Chapter 1

Introduction

Membrane domains and the polarized sorting of proteins and lipids: Who, where, when and how

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Polarized cells function as a barrier between distinct extracellular environments in the body, e.g. between the blood/underlying tissue on the one side and a lumen on the other. Instrumental to the specific functions required at the different extracellular environments (e.g. uptake, secretion, protection), the plasma membrane (PM) of polarized cells is divided into distinct domains, the basolateral domain facing the blood circulation (basal) and neighboring cells (lateral), and the apical domain lining the cavity or lumen. Each domain is characterized by a specific composition of proteins and lipids (Fuller and Simons, 1985), whereas tight junctional complexes prevent lateral diffusion and subsequent randomization of apical and basolateral enriched compounds. Intracellular sorting mechanisms are imperative to secure the polarized distribution of proteins and lipids and, in addition, to allow for redistribution of PM molecules (plasticity of polarity). A major challenge in current cell biology concerns the understanding of these mechanisms. Crucial towards such an understanding is *i)* the identification and characterization of intracellular pathways that lead to or emanate from the distinct PM domains, *ii)* the molecular mechanism(s) by which the cell sorts and targets proteins and lipids into specific PM-directed pathways and *iii)* the identification of intracellular sites where such polarized sorting is orchestrated.

Model systems to study membrane traffic in polarized epithelial cells

A variety of model systems have been developed and applied to investigate polarized membrane trafficking. These include, among others, whole organ studies, model cell systems and in vitro reconstituted cell-free transport assays. Of these, model cell systems are mostly used and have proven to be very useful. Madin Darby canine kidney (MDCK) epithelial cells provide one of the best-studied and generally adopted polarized cell systems. These cells can be grown as a tight polarized confluent monolayer on semi-permeable filter supports (Fig. 1a) in a relatively easy way. When grown to confluency on the filters, both the basolateral and the apical PM domain of the cells are directly accessible for experimental manipulation. Other cell types can also been grown and used in this way, including intestinal epithelial (Caco-2) and colon carcinoma cells (HT-29).

The polarized phenotype of hepatocytes is more complex and differs from the above mentioned epithelial cells in that the apical membranes of hepatocytes do not form a continuous planar surface. Instead, the hepatocellular apical membranes delineate a vesicular lumen, representing the bile canalicular (BC) space, which is enclosed between a pair of adjacent cells (Fig. 1b, Chiu et al., 1990; Sormunen et al., 1993; Zaal et al., 1994). For the study of membrane flow in hepatocytes, the hepatoma-derived cell line HepG2 has been proven to be a suitable and attractive model system (reviewed in Zegers and Hoekstra, 1998). HepG2 cells, when cultured under specific conditions of growth, regain their polarized phenotype, i.e. form apical, BC membranes, in a time-dependent manner. Similar to that of other epithelial polarized cells, the apical BC membrane of HepG2 cells is enriched in microvilli and separated from the basolateral PM by functional tight junctional complexes (Fig. 1b). It is thought that this separation is restricted to compounds in the exoplasmic leaflet only.

