

University of Groningen

Microvillus Inclusion Disease. Lessons about the apical plasma membrane.

Golachowska, Magdalena Renata

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:

2011

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Golachowska, M. R. (2011). *Microvillus Inclusion Disease. Lessons about the apical plasma membrane.*
s.n.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

CHAPTER 1

**The epithelial apical plasma membrane:
structure-function relationships and dynamics at
the molecular level**

Magdalena R. Golachowska and Sven C.D. van IJzendoorn

*Department of Cell Biology, section Membrane Cell
Biology, University Medical Center Groningen, University of
Groningen, The Netherlands*

Chapter 1

ABSTRACT

The differentiation of the epithelial cell's plasma membrane into an apical and basolateral domain is an evolutionary adaptation that allows multicellular organs and organisms to sense and defend themselves against potentially hostile external environments, and to vectorally transport molecules between these external environments and body tissues. The apical plasma membrane domain, only ~7 nm thick and up to 400 μm^2 , is the first physical line of defense against the outside world. The specialized functions that are allocated to the apical plasma membrane are facilitated by a distinct and highly dynamic structural architecture and macromolecular composition of the apical surface. Defects in the structural architecture and/or macromolecular composition lay at the basis of severe diseases. Here we discuss recently discovered biological principles and molecular mechanisms that regulate apical plasma membrane structure, maintenance and dynamics, and how these are/ may be affected in human diseases.

INTRODUCTION

An estimated 7 nm thick and up to 400 square meters of apical plasma membranes, provided by millions of epithelial cells, is a major selective physical barrier between the body and the outside world. Epithelial cells develop apical plasma membranes as part of an evolutionary conserved cell polarity program that endows virtually all eukaryotic cells with molecular machineries to create an asymmetric distribution of plasma membrane proteins and lipids, cytoskeleton, subcellular organelles and intracellular transport pathways. As such, the apical plasma membrane domain is readily distinguished from the basolateral plasma membrane domain with regard to 1) function, 2) macromolecular (i.e., protein and lipid) composition, and 3) structural organization. Recent years have provided exciting new insights into the structure-function relationships, formation, maintenance and dynamics of the epithelial apical plasma membrane, often in relation to human disease, and the underlying cell biological principles and molecular mechanisms.

Protein and lipid composition of the apical plasma membrane

The apical plasma membrane is defined as the region of the plasma membrane located at the apex of the epithelial cell that is separated, in vertebrates, from the basal-lateral region by a ring of tight junctions. Typically, the protein and lipid composition of the epithelial apical plasma membrane is distinct from that of the basolateral plasma membrane. The exclusive localization or enrichment of selected proteins and lipids allows cells to protect the apical plasma membrane against a potential hostile external environment (e.g., formation of a mucus layer in the intestine or the “bicarbonate umbrella” in the bile ducts) and at the same time to selectively internalize and secrete components at this environment. The apical plasma membrane domain is generally enriched in proteins and (sphingo)lipids containing a high proportion of carbohydrate molecules (readily visualized by Periodic acid-Schiff staining) and cholesterol (Fig. 2). Sphingolipids and cholesterol can form detergent-insoluble microdomains or rafts

Chapter 1

that cluster selected apical proteins and may modulate their activity (Paladino et al., 2004). Lipid rafts may act as a primary sorter for newly synthesized *O*- and *N*-glycosylated (Potter et al., 2006) and/or GPI-anchored (Lisanti et al., 1988; Brown & Rose, 1992; Matter & Mellman, 1994; Yeaman et al., 1997; Mellman and Warren 2000) apical plasma proteins by facilitating their incorporation into microdomains at the Golgi apparatus (Meiss et al., 1982; Hannan et al., 1993; Paladino et al., 2004) that, upon MAL proteolipid-facilitated clustering into larger oligomers (Puertollano & Alonso, 1999; Cheong et al., 1999; Puertollano et al., 1999), give rise to apical plasma membrane-directed vesicles (van Meer & Simons, 1988; Klemm et al., 2009). The clustering ability of apical cargo has been proposed to recruit specific molecular motor proteins and facilitates cargo exit from intracellular sorting compartments and subsequent cargo trafficking to the apical plasma membrane. KIF1A, a microtubule-based motor, was shown to interact with lipid rafts upon dimerization (Klopfenstein et al., 2002), while actin motors, like myosins I, V and VI may oligomerize and perform some of the unconventional functions as tethering vesicles or actin-dependent membrane bending (Woolner & Bement, 2009). For a detailed description of the mechanisms that control the sorting trafficking of apical plasma membrane proteins we refer the reader to an excellent recent review (Weisz & Rodriguez-Boulan, 2009).

While sorting and polarized targeting of resident apical plasma membrane proteins appears a highly efficient process to enrich the apical domain with selected proteins and lipids, some resident apical plasma membrane proteins are delivered randomly to all regions of the membrane and subsequently selectively stabilized at the apical plasma membrane domain, while being displaced from the basolateral plasma membrane domain (random delivery and selective retention). Proteins may be retained or anchored via their interaction with other components of the membrane or with the underlying cytoskeleton, which will increase their time of residence in the apical membrane (Matter & Mellman 1994; Yeaman et al., 1997; Mellman and Warren 2000). The selective interaction with components of the cortical actin cytoskeleton or terminal web is believed to be responsible for the anchoring of cytoplasmic PDZ

