which interdigitate into the cytosolic leaflet [45,62]. In this way a transmembrane interaction with bile-specific PCs seems possible, but the likelihood of such a mechanism awaits experimental support. Nevertheless, when assuming that phospholipid excretion primarily depends on vesicular transport for delivery of bile-specific PC to the cytosolic leaflet, it is not unlikely that biliary lipids and components of the apical membrane are co-transported. Finally, it has been reported recently that the human MDR1 and the murine mdr1a P-glycoproteins, which mediate the removal of cytotoxic drugs and show high homology with the mdr2 protein, can translocate a range of different short-chain lipid analogues, including sphingolipids [91,178]. Whether these P-glycoproteins can translocate natural lipids or fulfil a physiological function in intracellular lipid transport remains to be determined.

CONCLUSIONS

The existence of extensive membrane traffic pathways in eukaryotic cells is now well recognized. Along these transport pathways, molecular sorting frequently occurs, the underlying mechanism of which has best been established for membrane proteins. Lipids are equally engaged in sorting processes, as has been particularly revealed in experiments in polarized cells. Thus lipids do not accompany proteins as inert bulk cargo, being natural constituents of the membranes of vesicles that carry secretory or membrane proteins. Rather, evidence is accumulating which suggests the specific 'recognition' of lipids and their recruitment into specific pathways. As revealed in hepatic cells, apical transport of lipids can occur via a direct (TGN to apical membrane) and an indirect (transcytotic) pathway, whereas most apical proteins reach that membrane domain in these cells via the transcytotic pathway. This further exemplifies the existence of lipid-specific sorting mechanisms, i.e., mechanisms that do not rely exclusively on co-transport with apical membrane proteins. A major challenge in lipid cell biology will be to unravel these mechanisms at the molecular level. Of interest are also observations which point to the relevance of local membrane lipid composition and those that signify specialized functions of distinct lipid species, i.e., sphingolipids and their precursor, Cer, which might regulate sorting, vesiculation and overall membrane flow. Whether, in this respect, sphingolipids could also play a (direct) role in governing membrane flow during the biogenesis of apical membranes, thus (co-)regulating the establishment of plasma membrane polarity, remains to be established.

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