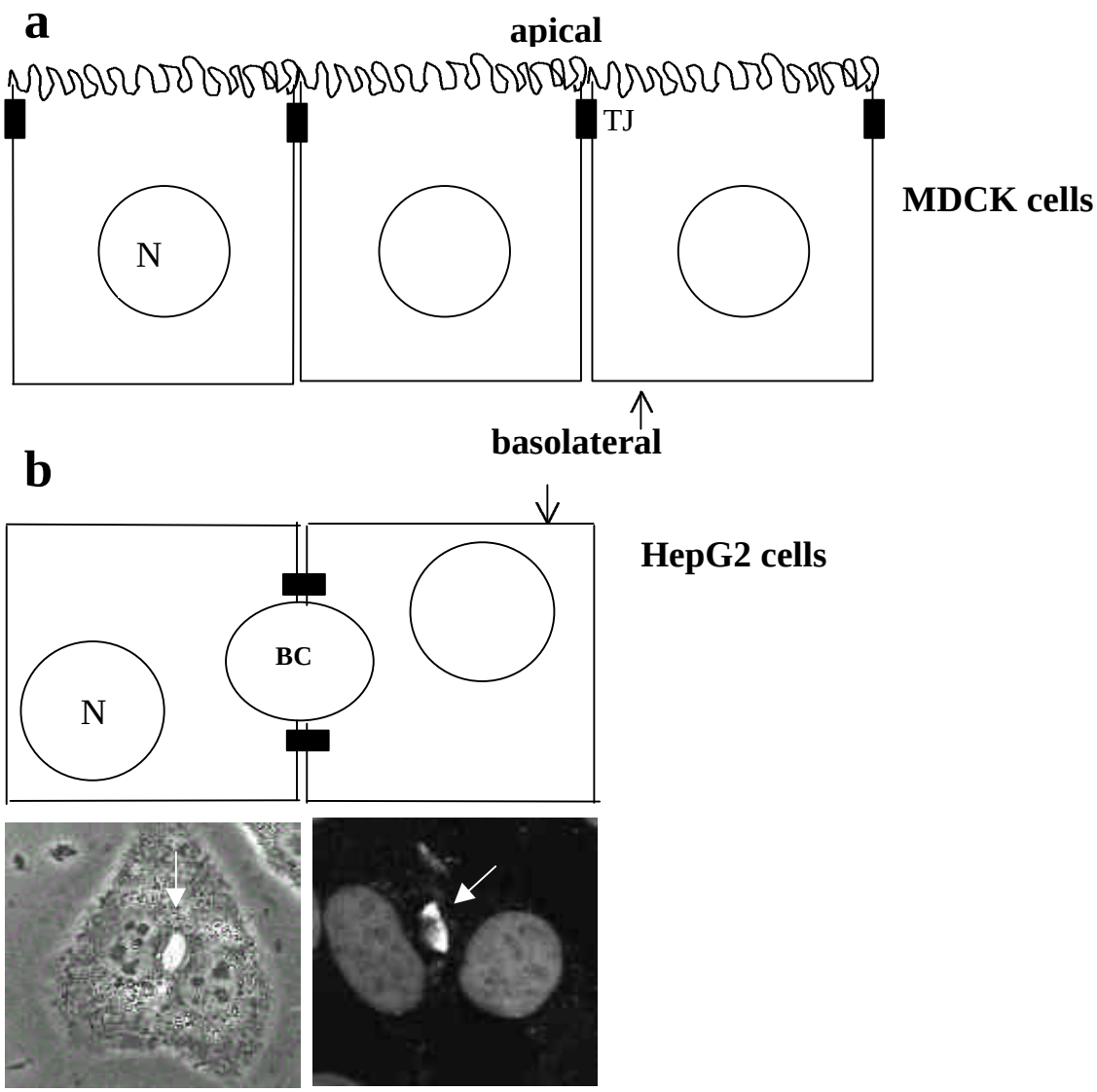

Figure 1. Model systems for polarized cells. (a) Filter-grown epithelial cells such as MDCK cells. (b) Hepatic HepG2 cells. The left photomicrograph shows a phase contrast image of two adjacent HepG2 cells with a BC in between (arrow). In the fluorescence photomicrograph on the right two adjacent HepG2 cells (with labeled nuclei), stained with an antibody against the tight junctional protein ZO-1 (arrow) are shown. TJ: tight junction. BC: bile canaliculus.

Intracellular sorting pathways

Intracellular sorting connected with PM homeostasis and biogenesis can occur in *trans*-Golgi network (TGN)-to-PM and transcytotic routes. For the polarized targeting of newly synthesized proteins and lipids, cells may employ either the direct route from the TGN to either PM domain or an indirect route, involving initial delivery to one PM domain and subsequent transcytosis to the opposite domain. The routes that will be predominantly taken for polarized delivery of a specific molecule depends on the cell type (Zegers and Hoekstra, 1998) and, in addition, may be dictated by specific conditions, such as degree of cell polarity