The apical plasma membrane structures and functions

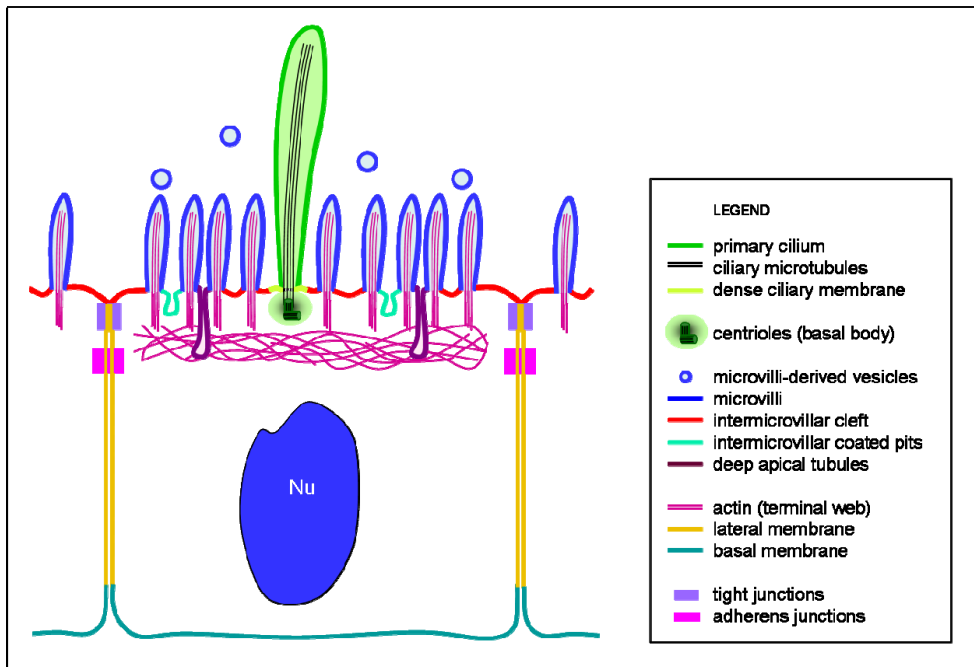


Figure 1. The subdomains of the epithelial apical plasma membrane. The apical plasma membrane can be structurally and compositionally subdivided in microvilli, cilia with dense membrane around the cilium base and the intermicrovillar zone which stretches in between the microvillar actin roots and can be further subdivided into the intermicrovillar cleft, intermicrovillar coated pits and deep apical tubules.

domain-containing transmembrane proteins, including gp135/podocalyxin (Meder et al., 2005), NHE-3 (Saotome et al., 2004) and CFTR (Swiatecka-Urban et al., 2002; Ostedgaard et al., 2003), at the apical plasma membrane (see also below). The (glyco)sphingolipids reside in the outer leaflet of the plasma membrane (Hoekstra et al., 2003) and are retained in the apical domain by tight junctions, multiprotein complexes composed of transmembrane proteins and cytosolic plaque proteins (Farquhar & Palade, 1963; Yap et al., 1997; Tsukita et al., 2001; Daugherty et al., 2004) that prevent their lateral diffusion to the basolateral plasma membrane domain (van Meer & Simons, 1986; Giepmans & IJzendoorn, 2009). The role of tight junctions in preventing the diffusion of apical plasma membrane proteins to the

Chapter 1

basolateral domain is debated (reviewed in Giepmans & van IJzendoorn, 2009). Tight junctions do not prevent the lateral diffusion of phosphoinositide lipids that reside in the inner leaflet. The polarized distribution of phosphatidylinositol 4,5-bisphosphate (PtIns (4,5)P₂ or PIP₂) in the apical plasma membrane domain and phosphatidylinositol 3,4,5-triphosphate (PtIns (3,4,5)P₃ or PIP₃) in the basolateral membrane (Gassama-Diagne et al., 2006; Martin-Belmonte et al., 2007), instead, is maintained by the localized activity of phosphatidylinositol 3-kinase (which converts PIP₂ to PIP₃) and phosphatidylinositol phosphatase (PTEN; protein and lipid phosphatase and tensin homologue deleted on chromosome ten; which converts PIP₃ to PIP₂) (Fig. 2) (Martin-Belmonte et al., 2007).

In conclusion, the epithelial apical plasma membrane domain displays a distinct macromolecular composition that is generated and maintained by intracellular sorting and targeting of these molecules, their local retention through interaction with other proteins or by tight junctions, and/ or their local synthesis.

Structural and compositional subdomains of apical membrane

Not all proteins in the apical plasma membrane show a completely overlapping distribution. Moreover, apical plasma membrane proteins show different lateral diffusion rates. Indeed, the development of novel microscopy techniques and macromolecular markers allowed a previously unrecognized organizational complexity of the apical plasma membrane to be revealed, and we can begin to sketch out the structural and molecular landscape of the epithelial apical surface. Generally, the epithelial apical plasma membrane can be structurally subdivided in several subdomains: microvilli, cilia and the intermicrovillar zone (Fig. 1). The intermicrovillar zone can be further subdivided into the intermicrovillar cleft, intermicrovillar coated pits that contain coated and uncoated endocytic vacuoles, and deep apical tubules. Selected proteins and lipids in the apical plasma membrane differentially localize to these structural subdomains which, for some of the proteins, has been correlated with their activity.

Microvilli

Microvilli are apical protrusions that are present in most epithelial cells and form a brush border that significantly increases the absorptive/secretory surface. Microvilli are particularly well developed in small intestinal epithelial cells, proximal tubule epithelial cells of the kidney and placental syncytiotrophoblasts, whereas epithelial cells of the pancreas, liver, and a number of commonly used epithelial cell lines display a lesser extent of microvilli number, size or organization. The microvillar membrane is highly enriched in rafts domains containing cholesterol, the ganglioside GM1 and the cholesterol binding protein prominin-1/CD133 (Janich and Corbeil, 2007; Corbeil et al., 2010), while it excludes another ganglioside, GM3 (Fig. 2) (Janich and Corbeil, 2007).

The typical appearance of microvilli is dictated by parallel actin bundles that project from a dense and complex meshwork of myosin-rich filaments below the apical plasma membrane, called the terminal web (Hanono et al., 2006). The terminal web is required for formation and stabilization of the microvilli-rich brush border and endows it with a contractile activity (Saotome et al., 2004; Mooseker et al., 1982). The apical plasma membrane is attached to the apical actin scaffold by cross-linking proteins belonging to the ezrin-radixin-moesin (ERM) family (Fig. 2) (Bretscher et al., 2002; McClatchey, 2003). Of all ERM proteins, ezrin shows the most restricted pattern of expression and is the only ERM protein detected in some epithelial cells, e.g. small intestinal epithelial cells (Berryman et al., 1993; Ingrassia et al., 2002). ERM proteins interact directly with F-actin via their C-terminal domain and with apical transmembrane proteins via their FERM domain (Hanono et al., 2006). ERM proteins also bind several cytosolic adaptors including EBP50/NHERF1 (ERM-binding phosphoprotein of 50kD/Na⁺/H⁺ exchanger regulatory factor), E3KARP (Exchanger 3 Kinase A Regulatory Protein) (Bretscher et al., 2000; Meder et al., 2005). Of these, those with PDZ domains (e.g., NHERF-1, -2) in turn interact with additional apical transmembrane proteins that also contain a PDZ-binding motif, including the Na⁺/H⁺ exchanger (NHE-3) (Fig. 2), cystic fibrosis transmembrane

