Implication of lipid microdomains in regulation of protein trafficking and epithelial cell morphology

THESIS
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by
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Date of oral exam: 31.07.2009
To Hau'i and my family

Annene, Babawo na Sinan a

Hau'i ye.
Nothing shocks me. I’m a scientist.

Harrison Ford (1942 - ) as Indiana Jones
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PUBLICATION


This study reports about interactions between prostate-specific membrane antigen (PSMA) and lipid microdomains isolated by different detergents (Tween-20-DRMs, Triton X-100-DRMs and Lubrol WX-DRMs) and thereby having different biochemical properties (different lipid composition). These different DRMs have revealed that distinct glycoforms of PSMA was associated with each of them, in various intracellular compartments and at the plasma membrane. I contributed to this study by performing the isolation of Lubrol WX-DRMs containing biotin-labelled surface proteins. I was thereby able to demonstrate an association of the mature PSMA molecules with the Lubrol WX-DRMs at the plasma membrane.

POSTER

GPI-Anchor Dictates Trafficking of Membrane Dipeptidase
Zeynep Hein, Nigel M. Hooper, Hassan Y. Naim
16th Meeting of German Veterinary Society Division Physiology and Biochemistry, Gießen (19-21/02/2006)

GPI-Anchor Dictates Trafficking of Membrane Dipeptidase
Zeynep Hein, Nigel M. Hooper, Hassan Y. Naim
Experimental Biology Annual Meeting, Washington, DC (28.04/ 02.05/2007)

Annexin-2 regulates membrane microdomain formation and influences viral entry into host cell
Zeynep Hein, Jörg Glende, Christel Schwegmann-Weßels, Georg Herrler, Hassan Y.Naim
Poster presentation for PhD Program „Veterinary Research and Animal Biology“ at the University of Veterinary Medicine, Hannover
ORAL PRESENTATION

*How does impaired membrane transport affect cellular defence mechanisms?*
Centrum for Infection Biology at the University of Veterinary Medicine, Hannover, Annual Meeting, Hannover (07/10/2006)

*Annexin-2 regulates membrane microdomain formation and influences viral entry into host cell*
Seminars in Biochemistry and Virology, Hannover (23/05/2007)

*How does Annexin A2 regulate epithelial polarity and viral entry?*
17th Meeting of German Veterinary Society Division Physiology and Biochemistry, Leipzig (09-11/03/2008)

*Annexin-2: A key player in the development of brush border membrane*
Seminars in Biochemistry and Virology, Hannover (19/11/2008)
LIST OF ABBREVIATIONS

SI units were used throughout this manuscript. All abbreviations mentioned in the text are listed below.

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<tr>
<td>AEE</td>
<td>apical early endosome</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ACE-2</td>
<td>angiotensin converting enzyme 2</td>
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<tr>
<td>anx</td>
<td>annexin</td>
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<tr>
<td>AP</td>
<td>apical</td>
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<tr>
<td>aa</td>
<td>amino acid</td>
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<tr>
<td>BBM</td>
<td>brush border membrane</td>
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<tr>
<td>BEE</td>
<td>basolateral early endosome</td>
</tr>
<tr>
<td>BL</td>
<td>basolateral</td>
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<tr>
<td>DPPIV</td>
<td>dipeptidyl peptidase</td>
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<tr>
<td>DRM</td>
<td>detergent-resistant membranes</td>
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<tr>
<td>EE</td>
<td>early endosome</td>
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<td>Fig.</td>
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<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>GDP-dissociation inhibitor</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine-nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GIT</td>
<td>gastrointestinal tract</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GPI-APs</td>
<td>GPI-anchored proteins</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>haematoxylin and eosin</td>
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<tr>
<td>K</td>
<td>keratin</td>
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<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MTOC</td>
<td>microtubule organizing center</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
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<tr>
<td>PI</td>
<td>phosphoinositide</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PI(3)P</td>
<td>phosphatidylinositol-3-phosphate</td>
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<td>PI(4)P</td>
<td>phosphatidylinositol-4-phosphate</td>
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<tr>
<td>PI(4,5)P₂</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
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<tr>
<td>PI(3,4,5)P₃</td>
<td>phosphatidylinositol-3,4,5-triphosphate</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
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<tr>
<td>PI5K</td>
<td>phosphatidylinositol 5-kinase</td>
</tr>
<tr>
<td>RE</td>
<td>recycling endosome</td>
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<tr>
<td>SI</td>
<td>sucrase isomaltase</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodiumdodecylsulphate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>TGN</td>
<td>trans-Golgi Network</td>
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<tr>
<td>WASp</td>
<td>Wiscott-Aldrich Syndrome protein</td>
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<tr>
<td>WB</td>
<td>Western blot</td>
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<td>wt</td>
<td>wild type</td>
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CHAPTER 1

INTRODUCTION
1. **INTRODUCTION**

In living organisms, homeostasis is maintained when cells transport and target proteins, lipids, and other organic components timely and accurately. All diseases emerge due to alterations in such elementary cellular processes caused by extrinsic or genetic factors. In the gastrointestinal tract (GIT), mutations in digestive enzymes or factors involved in their delivery to target locations in the cell often result in severe functional defects affecting the whole organism. Numerous GIT disorders including congenital sucrase isomaltase deficiency, cystic fibrosis, glucose-galactose malabsorption and congenital lactase deficiency (NAIM *et al.*, 1988; RIORDAN *et al.*, 1989; TURK *et al.*, 1991; KUOKKANEN *et al.*, 2006) result from mutations in proteins, which compromise the intracellular transport of GIT enzymes leading to their functional failures. Mutations in motor proteins and regulators of vesicular traffic are either the primary cause of gut anomalies (e.g. the myosin Vb mutation and rab 8 deletions leading to microvillus inclusion disease) or they result in predisposition to diseases, which affect the organism extensively (e.g. myosin IXb in Celiac disease and ulcerative colitis) (MONSUUR *et al.*, 2005; VAN BODEGRAVEN *et al.*, 2006; SATO *et al.*, 2007; MULLER *et al.*, 2008). During the last three decades, the discovery of heterogeneous distribution and clustering of lipids (termed lipid rafts by Simons and Ikonen in 1997) in biological membranes has brought a new dimension to cell biology. A large body of evidence has shed light onto biochemical and physical properties of lipid rafts in cells as well as their potential involvement in numerous cellular processes including protein and lipid sorting and signalling (SIMONS and VAN MEER, 1988; SIMONS and IKONEN, 1997; SIMONS and TOOMRE, 2000). In the light of these findings, the involvement of lipid rafts in metabolic and infectious diseases has become a central point of investigation. Today, lipid rafts have been shown to play key regulatory roles in diseases of central nervous, digestive and cardiovascular systems, metabolic disorders and bacterial and viral infections (SIMONS and EHEHALT, 2002). Advances in elucidating their contribution to processes such as cell growth and differentiation, protein and membrane transport and receptor mediated signalling support our understanding of pathomechanisms underlying diseases and offer new alternatives for therapeutic developments.
1.1 Cell differentiation and biogenesis of membrane domains

Intestinal mucosa is a suitable environment to study epithelial cell development since it allows the tracking of differentiating enterocytes along the crypt-villus axis. Enterocytes are examples of highly polarized columnar epithelial cells that acquire the ability to take up, process and transport nutrients across the intestinal mucosa as they proceed from the crypts towards the tip of the villi. Within 3-5 days, the stem cells located in the crypts divide and migrate towards the tip of an intestinal villus while they differentiate into mature enterocytes that eventually undergo apoptosis and are shed into the intestinal lumen (BABYATSKY and PODOLSKY, 1999). The exact impulses that are necessary to generate fully differentiated cells from proliferative cells constitute an extensively studied area of cell biology but are not yet fully understood.

1.1.1 Spatial cues and signalling mechanisms involved in enterocytic development

In the gut, enterocytic differentiation relies on numerous factors including transcription factors, integrin-mediated cell-substratum interactions and interactions with the extracellular matrix (KEDINGER et al., 1998; SIMON-ASSMANN et al., 2007) which renders this process rather complicated to study in vivo. Cell lines with intestinal origin on the other hand, serve as suitable model systems, in which individual steps of the maturation process can be investigated (NEUTRA and LOUVARD, 1989). Caco-2 cells are one of the best-characterized intestinal cell lines derived from the colorectal cancer of the human. Despite their colonic origin, they are able to differentiate spontaneously into small intestinal enterocytes in culture marked by the formation of the microvilli (MV), establishment of tight junctions and expression of small intestinal hydrolases such as sucrase isomaltase (SI) and alkaline phosphatase (ALP) (PINTO et al., 1983). Cell differentiation is negatively correlated with proliferation, beginning by downregulation of cell division cycle proteins, cyclins, transcription factors regulating G1-S phase transition and several proto-oncogenes (MARIADASON et al., 2002). Just like in vivo proliferative activity and transition to the postmitotic cell state are strictly controlled in Caco-2 cells by an interplay between several signalling pathways including Wnt, Notch and Hedhehog (SANCHO et al., 2004; SAAF et al., 2007). Establishment of cell-cell (via E-cadherin) and cell-matrix contacts (via integrins) inhibit proliferation and provide the spatial cues for cell polarization (VEGA-SALAS et al.,
1987; YEAMAN et al., 1999). This is followed by a global change in the cell morphology lead by the reorganization of cytoskeleton. At cell-cell contact sites, thin actin filaments branching from actin cables that circumscribe the perimeter of cells attach to E-cadherin-catenin complexes. This way, actin strengthens cell-cell contacts and is suggested to mediate the early formation of membrane domains by directing delivery of certain proteins such as Na/K-ATPase to cell-cell contacts (HAMMERTON et al., 1991). Microtubules also undergo redistribution upon cell adhesion. A dense network of short and randomly oriented microtubules starts to form in apical cytoplasm and longer bundles run parallel to lateral membrane with their plus ends facing basal membrane (BACALLAO et al., 1989). In this way, cytoskeletal tracts (microfilaments and microtubules) determine and reinforce the directional targeting of transport vesicles to distinct cellular sites (APODACA, 2001). Fidelity and efficiency of polarized delivery of proteins relies on an intact cytoskeleton (MRUK et al., 2005) and presumably protein scaffolds, which have been suggested to maintain the interaction between the transport vesicles, regulatory proteins (GTPases, kinases, phosphatases) and the cytoskeleton. Such scaffolds have been suggested to operate at cellular junction sites where e-cadherin forms focal attachment sites for actin via actin binding proteins like ankyrin, α-actinin and fodrin (NELSON et al., 2000). The same authors also have suggested that ZO-1 and its homologues play this scaffolding role at the level of tight junctions by linking the structural proteins occludin and claudin to actin and other cytoplasmic proteins including tyrosine kinases (Src, Yes) and GTPases (e.g. Rho, Rac) (DRUBIN and NELSON, 1996). The specific functions of such regulatory proteins that have been speculated to operate within these scaffolds are not known.

1.1.2 Formation of Brush Border Membrane

In enterocytes, the apical membrane architecture is specialized in order to expand the surface area that comes into contact with intestinal contents. This is accomplished by an array of finger-like protrusions of the membrane termed microvilli (plural for microvillus – MV). The fringed appearance of the apical membrane of enterocytes and kidney epithelia due to MV has lead to the term Brush Border Membrane (BBM), which now denotes the apical membrane of these cells. Although components of the microvillar core are well defined, the forces and mechanisms behind the assembly of the BBM components are poorly understood. The
microvillar core consists of 20-30 actin filaments that are bundled by villin, fimbrin (plastin I), espin and tethered to the surrounding membrane by the motor protein myosin Ia (MOOSEKER, 1985) and by ezrin (BRETSCHER, 1983). Unlike the ubiquitous actin bundling protein fimbrin, villin is restricted to microvilli and has been shown to bind the barbed-ends of actin filaments: this is suggested to initiate formation of the BBM (ATHMAN et al., 2002). By studies demonstrating their actin bundling ability and loss of microvilli due to their downregulation in vitro, both villin and fimbrin have been shown to contribute to the formation of microvilli (BRETSCHER, 1981; COSTA DE BEAUREGARD et al., 1995).

Villin and fimbrin also maintain the anchorage of minus ends of actin filaments in the microvillar core to the terminal web, which is a meshwork of actin filaments that are connected by myosin II, nonerythroid spectrins, α-actinin, and tropomyosin (FATH et al., 1990). A meshwork of intermediate filaments (IF) and microtubules underlie the terminal web. A recent study has drawn attention to the importance of interactions between actin bundling protein fimbrin with keratin 19 (a type I IF found also in intestinal epithelium) for the establishment of the terminal web and of MV (GRIMM-GUNTER et al., 2009). Although the mechanism for this interaction is not clear, it suggests that the formation of the BBM relies on very complex interactions, which, in an unknown order, regulate actin remodelling at the apical plasma membrane. Similarly, ezrin has been suggested to play a critical role in organizing the apical membrane of intestinal epithelium, manifesting its effects at the level of the terminal web. Although its deletion in mice did not result in overt anomalies in MV architecture (SAOTOME et al., 2004), its ability to promote microvillus formation was demonstrated in fibroblasts, which have developed finger-like protrusions after the introduction of ezrin (SHAW et al., 1998). The idea that ezrin might be involved in the earliest stages of MV development is supported by a finding that points to the interaction between IF and dormant (inactive) ezrin in Caco-2 cells (WALD et al., 2005). According to this hypothesis, in intestinal epithelium IFs recruit and accumulate ezrin to the vicinity of apical membrane, creating an ezrin pool available for activation upon polarization signals.

Intermediate filaments have been long regarded as simple and purely mechanic components of the cells. This notion has been challenged during the past few years by the new discoveries made in different systems. For example, keratin 8 (K8)-null mice have been found to mistarget apical proteins (AMEEN et al., 2001) and transitional depletion of K19 in Caco-2
cells has resulted in reduction of the number of the MV and disorganization of apical cytoskeleton (SALAS et al., 1997).