(Zurzolo et al., 1992). The TGN is well known to harbor several sorting mechanisms that govern the inclusion or exclusion into apically or basolaterally directed routes (Traub and Kornfeld, 1997), and much attention has been given to sorting in the direct, biosynthetic route. In addition, extensive, non-Golgi-mediated, transcellular movement of PM components occurs, either constitutive or regulated, in all polarized cells (Mostov and Cardone, 1995). Hence, it is readily envisioned that major polarized sorting events must also occur during transcytosis, presumably in endosomal compartments. Interestingly, sorting of molecules into cognate apical- and basolateral-type pathways, emanating from either the TGN (Yoshimori et al., 1996; de Vries et al., 1998) or endosomes (Mayor et al., 1998), also occurs in non-epithelial cells. This implies that highly regulated intracellular machineries must exist that connect the different vesicle populations (i.e. apical vs. basolateral, TGN- vs. endosome-derived) to the various transport routes that lead to different target PM domains.

Subcellular compartments in polarized transport pathways

An important step towards understanding the mechanisms of polarized sorting and targeting is the identification of traffic stations along the different traffic pathways where proteins and lipids are sorted and targeted to their preferred PM domains. In the biosynthetic pathway, the Golgi apparatus, and the TGN in particular, is a well-established organelle from which distinct apical and basolateral directed carrier vesicles bud, and thus appears a crucial compartment for polarized sorting and targeting. Experimental approaches combining pulse-chase incubations and subcellular fractionation have suggested that some proteins pass through post-TGN endosomal compartments prior to delivery to the PM (Ali and Evans, 1990; Leitinger et al., 1995; Sariola et al., 1995; Futter et al., 1995; Kipp et al., 1998). Unfortunately, the mapping of post-TGN compartments via which molecules pass en route to either PM domain in living cells has been difficult, mainly because of a lack of experimental designs to follow the fate of newly synthesized molecules. However, sphingolipid transport can be conveniently monitored by the application of fluorescently (C_6-NBD) labeled ceramide (Cer), which is metabolized to fluorescent sphingomyelin (SM) and glucosylceramide (GlcCer) in the Golgi (Lipsky and Pagano, 1983). SM and GlcCer analogs are then delivered to preferential PM domains (Simons and van Meer, 1988). Furthermore, the recent introduction of green fluorescent protein (GFP)-tagged proteins may prove to be a useful tool to monitor protein transport in living cells. Indeed, using GFP-tagged proteins some characteristics of biosynthetic protein carriers have been reported in non-polarized cells (Toomre et al., 1998; Hirschberg et al., 1998). In non-polarized COS cells, post-Golgi carriers containing GFP-tagged vesicular stomatitis virus glycoprotein (VSVG) protein were reported to fuse directly with the PM without intersecting with other membrane transport pathways in these cells (Hirschberg et al. 1998), suggesting that the TGN is the sole compartment where molecules are sorted in the biosynthetic transport pathways. However, in multinucleated myotubes, present during early myogenic differentiation, VSVG protein was targeted to peripheral vesicles where it co-localized with the regulatable glucose transporter (Rahkila et al., 1998), the latter being processed along the endocytic route (Wei et al., 1998). In addition,

time-lapse confocal microscopy in polarized hepatic cells suggested transit of GFP-mdr1 protein through sub-apical compartments in the TGN-to-apical PM pathway (Kipp et al., 1998). These data raise intriguing questions as to the function of distinct post-TGN compartments in polarized transport, and how they relate to other polarized (endo-/transcytotic) traffic routes.