Chapter 1

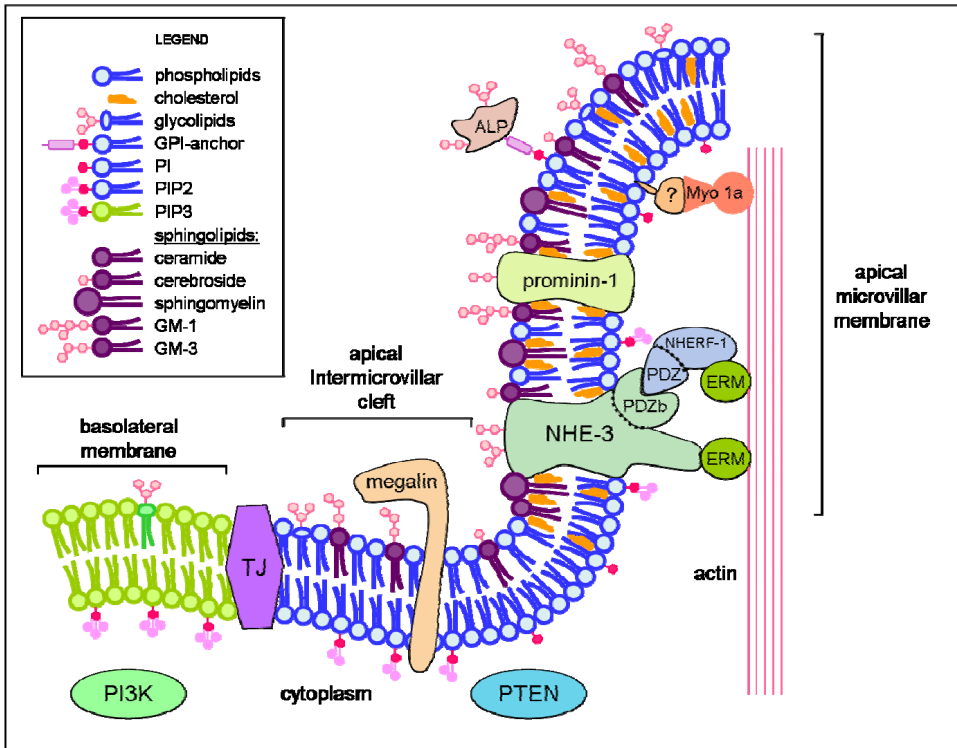


Figure 2. Differences in protein and lipid composition of the apical microvillar membrane, intermicrovillar cleft and basolateral membrane.

The apical plasma membrane domain is enriched in glycosylated proteins and lipids, and cholesterol. The outer leaflet of the apical membrane contains high level of sphingolipids, which together with cholesterol can form lipid rafts. The outer leaflet hosts also the glycosylated phosphatidylinositol (GPI)-anchored proteins, like ALP (alkaline phosphatase). The polarized distribution of outer leaflet components is maintained by tight junctions (TJ). The inner leaflet of plasma membrane is enriched in phosphatidylinositols (PI), with polarized distribution of PIP2 (apical) and PIP3 (basolateral) maintained by localized activity of two enzymes: PTEN, and PI3 Kinase, in apical and basolateral membrane respectively. The microvillar membrane exclusively contains prominin-1/CD133 a cholesterol binding protein and ganglioside GM-1. The intermicrovillar cleft exclusively contains megalin and ganglioside GM-3.

The microvillar membrane is attached to the apical actin scaffold by ezrin-radixin-moesin (ERM) family. The ERM proteins can bind the apical transmembrane proteins (e.g. NHE-3) directly or indirectly via PDZ domain-containing cytosolic adaptors (e.g. NHERF-1). The actin cytoskeleton is anchored to the microvillar membrane also through the myosin Ia (via yet unknown proteins or lipids), which participates in membrane shedding from the top of the microvilli and in brush-border-derived vesicles formation.

The apical plasma membrane structures and functions

regulator (CFTR), Crumbs-3, gp135/podocalyxin, platelet-derived growth factor receptor (PDGFR) (Reczek et al., 1997; Murthy et al., 1998; Yun et al., 1998; Short et al., 1998; Maudsley et al., 2000). ERM proteins thus provide complex scaffolds at the interface between the apical plasma membrane and the underlying actin network.

Ezrin plays a pivotal role in the formation of apical plasma membrane microvilli. ERM proteins exist in an “open”/active or “closed”/inactive state (Niggli & Rossy, 2008). The small GTPase RhoA is a well-known stimulus of ERM activation (Hirao et al., 1996; Shaw et al., 1998; Matsui et al., 1998). Proper ERM protein activity requires its phosphorylation and translocation to the apical plasma membrane-cytoskeleton interface. In the “open” phosphorylated conformation, ezrin interacts with proteins in the plasma membrane, F-actin, and multiple signaling molecules (Niggli & Rossy, 2008), creating a platform for the actin-rich brush border which is particularly prominent in intestinal epithelial cells (Saotome et al., 2004). Ezrin deficient mice show severe abnormalities in the shape of intestinal villi, fail to thrive and die within 3 weeks postnatal (Saotome et al., 2004). In addition, ezrin knockout in intestinal absorptive cells causes disorganization of the actin terminal web structure and formation of non-uniform, short and thick microvilli, which resemble immature microvilli of undifferentiated crypt cells prior to their migration and differentiation (Louvard et al., 1992; Saotome et al., 2004). Ezrin knock-out mice show a reduction in apical plasma membrane microvilli and basal infoldings in retinal pigment epithelium (RPE) accompanied by presence of microvilli-like inclusions (Bonilha et al., 2006). Also ezrin-binding proteins contribute to apical microvilli and brush border formation. Indeed, the knockdown of the ezrin-binding protein EBP50, the overexpression of an EBP50 mutant that do not bind to ezrin, or the overexpression of EPI64 mutants that do not bind EBP50 in JEG-3 cells (polarized human placental syncytiotrophoblasts) all lead to a reduced number of microvilli and the appearance of F-actin coated vacuoles in the cytoplasm (Hanono et al., 2006). Moreover, EPI64 is directly linked to Arf6-GTP, and overexpression of wild-type Arf6 or a dominant-active Arf6 mutant result in loss of microvilli and formation of vacuoles (Brown et al.,

Chapter 1

2001). In addition to ezrin, radixin-deficient mice are viable but develop hyperbilirubinemia as a result of a loss of the resident apical MRP2 transporter from bile canalicular membranes and loss of microvilli (Doi et al., 1999; Kikuchi et al., 2002).