In addition to the structural components of the MV, other actin-related proteins and phospholipids appear to regulate actin reorganization events at the apical membrane as well. Arp2/3 was the first protein complex to be shown to promote actin nucleation by creating filaments that grow at their barbed ends (MULLINS et al., 1998). Shortly afterwards, members of the WASp/Scar family (WASp_Wiscott-Aldrich Syndrome protein) were discovered and shown to assist Arp2/3 in the polymerization of new actin filaments from existing ones (HIGGS et al., 1999). More importantly, the Rho GTPase Cdc42 and phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) have been found to activate N-WASP (Neural-WASp) and Arp2/3 complex (ROHATGI et al., 1999). The Rho GTPase, Cdc42 is implicated in polarization processes as well. In yeast, it can promote polarization without the existence of any spatial cues through a self-organizing mechanism (WEDLICH-SOLDNER et al., 2003). In multicellular organisms, establishment of cell-cell contacts and E-cadherin based signalling is thought to activate Cdc42 (ARTHUR et al., 2002). Cdc42 does not only engage actin reorganization in cells but also causes the repositioning of Golgi apparatus and microtubule organizing centre (MTOC) upon events involving cell-cell contacting such as establishment of immunological synapse or wound healing (scratch induced migration) (ETIENNE-MANNEVILLE and HALL, 2001; CANNON and BURKHARDT, 2002). It is thereby able to determine the direction of the secretory pathway. Additionally, Cdc42 has been shown to bind coatamer complex and regulate vesicular trafficking between the ER and the Golgi apparatus (WU et al., 2000). Accumulating evidence supports the fact that Cdc42 is a central element in cell differentiation and polarized transport (ETIENNE-MANNEVILLE, 2004). Another Rho GTPase, RhoA, activates the MV component ezrin (MATSUI et al., 1999). This is maintained by the activation of phosphatidylinositol 4-phosphate 5-kinase (PI4P5K) which results in elevation of PI(4,5)P₂ levels. This has been suggested to activate dormant ezrin by its phosphorylation, leading to actin bundling activity, which is important for MV formation. Lipid second messenger PI(4,5)P₂ is a component of lipid microdomains (PIKE and MILLER, 1998), which are dynamic platforms within cellular membranes that allow local concentration of proteins and certain lipids (see Section 1.3 for detailed information). In the case of actin polymerization, it is likely that PI(4,5)P₂-rich domains serve
as an activation threshold reducer by increasing the local concentration of proteins that are involved in actin cytoskeleton modifications. This is probably maintained by bringing actin binding proteins and their activators (e.g. kinases, GTPases) in close proximity. The

Regulatory functions of PI(4,5)P$_2$ is not restricted to actin cytoskeleton. PI(4,5)P$_2$-rich domains also participate in microtubule plus-end capture and stabilization which is important for normal polarized motility (GOLUB and CARONI, 2005). PI(4,5)P$_2$ is not only a modulator of the cytoskeleton but an important determinant of the apical membrane. Martin-Belmonte and his colleagues (2007) have demonstrated how PI(4,5)P$_2$ and PI(3,4,5)P$_3$ are segregated to apical and basolateral membranes at the beginning of cell differentiation. Moreover, they have shown that annexin II, which binds PI(4,5)P$_2$, could recruit Cdc42 to the apical membrane. This results in recruitment of the Par6/aPKC complex by Cdc42, which together promote membrane polarity (MUNRO, 2006). In this setting, annexin II serves as a linker, bringing regulators of initial differentiation mechanisms such as Cdc42 and probably other candidates together with their substrates and/or regulators at distinct membrane regions that are rich in PI(4,5)P$_2$. This raises to questions to how annexin II acts in order to organize these dynamic interactions at the specialized membranes. It renders this protein very interesting as an opportunity to gain insights to epithelial cell differentiation by studying its behaviour in intestinal model systems.

1.2 Polarized sorting in epithelial cells

Most eukaryotic cells make use of spatial asymmetry to fulfil a variety of functions (differentiation, activation of immune response, absorption, directed membrane growth, transport of molecules across epithelial layers) (DRUBIN and NELSON, 1996) and to communicate with neighbouring tissues and cells. Epithelial cells are simple examples of polarized cells, which line cavities in the digestive, respiratory, or urinary system in mammals. These cells generally contain two distinct membrane domains. Their apical membrane domain faces the lumen of the cavity, whereas the basolateral (short for basal-lateral) domain contacts adjacent cells and the basement membrane (Fig. 1). The two domains are separated by tight junctions, which impede the diffusion of proteins and lipids to the other domain and maintain the integrity of the monolayer by connecting neighbouring cells with each other (TSUKITA et al., 2001). The identity of the discrete surface domains is maintained
despite the continuous addition and removal of membranes. The fusion of exocytic vesicles with the plasma membrane increases the surface area, and a fraction of the plasma membrane, and its associated proteins, are continuously internalized by endocytosis. The polarity of a cell depends on proper cell-surface attachment, establishment of intercellular contacts, polarization of cytoskeletal tracks and sustained and accurate delivery of protein and lipid components to relevant membranes (RODRIGUEZ-BOULAN and NELSON, 1989; NELSON and YEAMAN, 2001). The identity of the targeting signals and the cellular mechanisms decoding and processing them is an area of intensive research that seeks a comprehensive model for the polarized sorting of proteins and lipids.

1.2.1 Basolateral membrane and sorting determinants

The structure of the basolateral membrane resembles that of non-polarized cell membranes. It is rich in phosphatidylcholine (PC) and decorated with proteins responsible for nutrient uptake, cell adhesion, and growth control. Initially discovered basolateral sorting signals were tyrosine–containing primary amino acid sequences (e.g. NPXY or YXXφ) in the cytoplasmic domains of proteins (BREWER and ROTH, 1991; MATTER et al., 1992; MATTER et al., 1994). Such sequences form a secondary structure called a “tight β-turn” (COLLAWN et al., 1990). Additionally discovered basolateral sorting signals bearing no sequence similarity to

Figure 1. Schematic representation of a polarized epithelial cell. Apical membrane is characterised by microvillar protrusions. Adherens junctions (zonula adherens) are seen beneath the tight junctions, interconnected with bundles of actin (modified from ALBERTS et al., 1998).
tyrosine based motifs (di-leucine, PDZ-binding domains, single leucine and others) (AROETI et al., 1993; LE GALL et al., 1997; PAWSON and SCOTT, 1997; WEHRLE-HALLER and IMHOF, 2001) mostly share the common ability to form secondary tight β-turn structures as well. Tyrosine and di-leucine based basolateral sorting signals are recognized by the μ and β chains of the heterotetrameric adaptor protein complexes AP-1A, AP-1B, and AP-2 (OHNO et al., 1995; RAPOPORT et al., 1998; FOLSCH et al., 1999). All of these proteins adaptors are found in clathrin coated vesicles (AP-1A and AP-1B at the TGN and AP-2 at the plasma membrane) and promote clathrin assembly in vitro. Additionally, AP-3 and AP-4 have been described (ROBINSON and BONIFACINO, 2001) in basolateral membrane targeting as well. On the TGN membrane, AP-3 and AP-4 operate independently of clathrin, indicating a possible interaction with another scaffolding protein (ROBINSON, 2004).

1.2.2 Apical membrane and sorting determinants

The luminal domain of intestinal epithelia fulfils a variety of functions such as nutrient processing and uptake, transduction of extracellular stimuli, cellular immunity, and vectorial transport of ions and macromolecules. In order to enhance the functional capacity of the tissue, the apical surface of most epithelial cells is expanded vastly by a dense array of microvilli, which are membrane protrusions with an actin core (Fig.2). In intestinal cells, microvilli are connected to an underlying network of intermediate filaments, actin microfilaments, myosin II and other filamentous proteins which together are called the “terminal web” (HO, 1992). The terminal web merges with zona adherens, which runs across the cell like a belt, and actin microfilaments extending from terminal web contact tight junctions at intercellular borders. This highly organized structure promotes microvillar contractions and distributes mechanical stress that acts on a group of cells to the entire tissue (HULL and STAEHELIN, 1979). The microvillus membrane is decorated with hydrolases, membrane transporters, and many other glycoproteins. The apical targeting of all these functionally specific proteins relies on sorting signals. The first such signal established was the lipid anchor glycosyl-phosphatidylinositol (GPI) (LISANTI et al., 1988), which allows the attachment of proteins to the outer leaflet of the plasma membrane. Proteins carrying GPI-anchors are exclusively sorted to the apical membrane (LISANTI et al., 1990) and the replacement of the transmembrane anchor of a basolaterally sorted protein with a GPI-anchor
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is sufficient to retarget it to the apical membrane (LISANTI and RODRIGUEZ-BOULAN, 1990). In some cases, GPI anchoring is not the sole sorting determinant. Examples have shown that N-glycans are the primary apical sorting determinants in several GPI-anchored proteins (GPI-APs) and elimination of glycans results in random surface distribution of the protein (BENTING et al., 1999; PANG et al., 2004). In fact, N- and O-linked glycans are critical determinants for apical sorting (FIEDLER and SIMONS, 1995; YEAMAN et al., 1997). Sugar residues on proteins or lipids are recognized by a group of oligomeric proteins termed ‘lectins’. Several of them have been isolated from post-TGN vesicles carrying apically sorted proteins (FIEDLER and SIMONS, 1996; DELACOUR et al., 2005; DELACOUR et al., 2006). Lectins form cross-linked lattices with specific carbohydrates. Such lattices are suggested to promote accumulation of certain proteins or lipids (e.g. glycosphingolipids (GSLs)) and formation of stable clusters which can bud from - for example - TGN membranes as apical transport vesicles (DELACOUR et al., 2006; GARNER and BAUM, 2008). Other apical sorting signals reside on transmembrane domains of viral glycoproteins such as influenza virus neuraminidase and hemagglutinin or H,K-ATPase (KUNDU et al., 1996; SCHEIFFELE, 1997; DUNBAR et al., 2000). Additionally, cytoplasmic domains of proteins can determine apical localization (TAI et al., 1999). Existence of specific adaptors or receptors that recognize the latter two sorting signals remain to be elucidated.

The apical membrane of epithelia possesses a characteristic lipid composition. It is strongly enriched in glycosphingolipids (GSLs) and cholesterol (SIMONS and VAN MEER, 1988). GSLs are almost exclusively found at the apical membrane and readily self-associate, and together with cholesterol and saturated phospholipids, they often form discrete membrane microdomains. Termed ‘lipid rafts’ or ‘detergent resistant membrane microdomains (DRMs)’, these formations have long been suggested as apical sorting platforms (SIMONS and IKONEN, 1997). This hypothesis was supported by two observations: First, depletion of cholesterol and sphingolipids disrupted the apical delivery of marker proteins (KELLER and SIMONS, 1998; HANSEN et al., 2000). Second, certain apical proteins (e.g. most GPI-anchored proteins, sucrase isomaltase, and influenza hemagglutinin) are found in DRMs in post-Golgi carriers whereas basolateral proteins are not (SKIBBENS et al., 1989; BROWN and ROSE, 1992; ALFALAH et al., 1999). The distinct lipid composition and the physical properties (see Section 1.4) of lipid microdomains allow association of certain proteins via
their intrinsic properties (GPI-anchor, transmembrane domains) or adaptor molecules which enable, enhance, and stabilize these interactions between proteins and raft lipids.

### 1.2.3 Sorting pathways in polarized epithelia

The variety of sorting signals and the complexity of the communication between sorting platforms indicate that the polarized distribution of proteins and lipids is executed by diverse machineries. Some information on polarized transport pathways in epithelia comes from the studies performed in MDCK, Caco-2, and hepatocyte-derived cell lines. The main sorting events occur in the Golgi apparatus. Current observations suggest that membrane proteins are segregated at the level of TGN or even later (in endosomal compartments), and transported in distinct carriers to the apical and basolateral membranes (KELLER et al., 2001; RODRIGUEZ-BOULAN and MUSCH, 2005). As mentioned before, one main sorting criterion is the ability of proteins to interact with DRMs. This concept has been complemented with recent findings that point to the existence of discrete lipid microenvironments in the ER membrane (ALFALAH et al., 2005). Accordingly, DRM-associated proteins (almost all apical) and DRM-independent proteins (both basolateral and apical) are likely to be separated already in the early stages of the secretory pathway, shortly after their biosynthesis. This hypothesis is also supported by the previous discovery of signal-mediated sorting at the ER (NISHIMURA and BALCH, 1997).

The delivery of membrane proteins from the TGN to the cell surface is achieved by vesicular carriers. They originate from membrane tubules that extend from TGN, and they are coated either with clathrin lattices or a lace-like coat (LADINSKY et al., 1994). The mechanisms involved in the initiation of vesicle budding are intensively studied, yet elusive. It is suggested that in clathrin-coated regions of the TGN, the assembly of a coat-adaptor-protein complex can drive the extrusion and fission of vesicles (LUINI et al., 2008). In clathrin-free regions, the promotion of membrane curvature by the clustering of smaller DRMs (SCHUCK and SIMONS, 2004), the involvement of small GTPase Cdc 42 (MUSCH et al., 2001) and the pulling forces applied by kinesin and dynein moving on microtubules (POLISHCHUK et al., 2003) have all been suggested for vesicle budding. In addition to these, compelling evidence indicates a mechanism where precursors of post-Golgi carriers originate from the tubular domains of the TGN and are generated by the extrusion forces
applied by microtubuli upon their docking onto the TGN membranes (POLISHCHUK et al., 2003). Upon fission, post-Golgi carriers move towards the acceptor membranes, driven by kinesin and dynein GTPase motors (TAI et al., 1999; KAMAL et al., 2000). The interaction between motor proteins and cargo can either be direct (dynein light chain and rhodopsin), via adaptor proteins (mannose-6-phosphate receptor, AP-1 and KIF13A), or can occur due to colocalization of motor and cargo proteins in DRMs. Studies have shown that deletion of the sorting signals on proteins caused a TGN arrest of proteins both in polarized and non-polarizing cells which points to the signal-mediated Golgi exit of proteins as a general property of the exocytic pathway (MUSCH et al., 1996).

Under normal conditions, while proteins stick with their target membranes (e.g. SI is always apically targeted and Na/K-ATPase is always found at the basolateral membrane) polarized transport pathways vary by cell type. Pioneering membrane transport studies performed in MDCK cells have revealed that newly synthesised apical and basolateral

![Figure 2. Polarized sorting pathways for de novo synthesized proteins.](image)

Proteins reach Golgi apparatus after they are released from ER. Upon completion of post translational modifications, proteins exit TGN in transport vesicles. They are directly (I) delivered to either apical or basolateral membrane. In hepatocytes, some apical proteins are first sent to basolateral membrane (II), transcytosed and eventually targeted to the apical membrane of the neighbouring cell. Alternatively, proteins are first delivered to recycling endosome (RE) (III) and from there sorted to destination membrane.
proteins are delivered directly from TGN to the plasma membrane (RODRIGUEZ BOULAN and SABATINI, 1978; RINDLER et al., 1984; LE BIVIC et al., 1990b) (Fig. 3, arrows marked with I), whereas hepatocytes make use of a completely different transport mechanism by which apical proteins are first targeted to the basolateral membrane, then transcytosed, and finally delivered to the canalicular membrane of the neighbouring cell (BARTLES et al., 1987; SCHELL et al., 1992; BASTAKI et al., 2002) (Fig. 3 pathway (III)). In Caco-2 cells, both mechanisms are utilized (LE BIVIC et al., 1990a; MATTER et al., 1990). Subsequently, MDCK cells were also shown to employ transcytosis as an alternative apical delivery route for transfected and endogenous proteins (BRANDLI et al., 1990). The flexibility of targeting pathways that apply to a protein depends also on the cell type and the maturation state of the cells. For example, dipeptidylpeptidase IV (DPPIV) is transported either directly, via transcytosis, or by both pathways in MDCK, hepatocytes, and Caco-2 cells, respectively (LE BIVIC et al., 1990a; MATTER et al., 1990; CASANOVA et al., 1991b).

More recent findings indicate that endosomes constitute a further sorting step in protein trafficking in non-polarized and polarized cells. Many proteins transit recycling endosomes (REs) before they reach the cell surface (Fig.2) (e.g. polymeric Ig receptor (transcytosis), MHC Class II (basolateral), transferrin receptor (basolateral), GFP-YFP).

Figure 3. Protein transport pathways crossing endosomes. An MDCK cell is depicted as a model polarized epithelial cell containing only TGN and endosomes. Newly synthesised proteins can transit apical, basolateral or recycling endosomes (ARE, RE, RE) en route on their way to plasma membrane (solid lines). Indirectly, proteins can also be sent to cell surface and then internalized. Such proteins are initially contained in early endosomes (AEE and BEE). From EEs they are either directed to late endosomes (LE), degraded (dotted line), sent to common endosomes (dotted and interrupted line), or transcytosed. In common endosome, apical and basolateral proteins are sorted, and sent to relevant membrane domain. For basolateral proteins, such recycling can also occur readily in BEEs (dotted line).
(apical)) (ODORIZZI et al., 1994; ORZECH et al., 2000). However, for each protein investigated, discrepancies regarding the nature of the endosomes in different cell types have emerged. In non-polarized cells, REs are juxtanuclear and positive for rab11. Polarized cells have a more complex compartmentalization of the endocytic system, and the common recycling endosome (also termed subapical compartment (SAC)) (VAN et al., 2000) in these cells is rab11-negative. It is the main endocytic compartment which receives internalized apical and basolateral cargo from plasma membrane additional to cargo sent from the TGN. The rab11-positive endocytic compartment in polarized cells is the apical recycling endosome (ARE), which is a tubular structure underneath the apical membrane. Its discrete pH (CE and BRE; pH 5.8 and ARE; pH 6.5) (WANG et al., 2000) and the presence of rab11 indicate that the ARE is an independent sorting platform in polarized cells. The information on intracellular trafficking pathways that involve endosomes is summarized in Figure 3.