In comparison to the biosynthetic pathway, the intracellular compartments that comprise the endocytotic and transcytotic pathways have been characterized in considerable detail by electron and fluorescence microscopy, using internalized gold-, HRP- or fluorophore-labeled receptor ligands, fluid phase markers and/or lipids. These studies have revealed that basolateral to apical transcytosis involves an early passage through the basolateral early 'sorting' endosome (BEE). Here, transcytosing and recycling PM components are sorted from those that are destined for the degradative late endosomal-lysosomal pathway (Mellman, 1996; Mukherjee et al., 1997; Clague, 1998). Transcytosing proteins such as receptor-bound IgA (Apodaca et al., 1994; Barosso and Sztul, 1994), and (a part of) recycling PM receptors (Apodaca et al., 1994; Futter et al., 1998; Gibson et al., 1998) are then transferred to endosomal compartments that are typically oriented towards the apical PM domain (Apodaca et al., 1994; Barosso and Sztul, 1994). These compartments, termed sub-apical compartments (SAC; van IJzendoorn and Hoekstra, 1999), are accessible for proteins derived from both PM domains (Apodaca et al., 1994; Hughson and Hopkins, 1995; Knight et al., 1996; Odorizzi et al., 1996; Futter et al., 1998; Gibson et al., 1998). From the SAC, basolateral PM proteins such as the transferrin receptor (TfR) are targeted to the basolateral surface (Odorizzi et al., 1996; Futter et al. 1998; Gibson et al., 1998). Apical proteins are transported from the SAC to the apical surface, which may involve sub-apical intermediate compartments or apical recycling endosomes (ARE) (Gibson et al., 1998; Hemery et al., 1996; Brown et al., submitted). Hence, the SAC provides some highly interesting characteristics with respect to the processing of domain-specific PM proteins. Interestingly, endosomal compartments that are located in very close proximity of the apical PM have been described in a variety of polarized cells and have been implicated in the polarized, i.e. apical recruitment of molecules in a stimulus-dependent manner. Although many molecules may pass through the same subcellular compartments (e.g. BEE, SAC) during transcellular flow, individual transfer between such common compartments might involve distinct carrier vesicles (Schell et al., 1992). Sorting of molecules that display the same preferential target membrane not only adds an additional level of complexity, but also underscores the central role of the BEE and SAC. The compartments that are proposed to be involved in the different polarized sorting pathways are schematically depicted in figure 2.

Imperative to a correct analysis of the function of the distinct subcellular compartments and their inter-compartmental communication in the process of polarized trafficking, is the accurate definition of their boundaries. The characteristic kinetic properties that the different endosomes display with respect to the passage of molecules are helpful for such distinction. In addition, the use of different members of the small GTPase rab family, well-known modulators of specific intracellular traffic routes (Novick and Zerial, 1997) and

polarized sorting (Zacchi et al., 1998; Hunziker and Peters, 1998), in combination with specific polarized transport markers, may prove to be a powerful tool and pave the way to identify transport route-specific and transport routes-connecting compartments. In other words, to understand how and to what extent the different sorting pathways cooperate to secure cell polarity.

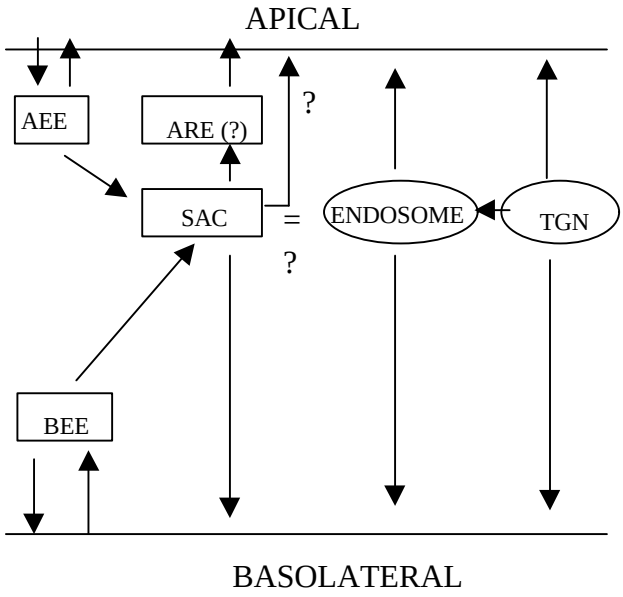

Figure 2. Schematic illustration of the compartments that may comprise the endo-/transcytotic pathway (squares) and the biosynthetic pathway (ovals) in polarized cells. Note that the arrows only show directions and might represent multiple pathways. {*AEE: apical early endosome, BEE: basolateral early endosome, SAC: sub-apical compartment, ARE: apical recycling endosome, TGN: trans-Golgi network.}*