Whereas ezrin is required for the formation of apical plasma membrane microvilli, ezrin deficiency does not interfere with the proper localization of apical and basolateral proteins and, hence, general epithelial surface polarity (Saotome et al., 2004). Ezrin was recently demonstrated to be one of the targets of the epithelial polarity-regulating Lkb1(Par4)/Strad/Mo25 complex in intestinal epithelial cells. Mo25 in this complex directly interacts with the serine/threonine kinase Mst4, and induces its translocation from the Golgi area towards a subapical membrane compartment where it phosphorylates and activates ezrin and initiates brush border formation (Klooster et al., 2009). Indeed, Mst4-depleted cells failed to recruit villin to the apical site of the cell and displayed an inhibition of brush-border formation (Klooster et al., 2009). By contrast, the polarized distribution of apical plasma membrane proteins and basolateral proteins was unaffected. However, the downregulation of Mo25 (which is upstream of Mst4) resulted in the inhibition of both brush border formation and cell surface polarity. These data indicate that the Mst4 and ezrin signaling pathway represents a branch of the Lkb1/Strad/Mo25-driven cell polarity program, which is specifically involved in apical brush border formation. In support of this, the expression of active ezrin in Ls174T cells or nonpolarized W4 cells results in the formation of a brush border in otherwise nonpolarized cells (Klooster et al., 2009). In conclusion, the presence or absence of apical microvilli can be uncoupled from general apical-basolateral polarity. Apical microvilli should therefore be considered as a structural and functional specialization of the apical surface, with ezrin as a key regulator.

Importantly, apical microvilli are not static structures. On the contrary, apical microvilli are highly dynamic and subject to growth and retraction over a period of minutes (Gorelik et al., 2003). The F-actin in microvilli, in which the barbed ends are

The apical plasma membrane structures and functions

associated with the tip, are tread-milling with the velocity of ~ 0.1 $\mu\text{m/s}$ (Waharte et al., 2005; Tyska & Mooseker 2002; Loomis et al., 2003). Moreover, the apical plasma membrane that covers the microvilli changes in short periods of time. In intestinal epithelial cells, unconventional myosin Ia anchors the microvillar plasma membrane (via yet unknown proteins or lipids) to the actin cytoskeleton, and moves along the actin bundles towards the tip of the microvilli while dragging the plasma membrane along (Fig. 2) (McConnell & Tyska, 2007; Nambiar et al., 2009; Tyska & Nambiar, 2010; Benesh et al., 2010). Accumulated membrane is subsequently shed from the top of the microvilli into the intestinal lumen (McConnell & Tyska, 2007). Brush-border-derived vesicles have previously been detected in the lumen of the small intestine (Black et al., 1980; Jacobs, 1983; DeSchryver-Kecsckemeti et al., 1989; Eliakim et al., 1989; Halhuber et al., 1994; van Niel et al., 2001). It is now evident that these are generated by apical microvilli via an active and regulated process. The microvilli-derived vesicles contain apical plasma membrane proteins, such as alkaline phosphatase and sucrase-isomaltase (McConnell & Tyska, 2007). Possibly, these microvilli-derived vesicles in the intestinal lumen may further increase the functional membranous surface area, and provide enzymes for food processing already in the lumen, before nutrients reach the actual surface of the enterocyte (Jacobs, 1983). It has also been speculated that the generation of apical microvilli-derived vesicles by neuroepithelial progenitors and other epithelial cells may have a role in tissue development and maintenance (Marzesco et al., 2005). As an alternative explanation, microvillar plasma membrane shedding may allow the enterocyte to continually modify its apical membrane composition in response to different demands in nutrient processing and absorption in the small intestine (Halhuber et al., 1994). Mice lacking myosin Ia, while viable, demonstrate a variety of defects, among which the most striking are herniations of apical membrane, irregularities in microvillar packing and abnormal variability in microvillar length (Tyska et al., 2005). It should be noted that the expression of *MYO1a* is restricted to gastrointestinal track and inner ear (Skowron et al., 1998; Skowron & Mooseker, 1999; Dumont et al., 2002; Donaudy et al., 2003),

Chapter 1

but other closely related class I myosins (Myo1b, Myo1c, and Myo1d) are also present in microvilli of intestine, kidney, liver and pancreas (Coluccio, 1997) and possibly contribute to the release of vesicles into the lumens of these organs (Beaudoin & Grondin, 1991).

The intermicrovillar zone

The intermicrovillar zone, located between the microvillar rootlets, is arbitrarily defined to extend 0.5-1 μm into the apical actin meshwork and can be subdivided into three zones: 1) the intermicrovillar cleft, 2) the intermicrovillar coated pits that contain coated and uncoated endocytic vacuoles and, 3) stable deep apical tubules (Yang et al., 2005). Interestingly, these microdomains are clearly visible *in vivo* in proximal tubules of (e.g. rat) kidneys (Yang et al., 2004), whereas most of the renal proximal tubule cells in culture show sparse microvilli and lack the well defined intermicrovillar coated pits microdomain (McDonough & Biemesderfer, 2003). The deep tubular invaginations of the apical plasma membrane are found in different cell types including neuronal, kidney proximal tubules and intestinal epithelia (Royle et al., 2003; Danielsen & Hansen, 2003; Hansen et al., 2003).

The intermicrovillar cleft is positive for the endocytic receptor megalin and excludes the cholesterol-binding proteins prominin-1 and prominin-2. The intermicrovillar cleft can also be distinguished from surrounding protruding subdomains by its lipid composition as it is relatively enriched in the ganglioside GM3 and relatively poor in cholesterol (Janich & Corbeil, 2007; Corbeil et al., 2010). The intermicrovillar coated pits might be distinguished with the clathrin adaptor protein AP2 (Yang et al., 2005), whereas the deep-apical tubules are positive for apical markers, annexin A2 (Danielsen et al., 2003), caveolin-1 and rich in cholesterol, and depleted of clathrin (Massol et al., 2005).