1.3 Lipid microdomains

In the last two decades, biological membranes have lost their image as a continuous phase, in which lipids act as a solvent for membrane proteins. Instead, it is now known that lipids are distributed asymmetrically between the outer and inner leaflets of the bilayer (VAN MEER, 1989) and that this imposes a different organization of membrane components on the lateral axis. According to this, the disparity between various lipid populations results in the separation of liquid and gel-like phases within one membrane. The gel-like phase consists of small membrane entities, which can move easily in the fluid remainder of the membrane. These dynamic structures are called lipid rafts (or lipid microdomains, membrane microdomains, DRMs).

1.3.1 Structure and function

In biological membranes, phospholipids with short and unsaturated acyl chains constitute the bulk of the membrane, which is fluid and disordered (also termed the liquid-disordered phase, or $l_d$). Glycosphingolipids and phospholipids with higher melting temperatures ($T_{m}$) and long, saturated acyl chains, in contrast, can form highly ordered (liquid-ordered, $l_o$) membrane microdomains (SILVIUS et al., 1996; BROWN and LONDON, 1998). Cholesterol has a high affinity for the $l_o$ phase and fills the voids between bulky polar head groups, supporting the tight packing of the raft lipids (VAN DER GOOT and HARDER, 2001). It also contributes to
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the separation of the two phases by enhancing their immiscibility through its differential influence on the acyl chains of saturated and unsaturated lipids (reviewed by EDIDIN, 2003). One interesting component of DRMs is PI(4,5)P₂, which is unlikely to associate with such formations due to its highly unsaturated and short acyl chains (PIKE and CASEY, 1996). How this lipid is integrated into DRMs is still unclear, however, a growing body of evidence points to its critical function in membrane polarity (COMER and PARENT, 2007).

Interspersed in the bulk membrane, lipid microdomains are dynamic structures which can diffuse laterally to build clusters or segregate from bigger clusters (Fig.4). Such phenomena have been demonstrated in artificial membranes consisting of two or three lipid species (AHMED et al., 1997; MILHIET et al., 2001; LAWRENCE et al., 2003). Additionally, atomic force microscopy (AFM) studies have allowed different research groups to visualize discrete lipid microdomains in model membranes (e.g. dioleoylphosphatidylcholine (DOPC)/sphingomyelin) and estimate their size, which varies between 50 and 200 nm (PRALLE et al., 2000; SASLOWSKY et al., 2002).

The evidence on existence of lipid microdomains in vivo has been put into question by experimental information on their size and lifetime (HARDER et al., 1998; PRALLE et al., 2000; SHARMA et al., 2004). Nevertheless, it is reasonable to assume that the complexity of the lipid and protein content of biological membranes results in a great variety of the size and physicochemical properties of lipid microdomains. Most likely, the single universal physicochemical attribute of membrane microdomains is their ability to resist extraction with

![Figure 4. Model demonstrating two distinct physical phases in lipid bilayer.](image)

It is suggested that bulk membrane consisting of mainly polyunsaturated lipids has a liquid disordered (l_d) nature (membrane is in fluid state, black) whereas membrane microdomains consisting of tightly packed phospholipids with saturated acyl chains bear a liquid ordered (l_o) state (membrane in gel-like state, red). Since these tightly packed formations can move laterally in fluid membrane they are named “rafts” inspired by floating structures over water. Right panel shows AFM images of sphingomyelin clusters (red) in DOPC background (black) and raft associated protein placental alkaline phosphatase (PLAP) as spikes. Note the protruding edges of sphingomyelin islands due to longer and straighter acyl chains than phosphocholine (taken from HENDERSON et al., 2004).
non-ionic detergents (for example, Triton X-100) at 4°C (BROWN and LONDON, 1998). This initial demonstration of the existence of DRMs in cells has now become a widely established and adopted method for their isolation from biological samples and has led to the term “Detergent Resistant Microdomains, DRMs” (BROWN and ROSE, 1992) which encompasses several membrane populations that share the common ability to remain insoluble in various detergents. After Triton X-100, other non-ionic detergents such as CHAPS, Brij, Lubrol and Tween-20 were also used to isolate DRMs. The DRMs isolated by using these detergents - which have different solubilizing powers - have shown discrepant lipid and protein compositions (DROBNIK et al., 2002; SCHUCK et al., 2003; ALFALAH et al., 2005). This diversity is supported by the fact that membranes of different intracellular compartments are of different lipid compositions, and therefore DRMs are not necessarily restricted to the plasma membrane and can exist in other intracellular membranes as well. For example, several studies have confirmed the existence of DRMs in ER membranes by the isolation of early biosynthetic forms of proteins in DRMs from different cell lines (PALADINO et al., 2004; BROWMAN et al., 2006; HEIN et al., 2009). Furthermore, the discovery of plasma membrane proteins such as prominin and prostate membrane specific antigen (PSMA) in Lubrol-DRMs that are soluble in Triton X-100 has proven that two different kinds of membrane microdomains can coexist in the same membrane (ROPER et al., 2000; CASTELLETTI et al., 2008).

The constitutive protein components of DRMs carry either lipid anchors (GPI anchor, doubly acylated proteins, palmitoylated proteins) or signals or structural features in their transmembrane domains that enable their association with these highly ordered lipid domains. The association of some proteins with DRMs is regulated through ligand binding or oligomerization, and therefore they can move in and out of DRMs (HOLOWKA and BAIRD, 2001; PALADINO et al., 2004). Through their association with the cytoskeleton, proteins can stabilize DRMs by restricting lateral diffusion (NAKADA et al., 2003). Fluorescent tagging/labelling, cross-linking with ligands or antibodies combined by cold extraction with Triton X-100, or modulation of cholesterol levels has allowed microscopic visualisation of DRMs in living cells (STAUFFER and MEYER, 1997; HARDER et al., 1998; VARMA and MAYOR, 1998; HAO et al., 2001; VAINIO et al., 2002) and substantially complemented the biochemical evidence on their existence in vivo.
DRMs bring about local differences in the composition and biophysical properties of the bilayer that have functional consequences. The research on DRMs has in recent years focused more on the cellular processes that involve and require DRMs. One of the best-characterized types of DRMs are caveolae, flask-shaped invaginations of plasma membrane that are involved in clathrin-independent receptor-mediated endocytosis (KURZCHALIA and PARTON, 1999). Found in many cells, caveolae are regions of plasma membrane where the uptake of virus particles (PELKMANS et al., 2002) and cholera toxin (ORLANDI and FISHMAN, 1998) occurs and where receptors and other signalling-associated molecules like the epidermal growth factor receptor (EGFR), the Src family of kinases, phospholipase Cγ (PLCγ), and protein kinase C (PKC) α and β (reviewed by OKAMOTO et al., 1998) are located. The involvement of DRMs in signal transduction is, however, not restricted to caveolae. IgE signalling during allergic response (FIELD et al., 1995) and T-cell antigen receptor signalling (JANES et al., 2000) are fundamental immunological events in which DRMs serve as platforms for the concentration of signalling components and the initiation of downstream processes. DRMs are suggested as regions of membrane bilayer that concentrate receptor molecules upon ligand binding to amplify the initial signal required for related processes, and that, at the same time, provide insulation from the surrounding modulators that are present in the liquid-disordered phase (SIMONS and TOOMRE, 2000). The concentration of signalling complexes on DRMs is dynamic and reversible; endocytosis leads to removal, and the dissociation of interacting components and cytoskeleton can also influence the coalescence and segregation of DRMs.

After determining the distinct lipid composition (rich in glycosphingolipids) of the apical membrane in MDCK cells, Simons and van Meer (1988) have postulated that the heterogeneity among membrane domains could only be maintained by the differential sorting of lipids and packaging into distinct transport vesicles in the TGN. This was supported by the observation that apically sorted GPI-anchored proteins were associated with membrane domains that remained insoluble after extraction with Triton X-100 (BROWN and ROSE, 1992). This observation was extended to a large amount of apically sorted proteins, and it was suggested that proteins were included in such microdomains in the Golgi apparatus, where sphingolipid synthesis is completed and cholesterol is abundant (VAN MEER, 1989). Interestingly, in the following years, compelling evidence revealed that a GPI-anchor and the
resulting association of proteins with DRMs were not necessarily responsible for apical sorting (CASANOVA et al., 1991a; BENTING et al., 1999; PANG et al., 2004). On the other hand, cholesterol and sphingolipid depletion have resulted in the missorting of GPI-anchored proteins (KELLER and SIMONS, 1998). The addition of GPI anchors to basolateral proteins led to apical sorting in some situations as well (CARIAPPA et al., 1996). The complexity of hierarchy among sorting signals render it difficult to appoint the interaction between proteins and DRMs as an absolute prerequisite for apical sorting, since proteins with various posttranslational modifications (N- and O-linked glycans, GPI-anchor, acylation) can associate with lipid microdomains. Nevertheless, the ability of DRMs to provide platforms in membranes where proteins and lipids can be ‘distributed’ into certain populations cannot be ruled out. Such sorting via DRMs has been observed in the ER membranes of yeast and mammalian cells (SUTTERLIN et al., 1997; BAGNAT et al., 2000; SARNATARO et al., 2004). Although the ER lacks sphingolipids (except for their precursor, ceramide), and only low amounts of cholesterol are available, DRMs that contain ER forms of proteins have been isolated from cells. This observation has required the employment of milder non-ionic detergents, such as Tween-20, since detergents like Triton X-100 or CHAPS with higher solubilizing strength are not likely to be selective for lipid microdomains in the early secretory pathway.

1.3.2 Lipid microdomains in disease

The discovery of lipid rafts as operational platforms for intra- and intercellular events such as signal transduction, protein sorting, and membrane transport has drawn attention to their potential involvement in disease–related processes. To date, lipid microdomains have been implicated in infectious, metabolic, and prion-related diseases and in cancer (for a comprehensive review, see SIMONS and EHEHALT, 2002). For example, in HIV infection, the enveloped virus particle exploits DRMs on host cell surface to enter the cell (CAMPBELL et al., 2001). Just like HIV, influenza virus proteins are sorted to the apical membrane for envelope assembly and virus budding, and disruption of DRMs by cholesterol depletion interferes with these processes (SCHEIFFELE et al., 1999). Viruses, bacteria, and parasites also modulate host cell signalling to enter and exit host cells. For example, the DRM-associated protein Nef from HIV promotes infectivity of the virus (ZHENG et al., 2001).
Bacteria are known to co-opt DRM-mediated endocytic pathways to invade host cells by avoiding acidic lysosomes and consequent degradation (ZAAS et al., 2005). E.coli can initiate actin pedestal formation for the uptake into host cells by mimicking a host cell receptor that becomes phosphorylated by a DRM-associated Src-kinase (PHILLIPS et al., 2004). Many bacteria trigger MAP kinase activation and cytokine production by stimulating host cells via LPS and its GPI-anchored receptor, which resides in DRMs (SOLOMON et al., 1998). *Plasmodium falciparum* (a protozoon causing malaria in humans) has also been shown to enter erythrocytes by a DRM-mediated mechanism (LAUER et al., 2000).

Apart from infections, DRMs are implicated in important metabolic disorders such as Alzheimer’s disease, Parkinson’s disease, lysosomal storage diseases, diabetes, neuropathies and chronic inflammation. Mechanisms underlying such pathologies are diverse but the contribution of DRMs seems to be universal; creating discrete pools of proteins in membranes where they can interact with their effectors or substrates. Therefore, modulating this segregation might be the solution to individual problems.

To date, involvement of DRMs in critical disease–related cellular events has been demonstrated mostly indirectly, via depletion or inhibition of DRM components, cholesterol, and sphingolipids. Co-patching with DRM-proteins was possible for some of viral proteins as well as proteins related to metabolic disease. Nevertheless, as the methods for detecting DRMs expand and their cellular functions are elucidated, research on DRMs offers substantial understanding of pathological mechanisms and therapeutic options.

### 1.4 Membrane dipeptidase and annexin II: two model proteins

This study aims to elucidate different functional aspects of DRMs in mammalian cells which require investigation of several cellular processes. More specifically, the involvement of DRMs in anterograde protein transport in early secretory pathway, protein sorting and cell differentiation have been investigated. Therefore, several model proteins were employed to study these diverse functions of DRMs in model cell systems. The results of this study have been organized as two independent manuscripts (of which one has been already published) and together aim to elucidate functional properties of DRMs. The first manuscript focuses on the DRMs in ER membranes and points to their function as platforms for a set of proteins that are efficiently transported out of the ER. In this study, wild type membrane dipeptidase
(MDP), which is a DRM-associated protein, and its anchorless variant (MDPΔGPI), which is excluded from DRMs were used to investigate ER to Golgi transport kinetics. The second manuscript aims to define the specific functions of annexin II, a calcium dependent lipid binding protein, which has been previously shown to concentrate at membrane regions rich in DRM-lipids (OLIFERENKO et al., 1999) and suggested to act as a stabilizer of the membrane microdomains by linking them to cytoskeleton (HAYES et al., 2006). The investigation of specific functions of DRM-associated annexin II in Caco-2 cells was conducted by a comparative analysis of wild type cell line with the mutant version, in which expression of annexin II was stably downregulated by RNAi technology. It was thereby possible to relate the outcomes of annexin II depletion to the contribution of DRMs to processes like cell differentiation and polarized sorting.