Polarized sorting mechanisms

Polarized sorting and targeting includes *i)* the formation of separate carrier vesicles that are enriched in PM-specific molecules, *ii)* transport to the specific PM domain, and *iii)* the docking and fusion of vesicles with the correct membrane domain (see Weimbs et al., 1997; Aroeti et al., 1998; Yeaman et al., 1998 and references therein). Instrumental to the formation of basolateral and apical vesicles is the clustering of proteins and lipids into separate domains within the membrane of the sorting compartments. Basolateral sorting signals of most transmembrane proteins are located in their cytoplasmic tails (Hunziker at al, 1991), which frequently contain critical tyrosine residues that are predicted to adapt a β-turn. In addition, the occurrence of a di-leucine motif may serve as a basolateral determinant (Matter and Mellman, 1994; Mostov and Cardone, 1995; Aroeti et al., 1998). It has been proposed that the β-turn is a fundamental feature for basolateral sorting (Weimbs et al., 1997). Indeed, the basolateral signal of the polymeric immunoglobulin receptor (pIgR) lacks tyrosine or di-leucine motives, but does display a β-turn. Some basolateral sorting signals, but not all, overlap with clathrin-coated pit localization signals. Interestingly, the LDL receptor contains both signals and each is by itself sufficient for basolateral sorting (Matter et al., 1992). Possibly, these functional redundant basolateral signals may allow for controlled inclusion of the protein in distinct (clathrin or non-clathrin-mediated) sorting routes. Clathrin is an important component of the machinery that is involved in the clustering of basolateral proteins in the plane of the lipid bilayer and/or vesicle formation of basolateral proteins (Heilker et al., 1996; Futter et al, 1998; Le Borgne and Hoflack, 1998). Clathrin-coated

domains are present on different cellular membranes including the TGN, the basolateral PM (Pearse and Robinson, 1990; Matter and Mellman, 1994) and, as recently discovered, endosomes (Stoorvogel et al., 1996; Futter et al., 1998; Okamoto et al., 1998). Hence, the question arises as to how the clustering of proteins via their intrinsic basolateral sorting signal relates to the different pathways (e.g. TGN-to-PM, endosome-to-PM, or TGN-to-endosome-to-PM), leading to the (basolateral) target membrane. Subtle differences in the amino acid composition surrounding the critical tyrosine residue allow discrimination between basolateral targeting and endocytosis of the protein (Matter et al., 1994). The non-tyrosine-based basolateral sorting signal of the TfR appears to be sufficient for basolateral sorting in both the biosynthetic and recycling route, but is distinct from the internalization signal. Moreover, mutations in the basolateral signal of TfR were identified that selectively impair basolateral sorting of internalized receptor from the endocytic pathway, but do not affect basolateral sorting of newly synthesized receptor. (Odorizzi and Trowbridge, 1997). An additional level of regulation for the intracellular processing of basolateral proteins from different membranes may be provided by the interaction with compartment-specific adaptor protein (AP) complexes. Indeed, AP-1 adaptor complexes that associate with clathrin on apical endosomes and the TGN, were reported to be immunologically distinct (Okamoto et al., 1998b). In addition, the adaptor protein subunits may display varying affinity for distinct signals. The specificity of binding of tyrosine-based sorting motifs to μ-chains of the adaptor complex was shown highly sensitive to the context in which the motif lies (Stephens and Banting, 1998). Moreover, adaptor protein subunits have been suggested to provide a mechanism for compartment-specific inclusion of proteins in clathrin-coated vesicles (Hirst and Robinson, 1998).

The lipid environment has also been reported to influence the interaction between adaptors and sorting signals (Rapoport et al., 1997). Moreover, different coat proteins such as clathrin, COPI and COPII, can induce vesiculation from cargo-free acidic phospholipid-containing liposomes (Matsuoka et al., 1998; Takei et al., 1998), thus bypassing recognition of proteinaceous sorting signals. Interestingly, the specificity of the acidic phospholipid requirement differs between each coat (Spang et al., 1998) and also differences in lipid head group composition and acyl chain saturation may influence coat recruitment (Matsuoka et al., 1998; Spang et al., 1998). The subcellular distribution of cytoplasmically orientated phospholipids and their possible involvement in the formation of apical and/or basolateral vesicles have not been addressed and remain to be investigated. On the other hand, the involvement of exoplasmic orientated lipids in polarized sorting have been addressed to a fair extent (see below).