The intermicrovillar zone, particularly the deep-apical tubules, may serve as specialized lipid-raft microdomains for the docking, fusion and budding of membranous vesicles, for which the dense architecture of terminal web lining the

The apical plasma membrane structures and functions

brush border and only small patches of non-microvillar apical membrane might pose a steric barrier (Hansen et al., 2003). Deep-apical tubules are proposed to participate in specialized forms of traffic such as recycling of synaptic vesicles (see review in Royle et al., 2003), and recycling and transcytosis in polarized epithelial cells (Danielsen & Hansen, 2003; Hansen et al., 2003). The deep-apical tubules may serve as storage pools for selected apical proteins, including the Na^+/H^+ exchanger NHE-3. NHE-3, together with the Na^+/Pi co-transporter 2 (NaPi_2) mediate sodium and water reabsorption in the proximal tubule and are actively transported from the tip of the microvilli towards the intermicrovillar zone in response to acute hypertension or treatment with parathyroid hormone (Yang et al., 2004) or the angiotensin-converting enzyme inhibitor captopril (Riquier-Brison et al., 2010) in rats. In contrast to NaPi_2 , which is subsequently endocytosed, NHE-3 is not internalized but remains inserted in the membrane continuous with the apical membrane, and there is little if any NHE-3 colocalization with the clathrin adapter AP2 (marker of the intermicrovillar cleft). The distinct fate of NHE-3 and NaPi_2 may reflect their partitioning into distinct membrane domains as NHE-3 but not NaPi_2 associates with cholesterol-rich lipid rafts (Riquier et al., 2009). The redistribution of NHE-3 coincides with a similar redistribution of myosin VI, an unconventional myosin motor that moves along the actin filaments towards the pointed ends of actin at the base of microvilli (Yang et al., 2005). During the translocation of the NHE-3 from the microvilli to the intermicrovillar zone the protein changes from its active 9.6S oligomeric brush-border form into an inactive 21S megalin-associated NHE-3 in dense tubules. The latter may serve as a reservoir of NHE-3 for its rapid regulation (Biemesderfer et al., 2001). Upon stimulation of the cells with angiotensin II, which stimulates sodium and water absorption, NHE-3 and NaPi_2 , concomitant with myosin VI, reenter the apical microvilli (Riquier-Brison et al., 2010). The deep-apical tubules were shown to consist of dense lipid-rafts and contain high levels of cholesterol, and may also function as a cell surface membrane reservoir for cholesterol and for rapid adaptive changes in the size of microvilli at the brush border. Depletion of cholesterol caused significant reduction in number of deep

Chapter 1

tubules which resulted in shortening of microvilli (Danielsen & Hansen, 2003, Hansen et al., 2003). Hence, the composition and dynamics of the intermicrovillar zone (in particular the deep apical tubules) appears to be closely related to that of the apical microvilli.

Cilia

Almost all of vertebrates' polarized cells contain a cilium – a long microtubule-based hair-like protrusion ($\sim 0.2 \mu\text{m}$ in diameter and $\sim 5 \mu\text{m}$ in length) that projects out from the centre of the apical membrane (Meder et al., 2005; Praetorius & Spring, 2005). Motile and non-motile cilia can be distinguished. The motile cilia are present in bundles on the cells' apical surface of trachea, intestines, ependymal cells lining brain ventricles and reproductive ducts, where they participate in moving the mucus, intestinal or cerebrospinal fluids or the cells, respectively. A single non-motile cilium (also called the primary cilium) is present on almost every mammalian cell, and serves as sensory organelle, providing chemo-, thermo-, photo- or mechano-sensation (Handel et al., 1999; Brailov et al., 2000; Gerdes et al., 2009). The primary cilium outgrowth is one of the final events in the polarization of epithelial cells monolayers (Bacallao et al., 1989). The discovery of the cilium dates back to 1898, but the importance of it was only recently acknowledged. The primary cilia coordinate a large number of cellular signaling pathways (e.g. Hedgehog, Wnt and planar cell polarity), and are closely related to cell division and differentiation (Michaud & Yoder, 2006; Satir & Christensen, 2007).

Although the cilium membrane is continuous with the surrounding apical plasma membrane, its protein composition differs significantly. For instance, the cilium plasma membrane and the area around the cilium is depleted of typical apical brush border proteins such as GPI-linked proteins (Vieira et al., 2006), gp135/podocalyxin, NHERF-2 (Meder et al., 2005) and contains membrane proteins that are not found in the other parts of the apical membrane, e.g. polycystin-1 and -2, which are implicated in polycystic kidney disease (Pazour & Witman, 2003). It is not understood how

The apical plasma membrane structures and functions

lateral diffusion of different proteins residing in the apical plasma membrane and cilium is prevented. However, there is evidence for an intramembraneous fence that shows unusually high condensation around the base of the cilium, as evidenced by staining with the Laurdan dye which intercalates between lipids and the emission spectrum of which changes as a function of membrane fluidity (Vieira et al., 2006). There is also evidence that cilium proteins use distinct apical plasma membrane-directed trafficking routes in comparison to other apical plasma membrane proteins (see below).

The vertebrates' cilium may require up to 1000 different polypeptides for its function (Gherman et al., 2006). In contrast to the surrounding actin-based apical microvilli, the cilium cytoskeleton is built by microtubular network, called "axoneme". The axoneme extends from the basal body, which is embedded in the cytoplasm and is built by subapical centriolar microtubular triplets. The basal body and axoneme are separated by a transition zone, which might also serve as a docking station for transport and motor proteins (Hong et al., 2003, Bartoloni et al., 2001).

The formation of the primary cilium is tightly linked to cell cycle progression and the polarization processes. The centrioles, forming the basal body for cilium outgrowth (Fig. 1), serve also as the anchoring structures for mitotic spindles. Although they might easily interconvert, there are no reports of the cells having both mitotic spindles and cilia at the same time. Actually, the formation of primary cilia occurs postmitotically in quiescent cells (Gerdes et al., 2009). When cells re-enter the cell division cycle, the cilium and basal body are disassembled (via a poorly understood process) and centrioles liberated to take the function of the organizing centre for the mitotic spindles (Badano et al., 2005). The initiation of cilium formation requires the deactivation of a complex of two centrosomal proteins Cep97 and CP110 (Spektor et al., 2007). The suppression of these proteins results in assembly of cilia-like structures, containing ciliary markers including glutamylated and acetylated tubulin, polaris/IFT88, polycystin-2 and centriole marker centrin, even in the cells that had not exited from the cell cycle (Keller & Marshall, 2008). By contrast, the

Chapter 1

overexpression of CP110 in quiescent cells suppressed the formation of the primary cilia (Spektor et al., 2007). During ciliogenesis, cilia elongate from the basal body to the distal tip through the addition of new axonemal subunits organized in macromolecular particles. Several basal body and ciliary proteins (such as inversin (Nürnberg et al., 2002)) colocalizes with polarity complexes (Par3/Par6/aPKC ζ and CRB3/PATJ1/PALS) at the cellular junctions and cilium, and participate in cilium formation. The polarity complexes are also necessary for cilium formation, as deletion of CRB3 inhibits ciliogenesis in MDCK cells (Fan et al., 2004) and deletion of Par3 or aPKC decreases the length of cilium.