1.4.1 Membrane dipeptidase

MDP (renal dipeptidase, microsomal dipeptidase, EC 3.4.13.19) is a zinc metallopeptidase that localizes to the brush border membranes of a variety of mammalian cells (LITTLEWOOD et al., 1989). It is involved in the renal metabolism of glutathione and the degradation of leukotrienes in the lung, and it exhibits β-lactamase activity that is used as a model in drug development studies against bacterial metallo-β-lactamases (KEYNAN et al., 1996). MDP is N-glycosylated (at positions Asn41 and Asn263), and it is attached to the membrane via a GPI membrane anchor at Ser368 (RACHED et al., 1990; BREWIS et al., 1995). Like with many other GPI-anchored proteins, this lipid anchor of MDP is responsible for its association with DRMs (PANG et al., 2004). The ability of GPI-anchored proteins to cluster as in discrete domains of the plasma membrane was discovered in early 90’s by Rothberg and her colleagues. They have shown that the folate receptor concentrates on discrete domains on the plasma membrane that are associated with caveolae (ROTHBERG et al., 1990). It has also been revealed that this association of folate receptor with caveolae depended on cholesterol. This finding has been supported by the observations that many GPI-anchored proteins like Thy-1, scrapie prion protein (PrPSc), and alkaline phosphatase (ALP) are clustered at plasma membrane, and that these regions of the membrane could be isolated by the non-ionic detergent, Triton X-100 (HOESSLI and RUNGGER-BRANDLE, 1985; BROWN and ROSE, 1992; TARABOULOS et al., 1995). Combined with the observation
that almost all GPI-anchored proteins were found at the apical surface, it was postulated that the Triton X-100 resistant domains were involved in the apical sorting of proteins, and that this occurred at the trans-Golgi membranes. However, further studies showed for many GPI-anchored proteins including MDP (PANG et al., 2004) that GPI-anchoring and subsequent association with DRMs was not the absolute sorting determinant for these proteins (see section 1.3.1 for references). In many cases (including MDP) N-glycans are responsible for apical targeting, and mutation of glycosylation sites results in missorting of the proteins. These findings have led to the general conclusion that the GPI-anchor was not solely sufficient to direct proteins to apical domain. Despite this, GPI-anchorage and DRM association seem to have other functional consequences. For example, Walmsley et al. (2001) have shown that the anchorless mutant of prion protein (PrPΔGPI) accumulated in the ER of human neuroblastoma cells. Although this mutant was secreted, the intracellular transport rate has been found to be 4-times slower than the wild type PrP. Moreover, the deletion of the GPI anchor attachment site has resulted in a defect in glycosylation, and prolonged residence of the protein in ER did not improve glycosylation. Similarly, in Trypanosoma brucei, forward transport rates decreased when an anchorless mutant of the variant surface glycoprotein (VSG) was expressed in procyclic cells (BANGS et al., 1997). Fusion of a GPI-anchor signal sequence from another surface glycoprotein to VSGΔGPI from T.brucei restored transport kinetics. Both studies argue that the bottleneck in forward transport is at the level of ER exit. It is plausible to speculate that lipid composition and the microenvironment around certain protein molecules in ER can determine the transport efficiency out of this compartment. This hypothesis is supported by observations in yeast that point to the importance of ceramide (the precursor of sphingolipids) for the enhancement of GPI-anchored protein transport (HORVATH et al., 1994). Thus, distinct COPII vesicle populations with different cargo might exist on ER membranes (MUNIZ et al., 2001). This separation is likely to occur based on the physical properties that are intrinsic to proteins. GPI-anchored proteins can integrate with highly ordered membrane domains, and other secretory proteins can be excluded from these formations and packed into the other vesicle type. The reason behind such segregation is not known, and the existence of DRMs on ER membranes and suitable methods for their isolation and analysis is a topic of growing interest.
1.4.2 Annexin II

Annexin II (annexin A2) is a member of the annexin multi-gene family of proteins, which are found in almost all eukaryotes. In vertebrates, 12 annexins and their splice variants constitute the annexin A family (A1-A11 and A13) (MORGAN and FERNANDEZ, 1997). Member proteins of the annexin family share the ability to bind acidic phospholipids in a Ca\(^{2+}\)-dependent manner and comprise the characteristic and highly conserved domains called “annexin repeats”. These approximately 70 amino acid-long domains are made up of five alpha helices and contain unique calcium binding sites, which allow annexins to dock onto membranes in a peripheral and reversible manner. Four annexin repeats (8 in annexin VI) build the compact disc-shaped annexin core. Calcium binding sites are located on the convex side where interaction with membrane lipids occurs. The concave side points away from the membrane and is accessible to interacting cytoplasmic elements (GERKE and MOSS, 2002). Preceding the annexin core, the N-terminal domain is the most variable part of annexins. This domain is subject to different post-translational modifications (e.g. phosphorylation, glutathionylation, myristoylation). Interactions with protein ligands are maintained via the N-terminal domain, whose exposure depends on Ca\(^{2+}\) binding as well (GERKE and MOSS, 2002). Interaction partners of annexins also vary greatly, which points to diverse and independent functions undertaken by individual members despite the high sequence homology among them (MOSS and MORGAN, 2004).

Annexin II (previously named calpactin for its ability to bind calcium and bundle F-actin) is a multifaceted member of the annexin family. It was the first annexin that was discovered to bind and bundle F-actin on a Ca\(^{2+}\)-dependent manner (GERKE and WEBER, 1984; GLENNEY, 1987). This discovery was followed by several studies reporting on annexin II as an important player in dynamic actin reorganization events at membranes sites containing raft lipids (e.g. PI(4,5)P\(_2\) and cholesterol) such as endocytosis (EMANS et al., 1993; HARDER and GERKE, 1993), macropinocytosis (MERRIFIELD et al., 2001), membrane attachment of *E.coli* (ZOBIACK et al., 2002), phagocytosis (PITTIS et al., 2003) and actin-dependent apical targeting of TGN-derived vesicles (JACOB et al., 2004). The activity of annexin II at these actin assembly points is probably tightly regulated, for example by phosphorylation at Tyr-23 in the N-terminal domain, which is catalysed by src-like kinases
and results in the inhibition of F-actin binding and bundling (HUBAISHY et al., 1995). Annexin II interacts with actin cytoskeleton indirectly as well. This is mediated by the interaction between annexin II and large F- and G-actin binding protein AHNAK. Although the purpose of this interaction is not clear, annexin II is thought to recruit AHNAK to the plasma membrane domains and thereby initiate actin polymerization at certain membrane sites (BENAUD et al., 2004). Apart from its contribution to the cellular functions involving dynamic actin reorganization events, annexin II has been shown to operate outside the endothelial cells as a co-receptor for plasminogen and tissue plasminogen activator (tPA) in fibrinolytic homeostasis (CESARMAN et al., 1994; HAJJAR et al., 1994). The wide range of functions undertaken by annexin II raises the interest to find a cohesive explanation for its mode of action at biological membranes.

Annexin II exists in cells as a cytosolic monomer and in a heterotetrameric complex with S100A10 (p11). In the polymeric constellation, two annexin II molecules are connected by the centrally located p11 dimer via annexin II N-terminal domains. This complex specifically binds membranes containing acidic phospholipids and/or membrane domains rich in cholesterol and PI(4,5)P$_2$ (HAYES et al., 2004; RESCHER et al., 2004). Depending on the mode of binding, it is suggested that annexin II$_2$-p11$_2$ can mediate fusion of two membranes (e.g. secretory vesicle – plasma membrane) by bringing them into close proximity (ALI et al., 1989; KNOP et al., 2004), cluster DRMs or link membrane domains and associated proteins to cytoskeletal elements or other protein ligands (RESCHER and GERKE, 2008). Ability of annexin II to interact with the DRM lipids with high affinity and specificity has drawn substantial attention to potential functions of this protein in conjunction with many emerging functions of the lipid microdomains in cellular processes. Biochemical analyses and cryoelectron microscopy studies with liposomes have revealed that annexin II was able to bind and aggregate membrane regions containing acidic phospholipids and cholesterol at low pH, independent of Ca$^{2+}$ (AYALA-SANMARTIN et al., 2001; LAMBERT et al., 2004). This non-conventional activity of annexin II has been linked to a great variety of dynamic membrane events involving F-actin assembly beneath membranes (see above for examples). Nonetheless, many questions remain unanswered regarding the mechanism and outcomes of aggregation and stabilization of PI(4,5)P$_2$ and cholesterol-rich membrane domains by annexin II.
Recently, annexin II was shown to participate in events that drive cell polarization (described as the generation of spherical MDCK monolayers with a lumen – cysts) (MARTIN-BELMONTE et al., 2007). Although the mechanism how annexin II modulates these events is not clear, interactions between annexin II, PI(4,5)P$_2$ and small GTPase Cdc 42 are suggested to play a decisive role for the formation of cortical actin cytoskeleton and apical membrane. This hypothesis was supported by the observation that downregulation of annexin II or expression of a dominant negative mutant form (Anx2CM) resulted in reduction of lumen formation in cysts and depletion of cortical actin. Such an effect after depletion of annexin XIII (an annexin implicated in apical exocytosis (GERKE et al., 2005) was also observed in MDCK cysts (TORKKO et al., 2008). In both cases, disruption of polarization was determined by the changes in cyst morphology and the redistribution of apical marker proteins and components of tight junctions (e.g. ZO-1). With the aim of pinpointing the exact function of annexin II, many studies have employed transient or stable depletion methods in model systems. The diverse outcomes of these approaches in mice and various cell lines are most likely due to different motivations and functional aspects that have been investigated by depletion of this protein. For example, annexin II-null mice displayed normal development and fertility except for subtle decreases in growth rates and body mass (LING et al., 2004). This study concentrated on fibrinolytic activity of annexin II and its effects on neoangiogenesis and other potential consequences of its depletion have not been investigated. Transient downregulation of annexin II in various cells lines has resulted in diverse phenotypes including missorting of apical proteins (JACOB et al., 2004), blockade of multivesicular endosome biogenesis (MAYRAN et al., 2003), inhibition of glial cell migration (TATENHORST et al., 2006) and delocalization of adherens junction protein vascular endothelial E-cadherin (HEYRAUD et al., 2008). To date, it has not been possible to investigate the long term effects of annexin II depletion on cells since such a cell line has not been established. Stable downregulation of annexin II in an epithelial cell line could create the phenotype which can be analysed under various conditions and might provide the opportunity to identify specific interaction partners and to dissect signalling pathways involving annexin II activity.
CHAPTER 2

ASSOCIATION OF A GPI-ANCHORED PROTEIN WITH DETERGENT-RESISTANT MEMBRANES FACILITATES ITS TRAFFICKING THROUGH THE EARLY SECRETORY PATHWAY
Research Article

Association of a GPI-anchored protein with detergent-resistant membranes facilitates its trafficking through the early secretory pathway

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Abstract

Membrane microdomains are implicated in the trafficking and sorting of several membrane proteins. In particular, GPI-anchored proteins cluster into Triton X-100 resistant, cholesterol- and sphingolipid-rich membrane microdomains and are sorted to the apical membrane. A growing body of evidence has pointed to the existence of other types of microdomains that are insoluble in detergents, such as Lubrol WX and Tween-20. Here, we report on the role of detergent-resistant membranes formed at early stages in the biosynthesis of membrane dipeptidase (MDP), a GPI-anchored protein, on its trafficking and sorting. Pulse-chase experiments revealed a retarded maturation rate of the GPI-anchor deficient mutant (MDP\textsubscript{ΔGPI}) as compared to the wild type protein (wtMDP). However, Golgi to cell surface delivery rate did not show a significant difference between the two variants. On the other hand, early biosynthetic forms of wtMDP were partially insoluble in Tween-20, while MDP\textsubscript{ΔGPI} was completely soluble. The lack of association of MDP\textsubscript{ΔGPI} with detergent-resistant membranes prior to maturation in the Golgi and the reduction in its trafficking rate strongly suggest the existence of an early trafficking control mechanisms for membrane proteins operating at a level between the endoplasmic reticulum and the cis-Golgi. © 2008 Elsevier Inc. All rights reserved.

Introduction

Ever since our understanding of cell membrane organization changed upon the discovery of cholesterol- and glycosphingolipid-rich membrane microdomains or detergent-resistant membranes (DRMs), many functional roles for these formations have been proposed. It has been shown that proteins involved in particular signalling events can be brought together at the cell surface via clustering of these microdomains [1]. It is also apparent that DRMs act as docking sites for pathogens and toxins on host cells [2]. Evidence for their role in cholesterol homeostasis has been shown by Heino et al. [3]. Besides these, a major function for membrane microdomains in the cell has been identified in the sorting of proteins in the secretory pathway. In polarized cells, a subset of apical proteins interacts with DRMs and their sorting occurs based on signals such as glycans [4], lipid anchors (e.g. glycosylphosphatidylinositol (GPI), acylation) [5] or signals residing in transmembrane anchors [6]. The sorting function of membrane microdomains within the secretory pathway occurs from the trans-Golgi network (TGN) onwards. Selective delivery of certain

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Abbreviations: BFA, Brefeldin A; DMEM, Dulbecco’s Modified Eagle Medium; DRMs, detergent-resistant membranes; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; MDCK, Madin Darby canine kidney; MDP, membrane dipeptidase; PrP, prion protein; TGN, trans-Golgi network

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proteins in TCN-derived vesicles enriched in certain lipids results in a distinct distribution of these molecules among membrane domains in polarized cells and maintains membrane identity and specialized functionality.

There is now rising interest in investigating the existence of similar membrane micro-organizations at earlier stages of the secretory pathway (i.e. endoplasmic reticulum (ER) and cis-Golgi) that would result in earlier sorting of proteins and lipids. These compartments are known to have a different lipid composition in their membranes which may influence also the biochemical properties of membrane microdomains occurring at these stages. Evidence for the apical/basolateral sorting of proteins at the level of the ER was shown by Alfalah et al. [7] where the non-ionic detergent Tween-20 was found to completely solubilize basolateral proteins, whereas apical proteins remained insoluble after extraction with this detergent. In yeast, it has also been shown that GPI-anchored proteins were selectively included in distinct vesicles budding from the ER [8], suggesting microdomain-associated sorting at ER exit sites. The consequences of selective inclusion of apical/GPI-anchored proteins into detergent resistant membranes (DRMs) during the early steps of protein transport remain to be elucidated.

DRMs are described as tightly packed formations of 25–50 nm estimated size [9,10] that can diffuse laterally in the relatively fluid membrane [11]. They display a distinct lipid content being enriched in long-chain saturated fatty acids (e.g. glycosphingolipids) and cholesterol [5]. A major biochemical feature of these formations is their resistance to extractability with non-ionic detergents. The initial detergent employed for the isolation of DRMs was Triton X-100, although other detergents followed including Brij96, Brij98, Lubrol WX and Tween-20 [12–15]. DRMs isolated using these detergents revealed discrete lipid compositions in comparison to classical Triton X-100 DRMs (for review, see [16]). Nevertheless, lipids found in these domains were chemically in agreement with the formation of a liquid ordered phase. In the ER, where low amounts of sterols and sphingolipids (albeit their precursors) are present, it is highly likely that DRMs are composed of other lipid components which constitute platforms where certain proteins can be incorporated.

Membrane dipeptidase (MDP) is a GPI-anchored protein that is preferentially localized to the apical membrane of kidney epithelia [17]. Porcine MDP has two N-glycosylation sites (Asn41 and Asn263) and the GPI-anchor is attached to Ser368 [18,19]. For MDP the N-glycans, rather than the GPI anchor, are responsible for its apical targeting [20]. DRM association is, however, maintained via the GPI anchor, excluding a sorting function for membrane microdomains in this case. It has been previously shown both in yeast and mammalian cell systems that loss of the GPI membrane anchor resulted in slower maturation rates [21,22], although the mechanism for this observation is not clear. We hypothesised that the lack of association of the GPI anchor deficient mutants with DRMs may be responsible for this retardation. In this study, we employed wild type porcine MDP (wtMDP) and its mutant lacking the attachment signal for GPI anchor addition (MDPΔGPI) in order to investigate the consequences of altered membrane microdomain association on transport along the secretory pathway. While wtMDP associated with DRMs at the level of the ER, MDPΔGPI was substantially excluded from these formations. MDPΔGPI displayed also a slower maturation rate, as mainly ER forms of this protein were present in the cells. These findings indicate an essential role for DRMs in the efficient and rapid transport of proteins through the secretory pathway, complementing other functions for these membrane microdomains that have been defined to date.

Materials and methods

Cell culture, transfections and biosynthetic labelling

Stably transfected Madin Darby Canine Kidney (MDCK) cells [20] and COS-1 cells were maintained at 37 °C and 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum and containing 0.5 U/ml penicillin and 0.1 μg/ml streptomycin. MDCK cells were transiently transfected with 3 μg of pdRsRec2-ER vector (Clontech, CA) using transfection reagent Nanofectin (PAA, Austria). COS-1 cells were transiently transfected with 5 μg cDNA of each of MDP wild type or MDPΔGPI [20] separately using DEAE-Dextran (Sigma) according to Naim et al. [23]. COS-1 cells were utilized in experiments 48 h after transfection. MDCK cells were grown to confluence before they were included in experiments. All cells were labelled with 100 μCi of [35S]-methionine for different time periods according to individual experimental procedures. In pulse-chase experiments, following pulse labelling proteins were chased with DMEM containing an excess amount of unlabelled methionine.

Lysis and isolation of DRMs

Total cell lysates were obtained by extraction with 1% (w/v) Triton X-100 in phosphate buffered saline (PBS) pH 7.4 for 90 min at 4 °C. This solution was supplemented with a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml antipain and 50 μg/ml trypsin inhibitor). Prior to immunoprecipitation, nuclei and debris were removed by a centrifugation step at 1500 g for 15 min at 4 °C. MDP was immunosolated from this supernatant (post-nuclear supernatant).