The best-studied apical sorting signal so far is the glycosylphosphatidylinositol (GPI) anchor which, when added luminally to proteins, suffices their apical targeting (Lisanti et al., 1989). In addition, proteins can also be directed apically by signals in the transmembrane domain (Kundu et al., 1996) or via N-glycosylation at the ectodomain (Matter and Mellman, 1994). Clustering of apical proteins in the plane of the lipid bilayer has been proposed to involve their association (either direct or indirect) with glycosphingolipid- and cholesterol

-enriched domains or ‘rafts’ in the exoplasmic leaflet of the TGN thus functioning as apical platforms (Brown and Rose, 1989; Simons and Ikonen, 1997). A similar raft-mediated sorting mechanism might also operate in recycling endosomes in non-polarized cells (Mayor et al., 1998). Interestingly, these recycling endosomes are believed to be the terminal station in the endocytic pathway prior to PM delivery and harbor machineries that can direct PM proteins to specific PM domains (Hopkins et al., 1994). Moreover, the recycling endosomes have been proposed to be analogous to the SAC in polarized cells (Apodaca et al., 1994; Zacchi et al., 1998; van IJzendoorn and Hoekstra, 1999). Hence, sphingolipid-mediated sorting events, similar as others (see above), are likely to occur in multiple subcellular compartments that target molecules to different PM domains.

The apical raft-hypothesis has been deduced from the observation that apical proteins and glycosphingolipids can be co-purified from detergent-insoluble (at 4°C) extracts on a sucrose density gradient (Brown and Rose, 1989). Intriguingly, also SM is highly detergent-insoluble in the cold but, like galactosylceramide and sulfatide, is preferentially transported to the basolateral surface (van Meer, 1993; van der Bijl et al., 1996). In addition, non-apical proteins have also been found detergent-insoluble in the cold (Weimbs et al., 1997). These data are difficult to reconcile with a function of rafts as exclusive apical sorting platforms. Polarized hepatic cells target most apical proteins, including GPI-anchored 5’ nucleotidase, first to the basolateral domain from where the proteins are subsequently targeted apically (Bartles et al., 1989; Schell et al., 1992), but sphingolipids can be transported directly from the TGN to the apical domain in these cells (reviewed in Zegers et al., 1998). In addition, apical-directed transport of a GPI-anchored protein that did not include the basolateral surface has also been reported (Ali and Evans, 1990). The hepatocyte thus raises intriguing questions as to the extent by which apical resident proteins are sorted along the indirect route and whether such sorting mechanism may involve basolateral sphingolipid-enriched domains, i.e. moving to and/or from the basolateral surface. In Fisher rat thyroid (FRT) cells, GlcCer is sorted basolaterally despite the apical sorting of some non-GPI-linked proteins (Zurzolo et al., 1993). In concanavalin A-resistant MDCK cells, GlcCer is sorted to the apical surface, whereas GPI-linked proteins, still detergent-insoluble, are sorted to both PM domains (Zurzolo et al., 1994), suggesting the existence of distinct detergent-insoluble fractions. Moreover, these results implicate that polarized sorting of GlcCer and (GPI-anchored) apical proteins are not necessarily related. Recently, it has been demonstrated that two functionally distinct sphingolipid domains could be separated from low-density detergent-insoluble membrane fractions that were either enriched in SM or GlcCer (Iwabuchi et al., 1998). Moreover, the GlcCer-, but not the SM-enriched fraction contained caveolin and a substantial higher amount of cholesterol was present in the GlcCer-enriched fraction. Taken together with the observed preferential targeting of GlcCer and SM to different PM domains (Simons and van Meer, 1988), and that cholesterol depletion as well as caveolin antibody blocking specifically interfere with apical-directed transport (Keller and Simons, 1998; Scheiffele et al., 1998), it is tempting to suggest that distinct sphingolipid domains might be available for sorting to distinct membrane domains. It is noteworthy that entry into detergent-insoluble

domains per se may not be sufficient for apical targeting, as was shown for influenza virus hemagglutinin (Lin et al., 1998). Interestingly, annexin XIIIb may be specifically involved in the apical targeting of sphingolipid-enriched domains (Lafont et al., 1998). Additional experimental approaches, preferably those allowing to determine direct tracking of different sphingolipids, may be helpful to elucidate the potential existence of separate sphingolipid domains.