Polarity complexes reorganize the cytoskeleton and polarized trafficking pathways. The formation of the cilium requires functional apical transport machinery and perturbation in it results in defects in cilium formation or length. This is exemplified by the knockdown of FAPP-2. FAPP-2, or phosphatidylinositol 4-phosphate adaptor protein 2, is a glucosylceramide-transfer protein that plays crucial role in glycosphingolipids synthesis and localizes in the trans-Golgi network through interaction with phosphatidylinositol 4-phosphate and the small GTPase ARF1 (D'Angelo et al., 2007; Godi et al., 2004) where it participates in the sorting and apical delivery of newly synthesized proteins and lipids. FAPP2 knockdown inhibits cilium formation in Madin-Darby canine kidney (MDCK) cells concomitant with a transient accumulation of subapical transport vesicles in the vicinity of the microtubule organizing centre was observed (Vieira et al., 2006). Interestingly, FAPP2 deficiency does not generally perturb polarized trafficking of apical (gp135/podocalyxin) neither basolateral (gp58/ Beta-subunit of Na⁺K⁺-ATPase and E-cadherin) proteins, and does not affect the barrier function of tight junctions. However, galectin-3 and raft-associated Forssman glycolipid (FGL), which in control cells are located exclusively on the apical plasma membrane, were (partially) missorted to the basolateral membrane (Vieira et al., 2006). In addition to FAPP-2, vesicular transport of newly synthesized ciliary proteins from the Golgi apparatus is facilitated by the small GTPase Rab8 which, in turn, is activated by a complex of

The apical plasma membrane structures and functions

Rabin8 and BBS1 proteins in the BBSome in the cilium basal body. GTP-Rab8-marked vesicles are transported to the base of the cilium where they may dock at the ciliary membrane and/or are transported up along the axoneme with help of the motor protein kinesin-2 (and KIF3A, KIF3B, KAP3) complexed with at least 10 intraflagellar transport (IFT) particles B (Rosenbaum & Witman, 2002; Scholey, 2008). The retrograde intraflagellar transport from the tip of cilium towards the cell body is facilitated by a motor protein dynein with a complex of 6 IFT particles A (Rosenbaum & Witman, 2002; Scholey, 2008). The Rab8-dependent trafficking route is taken by, among others, newly synthesized rhodopsin (Moritz et al., 2001). Rhodopsin is transported from the Golgi apparatus to the photosensitive outer segment of photoreceptor cells through a connecting cilium, an outgrowth of the inner segment that is the equivalent of the apical membrane in epithelial cells (Deretic & Papermaster, 1993; Sung & Tai, 2000). Rhodopsin fuses with a specialized site surrounding the cilium (Sung & Tai, 2000) and diffuses along the ciliary membrane, but might also be propelled into the outer segment by cytoskeletal motors and IFT particles B (Beech et al., 1996). Rab8a was shown to interact directly with cenexin/ODF2, a centrosomal and basal body component (Yoshimura et al., 2007) and loss of Rab8 results in the accumulation of vesicles at the base of the photoreceptor connecting cilium (Moritz et al., 2001).

Defects in cilia-assembly are associated with various genetic disorders characterized by defects in the growth and polarity of epithelial cells. The loss of ciliary components such as KIF-3A results in the loss of cell polarity and defective ciliogenesis in vitro (Fan et al., 2004; Lin et al., 2003), and mutation in Tg737/Ift88 (polaris/Ift88) mislocalizes the epidermal growth factor receptor (EGFR) to the apical membrane of renal cysts in mice (Taulman et al., 2001). Defects in the sensory function of primary cilia are pronounced in polycystic kidney disease (PCD) (Pazour & Witman, 2003), liver and pancreas diseases, nephronophthisis, Bardet-Biedl syndrome (Badano et al., 2006), Alstrom syndrome, Meckel-Gruber syndrome, most of which represents with kidney and gonadal abnormalities, pancreatic and hepatic

Chapter 1

fibrosis, obesity, diabetes, polydactyly, brainstem malformation and mental retardation, situs inversus and retinal degeneration (for comprehensive reviews, see Fliegauf et al., 2007; D'Angelo & Franco, 2009). Most of these disorders are caused by mutations in genes encoding for proteins that localize and function at the cilium, basal body and/or centrosome, and participate in Rab8-dependent vesicular transport from the Golgi apparatus to the ciliary plasma membrane (Nachury et al., 2007). In addition, defects in primary cilium are associated with numerous cancers, where the ligand-induced activity of Sonic hedgehog (Shh) and platelet-derived growth factor receptor- α (PDGFR- α) signal transduction pathways are altered (Yu J et al., 2003; Michaud & Yoder, 2006).

In summary, the apical plasma membrane consists of structurally distinct subdomains (Fig. 1). Selected apical proteins and lipids are actively segregated and retained in/at these structurally distinct apical plasma membrane subdomains. There is a dynamic bidirectional lateral movement of proteins between some of these subdomains which involves the action of nonconventional myosins. In some cases this is regulated by external cues and likely to be physiologically relevant. The apical plasma membrane domain is a highly dynamic organelle as microvilli can grow and shrink in response to stimuli and produce extracellular membrane vesicles. Deep apical tubules may be used for temporal storage of proteins and lipids and/or, as they penetrate the terminal web, for efficient interaction with the intracellular environment, e.g., other organelles or transport vesicles.