For isolation of DRMs a well established extraction method was adapted [5]. [35S]-methionine labelled cells were collected into detergent-containing buffer solution (1% (w/v) Triton X-100 in phosphate buffered saline (PBS) or 1% (w/v) Tween-20 in PBS) supplemented with protease inhibitors and homogenized by passing through a Luer 21-gauge needle 15–20 times on ice. After 2 h at 4 °C the lysates were centrifuged at 1500 g to remove debris and nuclei. The supernatant was subjected to ultracentrifugation at 100,000 g for 90 min to yield a supernatant and a pellet fraction. Pellets containing DRMs were resuspended in 1% Triton X-100 containing 0.1% SDS and used in immunoprecipitation experiments. The supernatant fraction was used directly.

Floatation method to isolate DRMs was adapted from Pang et al. [20]. For this, cellular detergent extracts (0.5% Triton X-100) were passed through the Luer 21-gauge needle. The lysate was adjusted to 40% sucrose by mixing with the same amount of 80% sucrose and layered beneath a 5–30% discontinuous sucrose gradient. Following centrifugation for 18 h at 100,000 g in a SW-40 rotor (Beckman Instruments), gradients were harvested in 1 ml fractions from the top of the tube. Individual fractions were concentrated with three volumes of 96% ethanol overnight at –20 °C. Protein pellets were obtained by centrifugation at 13,000 g for 15 min and dried prior to use. Dry pellets were resuspended in sample loading
buffer (4% (w/v) SDS, 20% glycin, 100 mM TrisHCl pH 6.8, 0.02% bromophenol blue) and boiled at 95 °C for 5 min. Samples were subjected to SDS-PAGE and gels were blotted onto PVDF membranes (Amersham Biosciences, Munich, Germany). Blots were overlaid with anti-MDP antibody to detect the distribution of this protein along the gradient. Floating fractions containing detergent resistant membranes were detected using a monoclonal antibody against flotillin-2 (Santa Cruz Biotechnology). A non-microdomain marker protein, RhoA, was detected with a monoclonal antibody (Santa Cruz Biotechnology).

**Immunoprecipitation, western blotting and enzymatic deglycosylation**

Post nuclear supernatants of cell lysates, pellets and supernatants obtained from ultracentrifugation steps were pre-cleared with protein A-Sepharose according to Jacob et al. [24]. Supernatants were immunoprecipitated with the polyclonal anti-porcine MDP antibody [17] and antigen–antibody complexes were recovered by the addition of Protein A-Sepharose. Samples were resolved by SDS-PAGE on 10% polyacrylamide gels. Proteins were detected using a phosphorimaging device (Bio-Rad). Where indicated, band intensities were quantified by Quantity One imaging program (Bio-Rad). For western blot analysis, gradient fractions were precipitated overnight at −20 °C with 96% ethanol and centrifuged at 1500 g to collect the protein pellets. The pellets were boiled in sample loading buffer and loaded onto 10% polyacrylamide SDS gels. Proteins were transferred to Hybond P [poly(vinylidene difluoride)] membranes (Amersham Biosciences, Munich, Germany). [35S]-labelled immunoprecipitates were deglycosylated using endo-β-N-acetylglucosaminidase H (endo H) as previously described [25] and subjected to analysis by SDS-PAGE.

**Fluorescence microscopy**

MDCK cells stably expressing wtMDP and MDPΔGPI (denoted MDCKwtMDP and MDCKMDPΔGPI respectively) were grown on coverslips and transfected with pDsRed2-ER vector that codes for an ER protein marker (Clontech, CA). After 48 h, cells were washed with PBS and fixed in 4% paraformaldehyde, quenched with 50 mM NH₄Cl and permeabilized with 0.5% saponin in 1% BSA. Both transfected and non-transfected MDCKwtMDP and MDCKMDPΔGPI cells were then processed for indirect immunofluorescence with anti-MDP antibody and anti-GM130 antibody (BD Biosciences, CA) as a marker for the Golgi apparatus (only non-transfected cells). In non-transfected cells Alexa Fluor 488 was used as a secondary label.

**Fig. 1** – Transport kinetics of wild type MDP and its anchorless variant. (A) MDCK cells stably expressing either wtMDP or MDPΔGPI were grown to confluence and labelled with [35S]-methionine for 120 min. The cells were then solubilized for 90 min with Triton X-100 (1% in PBS) in the presence of protease inhibitors and immunoprecipitation was performed with the anti-MDP antibody. Following immunoprecipitation, enzymatic deglycosylation was performed on the beads with Endo H or left untreated as control. Isolates were resolved on 10% polyacrylamide SDS gels and visualised by phosphorimaging. Band intensities were quantified by Quantity One imaging program (Bio-Rad). (B) Confluent MDCK cells stably expressing either wtMDP or MDPΔGPI were labelled with [35S]-methionine for 30 min and chased in the presence of excess unlabelled methionine for the indicated time. Cells were then solubilized with 1% Triton X-100 in PBS for 90 min at 4 °C and the lysates immunoprecipitated with the anti-MDP antibody. Isolates were resolved on 10% polyacrylamide SDS gels and visualised by phosphorimaging. (C) MDCK cells were continuously labelled with [35S]-methionine for 3 h and media were collected. MDP was immunoprecipitated with the anti-MDP antibody. MDP ΔGPI was found in the medium whereas no wtMDP was released into the medium after 3 h.
antibody to visualise the Golgi marker. In MDCK\textsubscript{wtMDP} and MDCK\textsubscript{MDP\negativedelta GPI} cells Alexa Fluor 568 was utilized as a secondary antibody to detect MDP. The fluorescence images were visualised using a Leica TCS SP2 confocal laser microscope with a 63× oil planapochromat lens (Leica Microsystems).

**Trypsin sensitivity assay**

MDCK cells stably expressing wtMDP and MDP\negativedelta GPI cDNAs were lysed with 1% Triton X-100 and post nuclear lysates were immunoprecipitated using polyclonal anti-porcine MDP antibody.

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**Fig. 2** – Intracellular distribution of wild type MDP and MDP\negativedelta GPI. (A) MDCK cells were transiently transfected with pDsRed2-ER (green) construct to label the ER. MDP was detected by indirect immunofluorescence (red). wtMDP displayed vesicular distribution in the cell and some labelling at the cell surface and showed almost no colocalization with the ER marker protein. MDP\negativedelta GPI displayed a reticular distribution in the cell and significantly colocalized with the ER marker. (B) Golgi apparatus was labelled with anti-GM-130 (green). For detection of MDP (red), polyclonal anti-porcine MDP antibody was used. At steady state, wild type MDP is distributed in the cell in vesicles and at the cell surface. No colocalization with GM-130 can be seen. MDP\negativedelta GPI, however, is concentrated in juxtanuclear areas and strongly colocalizes with the GM130 concomitant with an accumulation of this protein in this compartment.
Antigen–antibody complexes bound to Protein A-Sepharose beads were treated with 25, 50, 100, and 250 μg of trypsin at 37 °C for 30 min respectively. Reaction was stopped by boiling and the beads were loaded onto 10% polyacrylamide gels.

### Results

#### Transport kinetics of GPI-anchored MDP and its anchorless variant

It has been shown for two proteins, prion protein [22] and variant surface glycoprotein [26], that abolition of GPI-anchoring resulted in a retardation of their anterograde transport in the cell. We investigated whether the same phenomenon applied to MDP and its anchorless variant. MDCK cells stably expressing either wtMDP or its GPI-anchor deficient variant (MDPΔGPI) were pulse labelled for 120 min with [35S]-methionine, and MDP was isolated with anti-MDP antibody. Immunoisolates were treated with the indicated amounts of trypsin and resolved on polyacrylamide SDS gels.

Fig. 3 – Trypsin sensitivity assay. MDCK cells stably expressing wtMDP and MDPΔGPI were labelled with [35S]-methionine either for 30 min (upper panel) or chased for 30 min following 60 min labelling (lower panel). Following lysis with Triton X-100 (1% in PBS), MDP in postnuclear lysates was immunoprecipitated with the anti-MDP antibody. Immunoisolates were treated with the indicated amounts of trypsin and resolved on polyacrylamide gels.
protein released into the medium (Fig. 1C). This indicated that MDPΔGPI was partially retained in the ER with a consequent retardation in its conversion to the mature glycosylated form. Obviously, when the MDP protein has acquired its mature form, its biosynthetic characteristics, e.g. turnover are maintained regardless of the presence or absence of the GPI anchor, supporting the view that the difference in the life cycle of wtMDP and MDPΔGPI is restricted to the ER to Golgi transport.

**Intracellular distribution and compartmentalization of MDP**

In order to corroborate the biochemical evidence that MDPΔGPI resided in the ER for prolonged times, we investigated the steady-state intracellular localizations of both proteins with the help of organelle markers for the ER and the Golgi apparatus. First, MDCKwtMDP and MDCKMDPΔGPI cells were transiently transfected with pDsRed2-ER that encodes an ER protein marker and MDP was detected by indirect immunofluorescence. Confocal microscopic analysis revealed a strong colocalization of MDPΔGPI with the ER concentrated at the juxtanuclear region (Fig. 2A, lower panel), while wtMDP did not colocalize with the ER marker, displayed a vesicular pattern, and was also revealed at the cell surface. Labelling of the Golgi apparatus with anti-GM130 antibody demonstrated a strong colocalization of MDPΔGPI with the ER at the perinuclear region (Fig. 2B, lower panel) indicating that this protein resides both in the ER and the Golgi apparatus in contrast to wtMDP, which did not colocalize with GM 130 concomitant with a rapid exit of this protein from the Golgi. These data support the biochemical analyses and subsequently the view that both MDP protein forms are transport competent, albeit at different kinetics, whereby MDPΔGPI resides for prolonged times intracellular in the ER and the Golgi.

**Assessment of folding of MDP**

Retention of non-resident proteins in the ER is often a sign for misfolding or incomplete assembly of tertiary structures. In these cases, proteins are both accumulated in the cell and eventually degraded or display toxic effects to the cells and cause cell death. In order to exclude misfolding or other primary defects on MDPΔGPI synthesis, we performed a protease sensitivity assay using trypsin [27]. Stably transfected MDCK cells were labelled for 30 and 60 min. Following 60 min of labelling, proteins were chased in the presence of unlabelled methionine for 30 min. MDP was immunoprecipitated and samples were treated with increasing concentrations of trypsin (Fig. 3). We observed that major glycoforms of wtMDP and MDPΔGPI were resistant to trypsin indicating a correctly folded status for both of the variants.

**Association of MDP with detergent-resistant microdomains**

The GPI anchor is one of the major determinants for inclusion of a protein into DRMs [28]. In this regard, we expected to observe a difference between wtMDP and MDPΔGPI in terms of their

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### Table: Detergent extractability of wtMDP and MDPΔGPI during maturation

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### Table: Transport kinetics of wtMDP and MDPΔGPI to the cell surface

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association with Triton X-100 resistant membranes. MDCK cells stably expressing wtMDP or MDPΔGPI were lysed with Triton X-100 in the cold and subjected to ultracentrifugation to isolate Triton X-100 resistant membrane microdomains. Distribution of MDP among detergent insoluble "pellet" and the detergent soluble "supernatant" fractions was analysed by western blotting. Significant association of wtMDP with Triton X-100 DRMs was observed (Fig. 4A), whereas MDPΔGPI was completely excluded from the detergent-insoluble pellet. Enzymatic deglycosylation with Endo H revealed that only complex glycosylated wtMDP associated with the Triton X-100 resistant microdomains. The association of wtMDP and MDPΔGPI with DRMs was then studied in more detail by using buoyant sucrose density gradient centrifugation in the presence of Triton X-100 (Fig. 4B). Although a significant proportion of wtMDP associated with the flotillin-2 containing DRMs, none of the MDPΔGPI localized to these fractions. In view of these findings, we expected to detect other differences in the transport of these two variants.

We first investigated the kinetics of association of MDP with Triton X-100 DRMs in a pulse-chase assay (Fig. 5). wtMDP was present in the insoluble pellet fraction only in its complex glycosylated form; its immature form was present only in the detergent soluble fraction. In contrast, MDPΔGPI was excluded from the detergent insoluble pellet throughout the 2 h chase period, with both its immature and mature forms present exclusively in the detergent soluble fraction. Triton X-100 resistant membrane microdomains form at the level of the trans-Golgi network and plasma membrane [11]. Next we investigated whether this exclusion from Triton X-100 DRMs resulted in an impaired transport of MDPΔGPI after its release from the TGN. Therefore, surface immunoprecipitation of MDP in MDCK cells was performed (Fig. 6). Following a short labelling period (30 min) with [35S]-methionine, cells were chased for indicated times and proteins immunoprecipitated either at the cell surface for wtMDP or, in the case of MDPΔGPI, from the cell-free medium (Fig. 6 left panel). Interestingly, abolition of microdomain-association did not result in a significant retardation of the delivery of MDPΔGPI to the cell surface. Once the protein attained its complex glycosylated form, it reached the cell surface quite rapidly, independent from its integration into the membrane.

**Early association of MDP with DRMs along the secretory pathway**

Recently Alfalah et al. [7] showed that early biosynthetic forms of apically targeted proteins undergo polarized sorting. In fact sucrase-isomaltase, lactase-phlorizin hydrolase and aminopeptidase N as model proteins for apical sorting, were detected in Tween 20 insoluble fractions of cell lysates in their mannose-rich glycosylated forms, whereas basolaterally sorted proteins like the G protein of vesicular stomatitis virus, major histocompatibility...
complex class I, and CD46 were completely solubilized with this detergent [15]. We hypothesised that the association of early biosynthetic forms of MDP with Tween 20-DRMs might lead to the efficient transport of this protein. In order to restrict the cells to the mannose-rich forms of proteins, we labelled either stably expressing MDCK cells (Fig. 6) or transiently transfected COS-1 cells (data not shown) at 15 °C for only 20 min. Cells were lysed with Tween 20 and the detergent insoluble fraction was obtained via ultracentrifugation. A clear difference in extractability properties of wtMDP and MDPΔGPI was observed (Fig. 7A). Almost one third of total wtMDP was found in Tween 20-DRMs (Fig. 7B) indicating a clear association despite the low amount of protein that can be packed in these formations [15]. As for MDPΔGPI, this variant was essentially excluded from the Tween-20 DRMs (Fig. 7A).

To corroborate these findings, we utilized another method to create a similar environment in the cell with regard to the biosynthesis of glycoproteins. We used brefeldin A (BFA), a macrocyclic lactone from fungi that causes disorganization of Golgi stacks down to the level of cis-Golgi resulting in a block of anterograde membrane trafficking [29]. Mannose residues on proteins are no longer trimmed and addition of N-acetyl glucosamines does not occur. Transiently transfected COS-1 cells were treated with BFA for 2 h and lysed with Tween 20 in order to isolate detergent soluble and insoluble fractions. Essentially a similar pattern of protein distribution into DRMs was obtained (Fig. 8A). Quantification of the bands confirmed that a significant proportion of wtMDP was included in the detergent insoluble fraction, whereas a smaller proportion of MDPΔGPI was detergent insoluble (Fig. 8B). We also noticed a slight reduction in the size of the wtMDP that was detected in the Tween 20-resistant fraction. This could be due to a partial processing of the mannose-rich protein, possibly at the level of the ER to cis-Golgi. Together, the data indicate a role for DRMs in the sorting and transport of proteins from the ER to the Golgi apparatus, much earlier than at the level of the TGN.