Some proteins that contain a basolateral sorting signal are targeted apically when this signal is removed or inactivated, suggesting the presence of a recessive apical signal. Whether such apical rerouting includes incorporation into sphingolipid domains remains unclear. Apical sorting of the membrane protein enteropeptidase was reported not to involve detergent-resistant association with sphingolipid-cholesterol rafts (Zheng et al., 1999). Similar results were found for CD3-ε, a nonglycosylated type I membrane protein (Alonso et al., 1997). Interestingly, it has been suggested that a cytoplasmic sorting machinery may exist for apical targeting of proteins, analogous to that described for basolaterally targeted proteins (Chuang and Sung, 1998; Alonso et al., 1997). In this respect it is interesting to note that brefeldin A, which interferes with functional coat complexes, was shown to inhibit SAC-to-apical transcytosis of pIgR (Barosso and Sztul, 1994), consistent with the proposed role of clathrin-AP-1-containing coats in apical recycling (Okamoto et al., 1998). AP-3 was suggested to be involved in non-clathrin-mediated sorting of vesicle-associated membrane protein (VAMP), but not TfR, from endosomes to produce synaptic vesicles in neuroendocrine PC12 cells (Lichtenstein et al., 1998). Synaptic vesicle biogenesis displays features closely resembling the formation of apical intermediate compartments from the SAC, a process that precedes apical delivery of pIgR-IgA in MDCK cells (Gibson et al., 1998). Interestingly, in these cells, TfR is excluded from such apical structures by clathrin-AP-1-based retrieval from the SAC (Gibson et al., 1998).

It thus appears that a variety of signals are available that regulate the sorting of proteins by including them into specific domains, giving rise to apically or basolaterally targeted vesicles. Moreover, the inclusion (or exclusion) of proteins into vesicle populations with distinct preferential PM destinations can be regulated by posttranslational modifications. It has recently been shown that the pIgR can reach the basolateral PM irrespective of an interaction with AP-1, and it was suggested that post-translational modification of pIgR can modulate its recruitment into AP-1/clathrin-coated areas in the TGN, thereby regulating the efficiency of its basolateral delivery (Orzech et al., 1999). Furthermore, phosphorylation of a serine residue in the cytoplasmic tail of the pIgR prevents its basolateral recycling after endocytosis. Instead, the protein is sorted in the BEE into an apical directed pathway (Casanova et al., 1990). In developing postnatal rat retinal pigment epithelial cells, a basolateral-to-apical switch was observed which may be the result of suppressed decoding of specific basolateral signals (Marmorstein et al., 1998). In addition, a pIgR mutant, in which the serine was replaced by an aspartate to mimic the phosphorylation, was already targeted apically from the TGN in MDCK cells (Aroeti and Mostov, 1994). The formation and composition of the subsequently formed pIgR-containing apical vesicles (insertion into

specific lipid domains?) remains as yet unclear. In a recent study, transcytosing IgA-pIgR was suggested to associate with rafts posttranslationally, possibly in sub-apical compartments, prior to apical delivery (Hansen et al., 1999). This observation is supported by the notion that the association of proteins with rafts appears to be subject to regulation, for instance by protein palmitoylation (Arni et al., 1998; Melkonian et al., 1999), or the addition of a GPI-anchor. In oligodendrocytes, which posses specialized PM domains and cognate apical- and basolateral-type pathways (de Vries et al., 1998), GPI was added to some proteins concomitant with the maturation of these cells (Kramer et al., 1997). That cellular chaperone proteins may be involved to accommodate GPI-anchored proteins in a lipid raft is suggested by the time-dependent detergent-insolubilization of GPI-anchored proteins that were introduced into living cells (van den Berg et al., 1995). On a cautionary note, an altered polarized PM distribution following experimental manipulation of putative sorting signals may not necessarily be related to changes in polarized transport. This is illustrated by the observation that the cytoplasmic domains of the $\alpha 5\beta 1$ integrin are sufficient to mediate sorting by PM domain-selective degradation and stabilization (Gut et al., 1998). These data underscore the importance of visualizing polarized protein transport in living cells.