Structural and compositional remodeling of the apical plasma membrane by the apical plasma membrane recycling system

While the apical plasma membrane evidently displays considerable intrinsic dynamics to accommodate changing requirements, it is also intimately associated with the underlying endosomal system which plays an important role in shaping and remodeling the apical plasma membrane. The apical recycling system comprises a collection of functionally distinct compartments including apical early endosomes, the

The apical plasma membrane structures and functions

common recycling endosomes, and the apical recycling endosome (Fig. 3; see also van IJzendoorn & Hoekstra, 1999; Hoekstra et al., 2004; and Golachowska et al., 2010 for detailed reviews of the apical recycling system). Of these, the apical recycling endosomes are positioned closest to the apical plasma membrane domain (Goldenring et al., 1996) and can be marked by the presence of the small GTPase rab11a and its effector proteins (Rab11a-FIPs and myosin Vb), the presence of apical proteins and the absence of basolateral proteins. Apical recycling endosomes may contain apical plasma membrane components from different subcellular origins, and rapidly translocate to the apical plasma membrane in response to certain stimuli. In this way, apical recycling endosomes can adjust the number of receptors, channels and transporters in the apical plasma membrane with a high temporal resolution. This is well illustrated by the translocation of bile salt transporters from Rab11a-positive apical recycling endosomes to the bile canalicular plasma membrane of hepatocytes, concomitant with enhanced biliary secretion, in response to extracellular factors that stimulate the cAMP-protein kinase A (PKA) signaling cascade (Kipp & Arias, 2000; Kipp et al., 2001). Apical recycling endosome translocation appears to depend on actin filaments because cellular depletion of the Rab11a-effector and actin-based molecular motor protein myosin Vb or the expression of a dominant-negative motorless tail domain of myosin Vb in polarized Madin-Darby canine kidney (MDCK) cells (Lapierre & Goldenring, 2005), human epithelial colorectal adenocarcinoma Caco-2 cells (Ruemmele et al., 2010), human sub-bronchial gland Calu-3 cells (Swiatecka-Urban et al., 2007) and rat hepatoma/human fibroblast hybrid WIF-B9 cells (Wakabayashi et al., 2005) inhibit protein recycling to the apical plasma membrane and/or result in the accumulation of resident apical plasma membrane proteins in compartments in the subapical cytosol. An exciting possibility is that apical recycling endosome dynamics may be regulated by the primary cilium in polarized proximal tubule epithelial cells in the kidney. This may be suggested by observations that angiotensin receptors and NHE-3 translocate from condensed apical recycling endosomes, which cluster at the base of the primary cilium, to the apical

Chapter 1

plasma membrane in response to fluid flow, which is sensed by the primary cilium of these cells (Praetorius & Spring, 2003). Apical recycling endosome dynamics is also regulated by galectin-4. Galectin-4, which has a high affinity for lipid-rafts and N-linked carbohydrates, is secreted at the apical surface and, upon subsequent endocytosis and arrival in the apical recycling endosomes, stimulates the translocation of apical plasma membrane proteins to the apical surface via a mechanism that may involve raft clustering (Stechly et al., 2009; Schuck & Simons, 2004).

In addition to regulating the number of proteins at the apical surface, apical recycling endosomes can also structurally remodel the apical plasma membrane. For instance, in the acid-producing parietal cells of the stomach, H^+,K^+ -ATPase proton pumps are temporarily stored in the limiting membrane of vacuoles beneath the apical

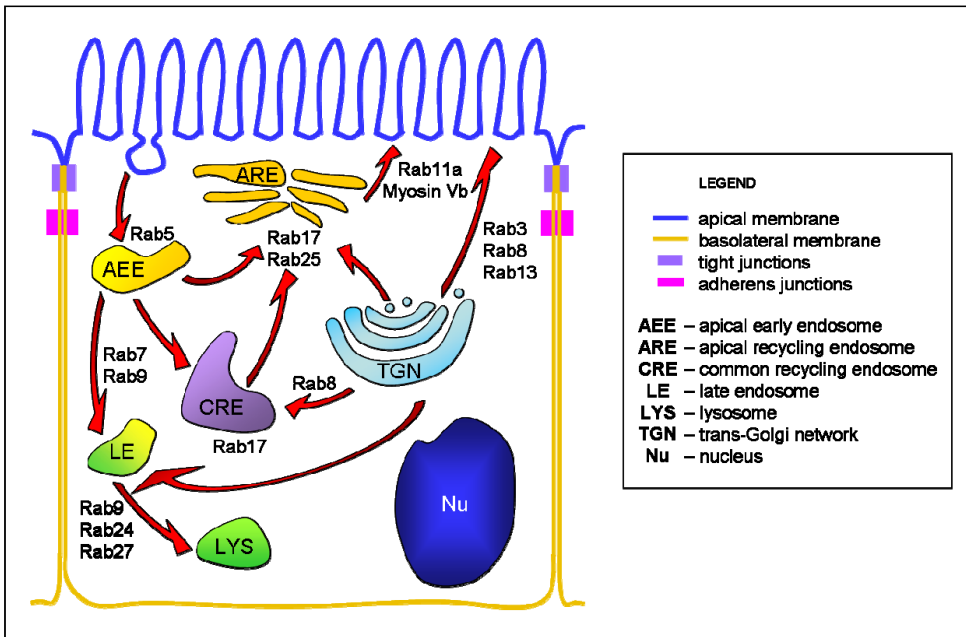


Figure 3. The apical (re)recycling routes in polarized epithelial cells. The apical recycling system comprises a collection of functionally distinct compartments including apical early endosomes, the common recycling endosomes, and the apical recycling endosome.

The apical plasma membrane structures and functions

surface. Following a stimulus (e.g., the presence of food, neuronal stimuli, etc.; often stimulating cAMP-PKA signaling) the vacuoles translocate and fuse with the apical plasma membrane, resulting in the efficient release and additional translocation of protons from the vacuoles into the external environment. Upon withdrawal of the stimulus, protons are no longer needed and the H⁺-ATPase proton pumps are re-internalized and stored again in vacuoles, already pumping there protons for the next digestion round. The massive translocation of Rab11a positive H⁺/K⁺-ATPase-containing vesicles to the apical plasma membrane (Forte et al., 1981) greatly expands the secretory surface which, concomitantly, is reorganized to form deeply invaginated canaliculi (Forte et al., 1977; Helander & Hirschowitz, 1972) with elongated apical microvilli. Such a structural reorganization allows a maximum secretory capacity of the apical plasma membrane. In opposition, the depletion of Rab11a or its effector myosin Vb in hepatocytes results in the loss of bile canalicular plasma membranes (Wakabayashi et al., 2005).