Discussion

In this study, using anchored and anchorless forms of the GPI-linked MDP, we show that the GPI anchor facilitates anterograde trafficking through the secretory pathway and that the critical step is at the level of exit from the ER. The GPI anchor facilitates the association of MDP with Tween 20-resistant DRMs in the ER. This association is required for the efficient exit of the protein from this compartment. In contrast, MDPΔGPI which lacks the GPI anchor associates less efficiently with the Tween-20 DRMs and is retained in its high mannose, immature form within the ER for a prolonged period of time. The ER is the cellular compartment where proteins acquire tertiary structure and initial post-translational modifications. Aided by ER-resident chaperones, newly synthesised proteins attain transport competent conformation and exit this compartment. Misfolded proteins are not delivered further but rather retained and eventually targeted to the 26S proteasome and degraded via the ERAD pathway [30]. In order to investigate whether retention or prolonged stay of MDPΔGPI was a result of misfolding, we employed a protease sensitivity assay. MDPΔGPI displayed an identical trypsin resistance pattern to wtMDP, excluding the possibility that the protein resided in the ER due to defects in tertiary/quaternary structure. Furthermore, MDPΔGPI leaves the ER and once it reaches the level of the TGN, its transport to the cell surface (i.e. its appearance in the cell medium) occurs at a similar rate as for the GPI-anchored wtMDP.

Previously, contrary to what might be expected, we showed that the GPI anchored form of the prion protein (PrP) was trafficked to the surface of mammalian cells approx. 4-fold faster than a mutant lacking the GPI anchor [22]. Like MDP, PrP is also N-glycosylated but in the latter case the lack of GPI anchoring blocks its N-glycosylation [31]. In contrast, removal of GPI anchoring from MDP has no effect on its N-glycosylation [32]. In the current study we confirm that the lack of the GPI anchor slows the forward trafficking of another mammalian protein, MDP, through the secretory pathway, indicating that this phenomenon is not restricted to PrP.

PrP was shown to associate with membrane microdomains in the early secretory pathway. Endo H sensitive forms of the protein were found in lighter fractions of density gradients obtained after Triton X-100 extraction [33]. When microdomain association of the immature protein was disrupted by cholesterol depletion, the maturation of PrP was slowed and the protein misfolded. Like PrP, we have shown that MDP associates with DRMs in the early secretory pathway, and that this association is required for the efficient maturation of the protein. Previously we have shown that TWEEN 20 DRMs are involved in the early sorting of multiple apical proteins in epithelial cells [7]. The present work indicates another critical role for these membrane microdomains at the level of the ER in the efficient trafficking of GPI anchored proteins along the secretory pathway.

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Appendix A. Supplementary data


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CHAPTER 3

THE DIVERSE ROLES OF ANNEXIN II IN TARGETING OF BRUSH BORDER PROTEINS AND IN INTESTINAL CELL POLARITY
The diverse roles of annexin II in targeting of brush border proteins and in intestinal cell polarity

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The abbreviations used are: ACE2, angiotensin converting enzyme 2; A2, annexin II; ApN, aminopeptidase N; BBM, brush border membrane; DRM, detergent-resistant membrane; DPP4, dipeptidylpeptidase 4; K19, (cyto)keratin 19; MDCK, Madin-Darby Canine Kidney; MTOC, microtubule organizing centers; PI(4,5)P₂, phosphatidylinositol (4,5) bisphosphate; PI(3,4,5)P₃, phosphatidylinositol (3,4,5) trisphosphate; PTEN, phosphatase and tensin homolog; SI, sucrase isomaltase; t-PA (tissue plasminogen activator); WASP, Wiscott-Aldrich Syndrome protein.
Summary
Functional intestinal epithelium relies on complete polarization of enterocytes marked by the formation of microvilli and the accurate trafficking of glycoproteins to relevant membrane domains. Numerous transport pathways warrant the unique structural identity and protein/lipid composition of the brush border membrane. Annexin II (Ca\(^{2+}\)-dependent lipid binding protein) is an important component of one of the apical protein transport machineries, which involves detergent-resistant membranes and the actin cytoskeleton. Here, we investigate in intestinal Caco-2 cells the contribution of annexin II to the sorting and transport of brush border hydrolases and role in intestinal cell polarity. Downregulation of annexin II in Caco-2-A4 cell line results in a severe reduction of the levels of the brush border membrane resident enzymes, sucrase isomaltase and angiotensin converting enzyme 2 as well as structural components such as ezrin. This reduction is accompanied by a redistribution of these proteins to intracellular compartments and a striking morphological transition of Caco-2 cells to rudimentary epithelial cells that are characterized by an almost flat apical membrane with sparse and short microvilli. Concomitant with this alteration is the redistribution of the intermediate filament protein keratin 19 to the intracellular membranes in Caco-2-A4 cells. Interestingly, keratin 19 interacts strongly with annexin II in wild type Caco-2 cells and this interaction occurs exclusively in lipid rafts. Our findings suggest a role for annexin II and K19 in differentiation and polarization of intestinal cells.

Introduction
Establishment of a polarized state is one of the major manifestations of the intestinal epithelium and it is fundamental to biogenesis of a functional gut. A unique structural feature of a polarized cell is the presence of distinct plasma membrane domains separated by tight junctions. The spatial arrangement of apical and basolateral domains warrants identity and functionality to intestinal tissue and includes accurate sorting of proteins and lipids to target membranes. Sustained and flawless function of the intricate machineries behind intracellular trafficking is essential for maintaining the homeostasis of the digestive tract and the organism itself. In the gut, one of the hallmarks of an intact and functional mucosa is the generation of microvilli at the apical membrane of enterocytes. These formations are decorated with digestive enzymes, intrinsic membrane proteins, receptor molecules, ion channels, and carrier protein complexes that are crucial for nutrient uptake. Additionally, actin based motor proteins like myosin Vb, myosin Ib contribute to the microvillus formation and delivery of
transport vesicles to the brush border membrane (BBM) and mutations in these genes result in perturbations in BBM structure (Hegan et al., 2007; Muller et al., 2008). Regardless of their colonic origin, Caco-2 cells spontaneously start to differentiate into small intestinal epithelial cells in culture (Pinto et al., 1983), including microvilli development and expression of enzymes actually found in the small intestine (Hauri et al., 1985; Kenny and Maroux, 1982). These characteristics render these cells a suitable model for studying the transport of intestinal proteins and cell differentiation in vitro.

Proteins that enter the secretory pathway are sorted by elaborate mechanisms. Polarized sorting is not only restricted to the discrimination between apical and basolateral proteins in the trans-Golgi network (TGN), but also among apically-sorted proteins (Jacob and Naim, 2001). In fact, distinct vesicle populations of different membrane lipid composition have been identified that exploit distinct cytoskeletal tracks. Expectedly, proteins packed into these vesicles differ in their biochemical properties as well. To date, proteins identified in a subset of post-Golgi vesicles with cholesterol-sphingolipid-rich membrane microdomains are galectin-3, alpha-kinase, and annexin II (A2) (Delacour et al., 2006; Heine et al., 2005; Jacob et al., 2004). Annexin II is a member of a family of cytosolic Ca\(^{2+}\)-binding proteins and has the ability to dock onto negatively charged membranes (Gerke and Moss, 2002; Gerke and Weber, 1984). A2 is a multifaceted protein, proven to play a role in events involving dynamic actin assembly at cellular membranes (Hayes et al., 2004; Hayes et al., 2006). For example, formation of actin pedestals at the enteropathogenic E.coli (EPEC) attachment sites on host cell membrane (Zobiack et al., 2002), actin-based macropinocytic rocketing (Merrifield et al., 2001), biogenesis of multivesicular endosomes (Mayran et al., 2003), cell motility (de Graauw et al., 2008; Hastie et al., 2008), and regulation of cell architecture (Benaud et al., 2004). Recently, Martin-Belmonte et al. (2007) have demonstrated that annexin II is involved in formation of cyst lumens of 3D cultures of MDCK cells. This process that is initiated by segregation of phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P\(_2\)) and phosphatidylinositol (3,4,5)-triphosphate (PI(3,4,5)P\(_3\)) via PTEN,(phosphatase and tensin homologue) continues with recruitment of A2 to PI(4,5)P\(_2\)-rich apical membrane, which then recruits Cdc42 (Martin-Belmonte et al., 2007). Altogether, these set of events result in organization of sub-apical actin cytoskeleton and formation of apical membrane.

Employing siRNA technology, we succeeded in creating clones of Caco-2 in which expression of annexin II was substantially downregulated to nearly 15% of normal situation. We examined one of these clones (Caco-2-A4) in detail in order to gain information about cellular processes like cell growth, differentiation, and sorting of apical proteins. Caco-2-A4...
revealed that in the absence of annexin II, cells grew normally but failed to differentiate into small intestinal epithelium, which is characterised by development of microvilli and a marked increase in expression of brush border hydrolases. In absence of annexin II, important structural components of the cytoskeletal network underlining the brush border membrane like keratin 19 and ezrin were also redistributed and diminished in brush border fractions. Despite these drastic alterations, tight junctions were normal and cell architecture remained unaffected. Our results indicate an important role played by annexin II in the initial steps of intestinal epithelial differentiation as an indispensable linker between cytoskeleton and brush border membrane components in differentiating cells, interacting with at least one member of intermediate filaments.

Results and Discussion

Creation of a knock-down cell line
Caco-2 cells were transfected with siSTRIKE™ U6 Hairpin Cloning System (Promega, Madison, WI) coding for anti-sense RNA directed against annexin II. Two constructs targeting two different locations on annexin II cDNA were used (positions 93-111 and 989-1007). Following selection with puromycin (5μg/ml), antibiotic resistant clones were tested for annexin II expression by western blotting using anti-annexin II antibodies (Figure 1A). Clone A4 in which siRNA targets sequences 93-111 of the annexin cDNA, revealed a substantial downregulation of annexin II to approximately 15% of wild type levels (Figure 1B). The clone A4 (indicated Caco-2-A4) was used in further experiments.

Expression levels of brush border proteins
To assess the consequences of downregulation of annexin II on proteins of the brush border membrane, cell lysates of wild type Caco-2 and Caco-2-A4 were prepared at day 5 after confluence, and the protein expression levels were investigated by western blotting (Figure 1A). The brush border enzyme sucrase isomaltase (SI) was found to be drastically downregulated (Fig.1A, right panel) whereas the expression of another brush border enzyme, dipeptidyl peptidase IV (DPPIV), remained unchanged. Over 90% of newly synthesized SI has been shown to be directly sorted to apical surface (Le Bivic et al., 1990; Matter et al., 1990) whereas DPPIV is targeted to the apical membrane in Caco-2 cells via a transcytotic
pathway, i.e. first delivered to basolateral membrane and then transported to the apical membrane, as well as a direct apical transport mechanism (Matter et al., 1990). Supported by previous evidence, this led us to assume that distinct trafficking mechanisms involved in the transport of apical or brush border proteins might be differentially regulated by the lack of annexin II. Sucrase-isomaltase has been oftentimes used as a differentiation marker of Caco-2 cells (Hauri et al., 1985; Zweibaum et al., 1983). It has been demonstrated that the expression of SI in several clones of Caco-2 cells is strongly associated with the differentiation state of these cells and reveals the highest expression variability among other enzymes like DPPIV, lactase-phlorizin hydrolase, alkaline phosphatase and aminopeptidase N (ApN) (Beaulieu and Quaroni, 1991). The low SI levels in Caco-2-A4 clone can be therefore directly linked to an incomplete polarity or a rudimentary polarized state of Caco-2-A4. Similar to SI, expression of angiotensin converting enzyme 2 (ACE2), which localizes to brush border membrane of Caco-2 cells (Ren et al., 2006), was downregulated in Caco-2-A4 as well. Brush border marker protein ezrin (villin-2) expression in differentiated cells has been shown to be approximately 3 times higher in comparison to proliferating Caco-2 cells (Pshezhetsky et al., 2007), there is also evidence from mRNA microarray analyses that ezrin expression increases in the early phases of cell differentiation and the levels are maintained as cells fully polarize (Halbleib et al., 2007). In our blots, clone Caco-2-A4 revealed lower levels of ezrin than in the wild type cells (Fig. 1A, left panel). Another brush border component, keratin 19 (K19) (Salas et al., 1997) revealed an increased band intensity in Caco-2-A4 cell line (see also later) and therefore a higher expression level. The levels of β-actin and calnexin were similar in the wild type and Caco-2-A4 cells.

**Cell differentiation in WT Caco-2 cells and Caco-2-A4**

Caco-2 cells spontaneously differentiate in culture to the enterocyte phenotype and the expression of hydrolytic brush border enzymes temporally increases after reaching confluence concomitant with the acquisition of a fully polarized state (Beaulieu and Quaroni, 1991; Hauri et al., 1985; Pinto et al., 1983; Wice et al., 1985). To monitor cell differentiation as a function of expression levels of brush border proteins Caco-2 cells were cultured to confluence and cell lysates were probed at different stages post-confluence (0, 3, 6, 9 days) for brush border resident enzymes and structural components of microvilli by western blotting. As expected, expression of SI increased progressively with time in wild type Caco-2 cells (Fig.2A, upper left). By contrast, the expression levels of SI in the Caco-2-A4 were drastically reduced and maintained essentially similar levels regardless of the time after confluence. In contrast to SI,
the pattern of expression of DPPIV was comparable in both cell lines as assessed by the steady increase in the expression levels in wild type Caco-2 and Caco-2-A4 cells. A profile of increased expression levels was also observed for ezrin in Caco-2 cells, peaking at day 6 postconfluence and remaining constant after this period (Fig. 2A, left panel). On the contrary, ezrin was gradually downregulated in Caco-2-A4, within the first 6 days, but revealed a marked increase at day 9 postconfluence. Ezrin is a component of apical F-actin-based scaffold, and it is one of the earliest proteins to be recruited to this scaffold (Bretschcher et al., 1997). Interestingly, Saotome et. al (2004) presented in vivo data to demonstrate that ezrin was not required for microvillus morphogenesis or epithelial polarity (Saotome et al., 2004). Instead, the function attributed to this protein is the organization of terminal web, which provides a platform for anchoring microvilli. This finding is also supported in vitro, where introduction of ezrin to fibroblasts caused actin containing protrusions resembling microvilli (Shaw et al., 1998). In light of these previous studies and our current findings, it is likely that reduction in ezrin levels is an indicator of undifferentiated state in Caco-2 cells.

We then investigated the expression of keratin 19 during 9 days following confluence. To date, contribution of K19 to the formation of microvilli is not clear. Keratin 19 knock out mice do not show any apparent phenotype, but deletion of both type II keratins, K18 and K19, is embryonic lethal (Tamai et al., 2000). Both type II keratins, K18 and K19, pair with keratin 8 in simple epithelium (Moll et al., 1982). Caco-2 cells lacking keratin 8 cannot recruit ezrin to the apical surface and consequently, the actin-ezrin scaffold fails to assemble (Wald et al., 2005). Further, a transitional interaction between ezrin and keratin 8 could be demonstrated that could be crucial for the establishment of microvilli. In our study, K19 levels did not vary during 9 days of confluence (Fig. 2A), while an increase in the intensity of this protein could be observed in Caco-2-A4 cells (Fig. 1, Fig. 2A). To evaluate the expression pattern of K19 in more detail, we compared its electrophoretic pattern by 2D gel electrophoresis combined with western blotting using anti-Keratin 19, A53-B/A2 antibody. The lysates derived from Caco-2-A4 revealed a distinct protein pattern comprising two strongly expressed protein spots, whereas K19 detected in Caco-2 cells revealed one single spot (Fig. 2B). First, this indicates that the expression level of K19 in Caco-2-A4 clone is higher than that in the wild type cells. Second, the appearance of a more basic spot is compatible with posttranslational modifications that alter the charge of K19, such as cycles of phosphorylation and dephosphorylation. While the effect of phosphorylation on K19 is unknown, phosphorylation of keratins in general can lead to an increased solubility and reversible disassembly of keratin filament structure (Ridge et al., 2005; Yano et al., 1991). It has been shown that a mutation at
the major phosphorylation site of K19, Ser35, results in defective filament assembly, while phosphorylation mutations in K8 and K18 did not affect filament assembly (Zhou et al., 1999). Accordingly, it is plausible to assume that unphosphorylated K19 would not be able to interact with its partners and a concomitant impaired IF assembly can lead to disorganization of initial cytoskeletal scaffold on which microvilli form. Evidence from the work of Wald et al. (2005) supports this assumption by indicating that ezrin-actin scaffold fails to assemble when K8 is absent from the cells. Furthermore, overexpression of this keratin sequesters ezrin in a subapical region where it can not interact with actin anymore. Whether this applies to K19 and ezrin remains to be elucidated.