Given the growing number of various sorting signals that each may be decoded in a regulated manner, it becomes rather difficult to envision how efficient polarized targeting can be ensured on a relatively short notice with a preferable limited number of protein/lipid clusters. This becomes even more apparent when considering that stimulation of polarized targeting (e.g. cAMP/PKA-enhanced apical targeting of sphingolipids and proteins (Hansen and Casanova, 1994; Roelofsen et al., 1998; Zegers and Hoekstra, 1997)) must be restricted to a subset of all cellular molecules and, preferably, compartments. In this respect it is noteworthy that ligand-receptor interaction at the cell surface was shown to elicit a signal transduction response that interfered with the trafficking of the protein complex but in a specific subcellular compartment, the SAC (Luton et al., 1998). Sequential sorting along transport routes that include (functionally) distinct subcellular compartments might relieve some of the ‘sorting stress’. Possibly, basolateral sorting of newly synthesized TfR may prove to be exemplary for such hypothesis as its basolateral delivery has been postulated to be orchestrated in the SAC (Futter et al., 1998). A challenge will be to unravel the interactions of different domains with yet be identified molecules that may function as a bridge to the different apical and basolateral directed transport routes.

Scope of this thesis

As outlined above, our knowledge of polarized sphingolipid transport is limited. In the work presented in this thesis, the polarized trafficking of sphingolipids in hepatic HepG2 cells and its regulation has been studied. By employing fluorescently tagged sphingolipid analogs, thus enabling to follow transport of different lipids in life cells, we have revealed the existence of bi-directional transcytotic pathways. Analogs of SM and GlcCer were found to be segregated in the apical to basolateral transcytotic pathway, a process that does not involve the Golgi apparatus. Thus, GlcCer displays a preferential basolateral localization, whereas SM is

preferentially targeted to the basolateral PM domain (Chapter 2). We have next analyzed the apical-to-basolateral transcytotic pathways in more detail by taking advantage of known membrane flow kinetics that allow to distinguish between endosomal compartments, in combination with other transport markers. In this way, we have pinpointed the involvement of a non-Golgi, endosomal compartment, located subjacent to the apical bile canalicular PM, which connects the basolateral and apical endocytic pathways. Indeed, apically derived sphingolipids co-localized with basolaterally endocytosed IgA in sub-apical compartments (SAC) in HepG2 that stably expressed the pIgR. It is demonstrated that sphingolipid sorting during apical-to-basolateral transcytosis is orchestrated in the SAC, an event that appears to be highly specific. Moreover, it is shown that transport of sphingolipids occurs by vesicular means (Chapter 3), raising important issues as to the membrane topology of sphingolipid sorting. We then further pursued the characteristics of the observed lipid segregation. Evidence is provided that strongly suggests that SM and GlcCer analogs are in distinct domains within the lumenal leaflet of the SAC. The polarized transport of these SAC-located domains appears to be differently regulated by modulators of membrane traffic and, interestingly, their direction of flow is subject to a molecular machinery that controls apical PM biogenesis (Chapter 4). Indeed, rerouting of the SM analogue from the SAC to the apical surface is dictated by the degree of HepG2 cell polarity, intracellular cAMP levels and protein kinase A activity, and may represent a physiologically highly relevant process (Chapter 5). Further characterization of SAC-to-apical traffic of SM and GlcCer revealed that the SAC-to-apical routes employed by SM and GlcCer in polarizing cells are distinguishable. Moreover, SAC-to-apical transfer of SM includes separate sub-apical intermediate compartments that, unlike the SAC, are dependent on microtubules for their spatial organization, and harbor rab11 (Chapter 6), a small GTPase that has been implicated in signal-induced apical deposition. Finally, data are presented in chapter 7 that suggest that in addition to transcytosing lipids, also newly synthesized sphingolipid analogs may pass through the SAC en route to the PM in MDCK cells. The role of the SAC in the establishment and maintenance of PM domain compositions is discussed in detail in chapter 8. Together, these studies have revealed novel insight into (polarized) sphingolipid sorting and the subcellular compartments involved in these events. In addition, these studies emphasize the suitability and the uniqueness of the HepG2 cell system to investigate apical PM biogenesis.