Consistent with the role of ezrin in controlling apical microvilli length (see above), apical membrane recycling in parietal cells was demonstrated to rely on a fast turnover of ezrin phosphorylation. Moreover, ezrin interacts with and is phosphorylated by PKA type I. Interestingly, in hepatocytes, the downregulation of another ERM protein, radixin, results in the loss of apical microvilli and the accumulation of associated apical plasma membrane proteins in Rab11a-positive apical recycling endosomes (Wang et al., 2006). ERM proteins, possibly also via interacting adaptor proteins such as NHERF-1/EBP-50, might therefore provide a molecular link between apical recycling endosome dynamics and structural remodeling of the apical plasma membrane in response to external stimuli.

Microvillus Inclusion Disease

The inability of intestinal cells to develop an apical brush border and the consequences it has on human functioning becomes particularly apparent in patients diagnosed with microvillus inclusion disease (MVID) (OMIM 251850). MVID is a

Chapter 1

rare hereditary enteropathy presenting with severe intractable diarrhea and malabsorption in neonates (Phillips et al., 1985; Cutz et al., 1989; Sherman et al., 2004; Goulet et al., 2004). At the cellular level, brush border atrophy with accumulation of lysosomal granules is observed in the apical cytoplasm of MVID enterocytes (Phillips et al., 1985, 1992; Ruemmele et al., 2006; Iancu et al., 2007). Apical brush border components are typically absent from the cell surface and accumulate in the apical cytoplasm of some cells (Ameen & Salas, 2000; Groisman et al., 2002). Indeed, the most striking phenotype is the appearance of microvillus inclusions in the apical cytoplasm of a variable number of enterocytes. The origin of these microvillus inclusions is unclear. They have been proposed to be the result of a massive autophagocytic internalization of the apical plasma membrane domain (Reinshagen et al., 2002). Autophagocytosis is a pro-survival process of cellular self-digestion of (redundant or faulty) proteins and organelles that occurs during nutrient deprivation, endoplasmic reticulum stress, developmental programmed cell death, microbial infection, and diseases characterized by the accumulation of protein aggregates. In MVID enterocytes the apical membrane can form large invaginations toward the cell interior which may enclose and detach creating an inclusion.

The large and differentiated vacuoles that contain microvilli and apical markers (sucrase-isomaltase) were negative for lysosomal markers (LAMP-1). However, additional lumens were detected that contained both sucrase-isomaltase and LAMP-1, suggesting that microvillus inclusions are eventually subject to degradation. Uptake experiments on fresh MVID intestinal biopsies revealed ferritin and ovalbumin accumulation in the microvillus inclusions, indicating that microvillus inclusions are related or linked to apical endocytic pathways and the apical endosomal system.

Because basolateral proteins display a normal polarized distribution at the surface of MVID enterocytes (Michail et al., 1998; Ameen & Salas, 2000) which appear normally arranged in monolayers with distinguishable cell-cell adhesion junctions, a defective intracellular trafficking of apical brush border proteins in MVID has been proposed to be part of the pathogenic mechanism (Ameen & Salas, 2000). The exact

The apical plasma membrane structures and functions

nature of such a traffic impairment remains obscure but may occur in the biosynthetic pathway via which newly synthesized brush border proteins are delivered from the trans-Golgi network to the cell apex. This pathway, at least for some brush border proteins, involves passage through Rab11a-positive apical recycling endosomes. In this regard it is interesting to note that the *de novo* (i.e., protein synthesis dependent) formation of microvillus inclusions has been observed in cultured human (intestinal) epithelial cells and has been proposed to be the result of homotypic fusion of post trans-Golgi network apical vesicles that are prevented from fusion with the cell surface (Vega-Salas et al., 1987, 1988).

Alternatively, but not necessarily mutually exclusive, the traffic defect may be in the apical recycling route via which apical brush border proteins are recycled back to this surface domain (Hoekstra et al., 2004). As described above, the small GTPase Rab11a and a family of Rab11a-interacting proteins (FIPs) (Hoekstra et al., 2004; Peden et al., 2004; Prekeris, 2003; van IJzendoorn, 2006; Prekeris et al., 2000) that also includes myosin Vb, regulate apical recycling in cultured epithelial cells (Lapierre et al., 2001; Swiatecka-Urban et al., 2007; Nedvetsky et al., 2007). Furthermore, apical recycling endosome dynamics have been implicated in the biogenesis of apical surface domains, although direct *in vivo* evidence for such a relationship has not been presented thus far (van IJzendoorn & Hoekstra, 1998; van IJzendoorn & Hoekstra, 2000; Wakabayashi et al., 2005; Li et al., 2007). Because of the specific loss of apical plasma membrane identity, MVID provides an outstanding opportunity to study the genetics and molecular dynamics that underlie apical surface development, and the potential connection to the apical endosomal recycling system.

Chapter 1

Outline of the thesis

The aim of this thesis is to elucidate the genetic cause and obtain insight into the pathogenesis of microvillus inclusion disease and, in this way, obtain insight into molecular mechanisms that control apical plasma membrane development and dynamics in general. Following this introduction to the molecular landscape, structure-function relationships and dynamics of the epithelial apical plasma membrane, we describe in Chapter 2 of this thesis how we have used homozygosity mapping and genomic DNA screening of three patients diagnosed with microvillus inclusion disease (MVID) to identify causative mutations. The gene that is responsible for MVID was identified and by performing immunohistochemistry on patients' material the resultant cellular consequences were investigated. In Chapter 3, we performed immunohistochemical analyses to determine whether the characteristic apical plasma membrane defects in MVID patients were restricted to specific parts of the intestine or, instead, a feature of epithelial cells along the entire horizontal axis of the intestine. In addition, we investigated the general structural and compositional architecture of the apical plasma membrane in the proximal tubule epithelial cells of the kidney of MVID patients who developed renal Fanconi syndrome and, in this way, obtained information with regard to organ specificity. In Chapter 5 we investigated the effect of an environmental factor, i.e., extracellular acidosis, on the dynamics of the apical plasma membrane, and in Chapter 6 we investigated the effects of extracellular acidosis on the structure and function of the Golgi apparatus. In Chapter 7 recent developments, which include data described in this thesis, with regard to the involvement of recycling endosomes in apical plasma membrane formation and epithelial cell polarity is reviewed.