**Ultrastructural analysis of Caco-2**

Establishment of cell-cell contacts in Caco-2 cells is followed by spatial organization of two distinct membrane domains. One characteristic feature of these cells is the formation of an array of cytoskeletal protrusions, microvilli, at the apical membrane. We wanted therefore to determine whether downregulation of annexin II elicited any alterations in the cell morphology. For this, we compared the Caco-2 with Caco-2-A4 cells at the ultrastructural level by electron microscopy. In both cases, cells at 7 days of confluence were analysed, during which time an increase in expression rates of SI, DPPIV, and ezrin has been observed. As shown in Fig. 3 dramatic differences between the two cell lines could be demonstrated with respect to the length of the microvilli, their distribution and density at the cell surface,. wild type Caco-2 cells revealed uniformly aligned and parallel distributed microvilli at the apical membrane, while the microvilli in Caco-2-A4 cells were rudimentary, disorganized, shorter and substantially fewer. Interestingly, microtubular structures were scattered in a disorganized manner occupying the cytoplasm. The microvillar appearance and organization in Caco-2-A4 at day 7 resembles early stages of confluent Caco-2BBe cells (Peterson and Mooseker, 1993) and is far behind the well-differentiated wild type by Caco-2 cells.

**Adherens and tight junctions**

Formation of cell-cell adhesion complexes is essential for an intact epithelial barrier. Mistargeting or disassembly of member proteins results in loss of polarity and dissolution of cell barrier. Recently, annexin II has been proposed to act as a linker in vascular endothelial adherens junctions between actin cytoskeleton, vascular endothelial cadherin-based complex and cholesterol-rich membrane domains (Heyraud et al., 2008). Given the dramatic morphological alterations in the brush border membrane in Caco-2-A4 cells, we set out to
further examine the cell-cell contacts and junctional structures in Caco-2-A4 lacking annexin II using the protein markers E-cadherin and ZO-1. E-cadherin is one of the major components of adherens junctions found at the lateral plasma membrane that mediates intercellular contacts by pairing with other E-cadherin molecules on neighbouring cells (Gumbiner, 2005). ZO-1 localizes to tight junctions and serves as a connecting element between junctional complexes and cytoskeleton (Stevenson et al., 1986). Cells grown on coverslips were fixed 6 days postconfluence, i.e. following accomplishment of cell-cell contacts, and labelled for the proteins of interest. In wild type Caco-2 cells, E-cadherin was strongly labelled at the lateral membrane (Fig. 4A). F-actin localized to the apical membrane and was also found to some extent at the cell boundaries. In Caco-2-A4 cells the subcellular distribution of E-cadherin was found unaffected (Fig.4B), the labeling intensity, however, was reduced. Likewise, a weaker actin labeling could be also demonstrated in these cells. Downregulation of annexin II had no marked influence on the localization of ZO-1, as assessed by the similar intensities in Caco-2 and Caco-2-A4 and its detection at the cell-cell junctions (Fig. 4C and 4D) and at the border of apical and lateral membranes (Fig. 4D lower panel, xz-axis). Together, the ultrastructural analyses and the subcellular distribution ultrastructural annexin II downregulation has affected more substantially the morphology of the brush border membrane development than the basal-lateral domains. Given the direct implication of annexin II in the membrane trafficking to the apical membrane, it is plausible to assume that the membrane integrity and homeostasis have been severely affected.

**Biochemical analyses of brush border development**

The drastic morphological alterations in the brush border membrane in Caco-2-A4 have been further substantiated biochemically by assessment of the levels of brush border and intracellular proteins in subcellular fractions of these cells in comparison to wild type Caco-2 cells. For this, brush border membranes were separated from intracellular and basolateral membranes through the discriminatory effect of divalent ions such as Ca$^{2+}$ or Mg$^{2+}$. Brush border membranes do not precipitate with these divalent ions and can be easily separated in a relatively pure form from other membranes by centrifugation. Usually the enrichment factor of the brush border membranes in the P2 fraction (see Materials and Methods) correlates with a well developed brush border membrane (Chantret et al., 1988; Schmitz et al., 1973). This factor is assessed by comparison the activities of the intestinal differentiation marker SI in the P2 fraction versus the total cellular homogenates. Brush border membranes prepared at different stages of confluence after establishment of the cell monolayer (0, 3, 6, and 9 days)
revealed the highest enrichment of the brush border membranes at day 6 of confluence (Fig.5). Here, the activity of SI in P2 was approximately 5 times higher than in homogenate. In the Caco-2-A4 cell line, on the other hand, the low SI activity levels were accompanied by a substantial reduction in the enrichment factor of this enzyme in P2 (Fig.5), which did not change 9 days postconfluence.

Next, the patterns of several proteins in the P2 and P1 fractions were analysed by western blots. In agreement with the enzymatic activity data, the SI band in the P2 fraction was several orders of magnitude stronger than its counterpart in the P1 fraction (Fig.6A). In Caco-2-A4 the overall protein intensity of SI in P2 and P1 was substantially reduced concomitant with reduced expression levels of SI. Despite the low protein and activity levels of SI in Caco-2-A4 cells an enrichment factor of roughly 2.9 of P2 could be calculated. SI is considered to be a relevant protein marker for differentiation of intestinal cells. The reduction in the expression levels of Caco-2 cells is therefore suggestive of incomplete differentiation pattern supporting the electron microscopy data (see Fig. 3). In sharp contrast to SI, significant differences were detected neither in the distribution patterns of DPPIV (among H, P1 and P2) nor in its expression levels in Caco-2 and Caco-2-A4 cells. Additionally, the enrichment factor of DPPIV in P2 was almost 2-fold lower than that of SI (Fig.6B). This lower expression level at the BBM could be explained by the trafficking behaviour of DPPIV in Caco-2 cells. In fact, DPPIV is transcytosed via the basolateral membrane to the apical membrane (Matter et al., 1990) and is therefore expected to be partially present at steady state at the basolateral membranes which are comprised by the P1 fraction. Obviously, DPPIV expression levels are independent of the differentiation state of intestinal cells. The microvillus protein component ezrin was recovered predominantly in P2 fractions in both cell lines (Fig. 6B). Strikingly, distribution of keratin 19 was affected most. Polarization of K19 begins early in Caco-2 cells, before the brush border begins to be organized (Wald et al., 2005). In wild type Caco-2 cells K19 was found exclusively in the brush border membrane since it partitioned into the P2 fraction (Fig.6A). In Caco-2-A4 the distribution of K19 shifted substantially from the brush border membrane in P2 and became predominantly located in P1 (Fig.6A and 6C). This observation points to a relocation of K19 from the subapical compartment. It is possible that the absence and/or inactivation (dephosphorylation) of K19 in this cellular area is responsible for perturbed microvillar organization. Compelling evidence has demonstrated an association of K19 with γ-tubulin, a component of microtubule organizing centers (MTOC) (Salas, 1999). Intermediate filaments below the apical membrane are thought to anchor MTOCs and establish a cue for polarized distribution of microtubuli.
Considering their early polarization and association with MTOCs it is plausible to suggest intermediate filaments as initial scaffolds serving various factors like actin-bundling elements (e.g. villin, fimbrin, ezrin), other cytoskeletal components (e.g. myosin Ia) and extrinsic membrane proteins that contribute to formation of microvilli. The keratins K19 and K21 reveal variable expression in the intestine and are reliable markers of the gut morphogenesis. While K19 is mainly expressed in proliferative crypt cells, K21 is exclusively found in well differentiated villus, goblet, enteroendocrine, and Paneth cells (Beaulieu and Quaroni, 1991). The elevated expression levels of K19 upon downregulation of annexin II in Caco-2-A4 supports the notion that this cell line is less differentiated than wild type Caco-2 cells thus strongly confirming the morphological data (see Fig. 4).

We further investigated the cellular distribution of actin in Caco-2-A4 and found that its enrichment in the brush border fraction decreased substantially in comparison to wild type Caco-2 cells (Fig. 6A, lowest panel and Fig. 6B). This is most likely due to reduction in number and size of microvilli, which contain considerable amounts of actin.

In addition to the apically located proteins we also probed the brush border fractions for purity using an intracellular protein marker, the endoplasmic reticulum (ER) resident protein calnexin. As expected, calnexin was completely absent from the P2 fractions of wild type Caco-2. On the other hand, the partial presence of calnexin in the P2 fraction of Caco-2-A4 cells points to an increased contamination of intracellular membranes in P2 and an exacerbated purification of P2 likely due to a rudimentary apical membrane.

**Keratin 19 – annexin II interaction**

There is a great deal of evidence indicating an essential role of annexin II as a cross-linking element between exo/endocytic vesicles and the plasma membrane (Drust and Creutz, 1988; Gerke and Moss, 2002; Liu et al., 1995; Senda et al., 1998). We investigated a possible interaction between annexin II and keratin 19, since intracellular compartmentalization of this protein was severely affected by the downregulation of annexin II. An interaction between annexin II and keratin 19 could be demonstrated in a co-immunoprecipitation experiment (Fig. 7). Here, a strong annexin II band could be recovered from the immunoprecipitates using an anti-K19 antibody. Importantly, annexin II was detected in the fraction representing Triton X-100 DRMs (detergent resistant membranes) (Fig. 7, lane DRM pellet: IP). Compared to the minor co-immunoprecipitated annexin II in the detergent soluble fraction (Fig. 7, lane Supernatant: IP), the result unequivocally indicate that the interaction between annexin II and keratin 19 occurs in DRMs. We were also able to demonstrate colocalization of these two
molecules in wild type Caco-2 cells (Fig.8). We used proliferating Caco-2 cells since it is more convenient to visualise cellular components and we sought an association between annexin II and keratin 19 at early stages of cell differentiation. We observed costained areas as a part of vesicular structures which were positive for annexin II. Once again, the colocalization pattern of both molecules supported a transient interaction between annexin II containing vesicles and K19 scaffold. Until now, only one report has demonstrated a colocalization of annexin 1 and cytokeratin 8 in lung adenocarcinoma cell line A549 (Traverso et al., 1998). This association was independent of an intact actin cytoskeleton and was restricted to an area below the plasma membrane. A functional explanation for this interaction has not been made until now. We speculate that annexin II can act as a docking element between the intermediate filaments and the actin scaffold at the base of microvilli. It is known that annexin II can interact with actin per se or heterotetrameric annexin II-p11 complex can indirectly bind actin via AHNAK (for a review see (Hayes et al., 2004)). All the functions attributed to annexin II in this respect are dynamic tasks such as fusion of membranes and actin remodelling. Upregulation of keratin 19 in the absence of annexin II indicates a strong connection between these molecules. We propose that in absence of annexin II, K19 fails to assemble properly and consequently, structural elements necessary for the brush border architecture can not be delivered to their target location. Although this delivery mechanism has been until present poorly characterised, a growing body of evidence based on knock-out and overexpression models points to importance of keratins in epithelial polarity and vectorial exocytosis (Oriolo et al., 2007). It is also possible that annexin II can link a kinase or its substrates which phosphorylates keratin 19. Potential enzymes responsible for phosphorylation suggested are PKC, calmodulin-dependant kinase and cAMP-dependent kinase (Ridge et al., 2005; Zhou et al., 1999). To date, information delivered on effects of phosphorylation of intermediate filaments concentrate on hyperphosphorylation of keratins, which leads to disassembly and destruction of these structures (Baricault et al., 1994; Ridge et al., 2005; Yano et al., 1991; Zhou et al., 2006). Reduction of phosphorylated K19 could provide feedback information and give rise to compensatory K19 expression.

Concluding Remarks
In this paper, we present findings on the effect of absence of annexin II in Caco-2 cells. To date, downregulation of annexin II in mice and cell culture has caused several outcomes that can not be correlated due to the diversity of the investigated functions. Annexin II knock-out mice were created in order to investigate the function of this protein as a cofactor of
plasminogen and tissue plasminogen activator (t-PA) (Ling et al., 2004). The knock-out mice were viable and did not display any particular defects except for fibrin homeostasis. Other RNAi-based transitional downregulation approaches in cultured cells emphasise the effect of annexin II on actin cytoskeleton in cellular processes including apical delivery of post-TGN vesicles (Jacob et al., 2004), cortical actin organization via AHNAK protein (Benaud et al., 2004), cell migration (Babbin et al., 2007; Tatenhorst et al., 2006), dynamic actin based activities (e.g. macropinocytosis (Merrifield et al., 2001)) at the plasma membrane (Hayes et al., 2006), distribution of recycling endosomes (Zobiack et al., 2003) and formation of epithelial adherens junctions (Heyraud et al., 2008; Yamada et al., 2005). In the light of our results, we argue that annexin II is an essential component of a machinery that drives cell polarity and differentiation. This protein plays a key regulatory role in the organization of sub-apical cytoskeleton and related pathways during establishment of apical membrane in epithelial cells. Annexin II concentrates at PI(4,5)P$_2$-containing membranes (Rescher et al., 2004) and provides contact sites for actin cytoskeleton at cellular membranes. Apart from being a second messenger, PI(4,5)P$_2$ was shown to act as a critical modulatory cofactor in signalling pathways such as Cdc42-WASP-Arp2/3 and Rho-ERM involving actin recruitment and organization (see reviews (Caroni, 2001; Sechi and Wehland, 2000)). Annexin II is involved in both of these pathways that mediate cell polarity, adhesion and motility (Martin-Belmonte et al., 2007; Rescher et al., 2008). Whether annexin II contributes to these processes primarily as a member of signalling cascades or as an organizer of lipid microdomains is not clear. It is more likely that annexin II is a non-redundant lipid-binding and clustering element that stabilizes signalling platforms at cell surface (Gerke et al., 2005; Oliferenko et al., 1999; Rescher and Gerke, 2004). Absence of annexin II can lead to impaired aggregation of PI(4,5)P$_2$ at the plasma membrane. Ezrin and WASP require PI(4,5)P$_2$ for their activation and following downstream events depend on activation of these key components. Thus, these pathways can not be initiated when annexin II is not there to bind and stabilize membrane microdomains.

To date, reported interactions between annexins and intermediate filaments are restricted. Such a mutual action between annexin II-p11 heterotetramer and type III intermediate filaments was reported in glial cells (Bianchi et al., 1994). Our study revealed that distribution of keratin 19 was severely affected in the absence of annexin II in Caco-2 cells. We were also able to demonstrate a colocalization of annexin II and keratin 19 in wild type Caco-2 cells. Contribution of K19 to organization of apical submembrane cytoskeleton and formation of apical pole has been readily demonstrated in Caco-2 cells (Salas et al., 1997). Although we do
not know how annexin II influences the apical localization of K19, it is evident that an interaction between these two molecules is necessary to maintain the scaffold on which brush border membrane depends.

Although these initial findings require further biochemical investigations, we consider such a link between a cytoplasmic lipid-binding protein and intermediate filaments as a promising topic underlying cell differentiation.

Taken altogether, reduction of brush border associated enzymes and altered expression levels and posttranslational modifications of proteins involved in brush border formation, such as ezrin and keratin 19 supports the notion that annexin II is a key player in cell differentiation and an indispensable element of apical membrane domain.

**Materials and Methods**

**Reagents and antibodies**
The mAb anti-annexin II antibody was a kind gift from Volker Gerke (Osborn et al., 1988), mAb anti-SI antibody HBB 3/705 and mAb anti-DPPIV antibodies were obtained from Drs. Hans-Peter Hauri and Erwin Sterchi (University of Basel and University of Bern, both Switzerland), mAb anti-actin, and polyclonal anti-ezrin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-keratin 19 Ab, A53-B/A2, was purchased from Sigma (Munich, Germany). The mAb anti-zona occludens 1 (ZO-1) was obtained from Zymed (San Francisco, CA). Phalloidin coupled to rhodamin and secondary antibodies coupled to Alexa Fluor dyes were purchased from Molecular Probes (Invitrogen, Karlsruhe, Germany). The mAb anti-calnexin and mAb anti E-cadherin antibodies were purchased from BD Biosciences (Heidelberg, Germany). Horseradish peroxidase-coupled goat anti-rabbit and goat anti-mouse and rabbit anti-goat antibodies were from Dako (Glostrup, Denmark). Hybond™-P PVDF membrane was obtained from Amersham Biosciences (Freiburg, Germany) and ECL from Pierce (Thermo Fisher Scientific (Bonn, Germany). Cell culture media, fetal calf serum and antibiotics were purchased from PAA (Pasching, Austria).

**Cell culture**
Caco-2 cells of human adenocarcinoma origin were cultured in humidified atmosphere containing 5% CO₂ in air at 37°C in Dulbecco’s Modified Eagle’s Medium (DMEM)
containing 4.5g/L glucose in the presence of fetal calf serum (10% v/v) and penicillin-streptomycin (100U/ml and 0.1mg/ml respectively). Cells were seeded at high density (5-6 x 10^4 cells/cm^2) and confluence was estimated by microscopy.

**Downregulation of annexin A2 expression in Caco-2 cells**
Caco-2 cells were stably transfected with plasmids (siSTRIKE™ U6 Hairpin Cloning System, Promega, Madison, WI) coding for anti-sense RNA directed against annexin II. Two different constructs targeting two different locations on annexin II cDNA were tested (position 93-111 and 989-1007). Cell line A4 (indicated thereafter Caco-2-A4) used in this study is a clone of Caco-2 cells stably transfected with anti-sense annexin II construct directed against nucleotides in positions 93-111.

**Preparation of brush border membranes**
Brush border membranes of Caco-2 cells were isolated by the modified (Sterchi and Woodley, 1980) divalent cation precipitation method (Schmitz et al., 1973). Briefly, the cells were homogenized using a Potter-Elvehjem homogenizer in the hypertonic homogenization buffer (Tris-HCl 2mM, mannitol 50 mM pH 7.1) supplemented with a mixture of protease inhibitors. The homogenates were passed through a Luer-21 Gauge needle 20 times/ml and CaCl_2 was added to a final concentration of 10 mM. Following 30 minutes gentle agitation at 4°C, the homogenates were centrifuged at 2,000 x g for 20 minutes to obtain basal-lateral and intracellular membranes. Finally the supernatant was centrifuged at 25,000 x g for 30 minutes to yield the brush border membranes. Where appropriate solubilization of the membranes was performed with 1% Triton X-100 in PBS for 2 h in the cold followed by high speed centrifugation and the supernatant was further used in biochemical analyses.

**Immunoassays**
In co-immunoprecipitation experiment, wild type Caco-2 cells were solubilized with Triton X-100 (1% w/v in PBS) for 2h at 4°C and a postnuclear supernatant (lysate) was obtained by a short high speed centrifugation. The supernatant was subjected to ultracentrifugation (100,000 x g for 1h). Supernatant (S1) was used in immunoprecipitation as it was and the pellet (P1) (DRM) was solubilized further with deoxycholate (0.5 % in PBS) at 4° for 1h. This lysate was centrifuged briefly to separate insoluble particles (P2) and the antibody for immunoprecipitation was added to its supernatant (S2).
For indirect immunofluorescence, cells were fixed in 4% paraformaldehyde (in PBS, pH 7.4) at room temperature. Quenching was performed 2 times for 10 minutes in ammonium chloride (50 mM NH₄Cl in PBS). Blocking and addition of the antibodies were performed in PBS containing 1% bovine serum albumin and 0.5% saponin. Preparations were visualized using a Leica TCS SP2 confocal laser microscope with a 63x oil planapochromat lens (Leica Microsystems).

*Transmission electron microscopy of ultrathin cryosections.*

For detection of brush border membrane and underlying actin network, epon sections were processed with standard methods. Mucosa samples were fixed in 1% glutaraldehyde (in medium for 10 min at 37°C) and washed with 0.1 mol/L sodium cacodylate buffer and postfixed in osmium tetroxide (OsO₄). Samples were dehydrated through a series of graded ethanol washes and embedded in epon. Sections were cut, placed onto mesh copper grids, stained with uranyl acetate and lead salts. Grids were analyzed using a Philips EM 400 T transmission electron microscope (Kassel, Germany).

*Sucrase activity assay*

Sucrase activity in the homogenates, intracellular and basolateral membranes (P1, pellet 1 fraction) and brush border membranes (P2, pellet 2 fraction) was measured using hexokinase method (Gluco-quant, Roche Diagnostics, Mannheim, Germany).

*Quantification of band intensities*

Specific bands on PVDF membranes detected by antibodies were visualised by a ChemiDoc XRS Molecular Imager (BioRad, Hercules, CA) device. Digital images obtained from imager were quantified by using image processing and analysis software ImageJ (1.37v).

**Acknowledgements**

We would like to thank Hans-Peter Hauri (Biozentrum Basel, Switzerland) and Erwin Sterchi (University of Bern, Switzerland) for the gifts of anti-sucrase-isomaltase antibodies. The excellent technical assistance of Gabi Wetzel is highly appreciated. This work has been supported by a grant from the Deutsche Forschungsgemeinschaft (DFG) to H.Y.N. (SFB 621: Pathobiology of the Intestinal Mucosa).
References


**Figure legends**

**Figure 1. Expression analysis of brush border components in wild type Caco-2 and Caco-2-A4 cells.** (A) Equal amounts of total protein from lysates of Caco-2 cells was loaded for each lane on SDS gels and blotted. Protein amounts in rows are 50 μg for annexin, 20μg for ezrin, K19, actin, DPP4 and calnexin, and 75μg for SI, respectively. Star (*) indicates the specific 40 kDa keratin 19 band (B) Band intensities of annexin from independent gels were quantified and demonstrated in column chart (means ±SD, n=4). Annexin II amount in wt Caco-2 cells was set to 100% and relative expression rates in clones are displayed.

**Figure 2. Brush border development during first 9 days after confluence and 2D analysis of keratin 19.** (A) WT Caco-2 and Caco-2-A4 cells were lysed on indicated days post-confluence; samples containing equal amounts of total protein were separated by SDS-PAGE. Except for SI, 20 μg total protein was loaded in each lane. For the detection of SI, 50 and 100 μg protein was loaded from WT Caco-2 and Caco-2-A4 cells, respectively. Star (*) indicates the specific 40 kDa keratin 19 band. (B) 2D-gel electrophoresis and western blotting of K19. 50 μg of WT Caco-2 and Caco-2-A4 lysates were subjected to 2D-gel electrophoresis (Isoelectric focusing, pH range from 3 to 10 and electrophoresis on 10% slab gels). Gels were blotted on PVDF membranes and K19 was detected by monoclonal anti-keratin-19 antibody.

**Figure 3. Ultrastructural analysis of brush border membrane.** 6 days confluent Caco-2 cells were fixed with glutaraldehyde and ultrathin sections were prepared for transmission electron microscopy. Arrowheads in left panel indicate actin rootlets at microvillus base. In right panel arrows are directed against microtubules. Abbreviations: N, nucleus; mt, mitochondrion.

**Figure 4. Localization of junctional proteins.** (A,B) 6 day confluent WT Caco-2 (A) and Caco-2-A4 (B) monolayers were fixed and E-cadherin (green) and actin (red) were detected by indirect immunofluorescence. (C, D) ZO-1 (green) and actin (red) were immunolabelled in monolayers on post-confluent day 6 (WT Caco-2 (C) and Caco-2-A4 (D)) with monoclonal anti-ZO-1 antibody and phalloidin-rhodamin, respectively. Scale bars 20µm.

**Figure 5. SI activity at the brush border membrane.** Enzyme activity from brush border fraction (P2) and homogenate (H) was determined and relative activity (mM glucose/μg protein produced in P2 divided by mM glucose/μg protein produced in H) was calculated and displayed as bars (means ±SD, n=3)

**Figure 6. Biochemical analysis of the distribution of brush border enzymes and marker proteins.** (A) Brush border membranes were isolated by calcium chloride precipitation
method. Protein amounts in homogenate (H)(total membranes and cytosol, microsomal, internal membranes (P1) (membrane bound intracellular components and basolateral membranes) and, brush border membranes (P2) were determined and equal amounts were loaded on SDS gels for each protein investigated (SI, 75µg; DPP4, ezrin, keratin 19, calnexin, actin, 20µg). Star (*) indicates the specific 40 kDa keratin 19 band. (B) Band intensities from H and P2 fractions were quantified and mean intensity in P2 was divided by mean intensity in H for corresponding proteins. Results are demonstrated in column chart (means ±SD, n=3 or 4). (C) Keratin 19 band intensities were quantified, amount of keratin 19 in homogenates was set to 1. Relative amounts calculated are displayed. Data are mean ratios of 4 independent experiments ±SD.

**Figure 7. Co-immunoprecipitation of keratin 19 and annexin II from wild type Caco-2 lysates.** Caco-2 cells were solubilised with 1% Triton X-100 in PBS and ultracentrifuged. The pellet (DRM) was retained and solubilised with 0.5% DOC and brought to the same volume as the supernatant of the same centrifugation. Immunoprecipiation of the supernatant (indicated S1) and DRM (indicated P1) with anti-K19 was performed followed by Western blotting with anti-annexin II antibodies. The samples analysed by Western blotting (from left to right) were: total cell lysates, immunoprecipitated supernatant S1 with anti-K19, immunoprecipitated DRM with anti-K19, DRM pellet solubilized prior to IP (S2) and DRM pellet after IP (P2).

**Figure 8. Intracellular localization of keratin 19 and annexin II in Caco-2 cells.** Wild type Caco-2 cells were fixed with paraformaldehyde and keratin 19 (green) and annexin II (red) were labelled by indirect immunofluorescence. Note the annexin II positive vesicles partially overlapping with K19 filaments (yellow) (white arrowheads). Scale bars 20µm.
Hein et al. Figure 2

<table>
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B

Hein et al. Figure 2
Hein et al. Figure 4D

merge

Caco-2-A4

actin

ZO-1
Relative SI activity in WT Caco-2 cells

Relative SI activity in A4 cells
Hein et al. Figure 6

A

<table>
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B

Enrichment in P2

wt □ a4

mean intensity P2/mean intensity H

C

K19 distribution

relative protein amount

H | P1 | P2 | H | P1 | P2
WT Caco-2 | Caco-2-A4

Hein et al. Figure 6
Total lysate
Supernatant (S1) : IP
DRM pellet (P1) : IP
DRM pellet (S2) : total
DRM pellet (P2) : after IP

IP: anti-K19

WB: anti-annexin II

Hein et al. Figure 7
CHAPTER 4

DISCUSSION
4. DISCUSSION

This study initially set out to investigate the significance of protein-lipid interactions in the pathobiology of intestinal mucosa. To this end, cell models that show enterocytic properties (growth in monolayers, polarized sorting of proteins and lipids, tight junction formation) were employed. Analyses regarding transport behaviour of MDP and its mutants (a GPI-anchored and DRM-associated protein) in MDCK cells and outcomes of depletion of annexin II (a DRM-binding and regulating protein) in Caco-2 cells have been conducted to dissect several functional aspects of DRMs in mammalians, which have not been studied or clarified to date. Surprisingly, the results of this study have revealed information addressing at least two distinct topics of cell biology (protein sorting and transport and cell differentiation), which require to be discussed in detail separately.

4.1 Roles of DRMs in early protein sorting events in vesicular transport

In the first part of this work, using the GPI-anchored protein membrane dipeptidase, I was able to demonstrate that its interaction with DRMs in the ER was crucial for its rapid and efficient exit from this compartment. DRM-association of MDP is maintained via GPI-anchor (PANG et al., 2004) and the loss of this association (situation demonstrated by the anchorless MDP variant) results in a significant reduction in forward transport rate and accumulation of MDPΔGPI in the ER. Such a phenomenon applied also to anchorless forms of other GPI-APs: variant surface glycoprotein (VSG) from T.bruceti and prion protein, which were detected to accumulate in the ER and to be trafficked 4-5 times slower than the wild type protein (MCDOWELL et al., 1998; WALMSLEY et al., 2001). Retention in the ER was not a result of any defects in the protein conformation, which might be expected to occur due to impaired post-translational modifications that can be linked to the loss of membrane anchor. All these variants were secreted from the cells and considering that the Golgi to cell surface transport rates were similar in the case of wild type and anchorless MDP, the rate limiting step in the anterograde trafficking of such proteins is likely to be restricted to ER-cis-Golgi. GPI anchoring has been shown to be a crucial determinant for the efficient exit from the ER in different organisms (DOERING and SCHEKMAN, 1996; SIMONS and IKONEN, 1997). In yeast, Muniz and his colleagues (2001) have demonstrated that GPI-APs exit the ER in different vesicles from other secretory proteins. These vesicles contain several tethering
factors (v-SNARES from the ER) and a Rab GTPase from the ER, which implicates that a distinct sorting mechanism for GPI-APs in the ER might exist (MORSOMME and RIEZMAN, 2002; MORSOMME et al., 2003). Additionally, ongoing sphingoid base synthesis has been shown to be necessary for the ER-exit of GPI-APs in yeast (SUTTERLIN et al., 1997) and ceramide has been found to stabilize membrane association of GPI-APs (WATANABE et al., 2002). Ceramide has been suggested to exert its selective and stabilizing function in the membranes through promoting the formation of membrane microdomains in the ER since it has been shown to induce membrane microdomain formation in vitro (XU et al., 2001). Considering the low concentrations of cholesterol and sphingolipids in the ER (JECKEL et al., 1990; LANGE, 1991), it is likely that membrane microdomain formation in this compartment can be promoted by ceramide which has very long saturated acyl chains (C26) in yeast (DICKSON, 1998; MUNIZ and RIEZMAN, 2000). In mammals, ceramide acyl chains are shorter (C18-C24), and packing and organization of membrane lipids through membrane thickening is not predicted to be possible. This was a reason why the existence of microdomains on ER membranes has remained a conundrum. Additionally, Triton X-100-DRMs have revealed only the mature forms of proteins, indicating that an interaction could only take place in the TGN and at the cell surface (BROWN and ROSE, 1992; SIMONS and IKONEN, 1997). In 2004, Sarnataro et al. have isolated early biosynthetic forms of the PrP(C) (cellular PrP – wild type PrP) in DRMs from Fisher rat thyroid cells (FRT) and have argued that the cellular conformation of this protein was stabilized by its early association with the cholesterol-rich membrane microdomains in the ER. This was followed by the discovery of several ER-resident proteins which partitioned into Triton X-100-DRMs. Erlin-1 and erlin-2 and a number of enzymes involved in the synthesis of GPI anchor have been shown to reside in distinct membrane domains of the ER, which could be isolated using Triton X-100 (PIELSTICKER et al., 2005; BROWMAN et al., 2006). Although the results of these studies are somewhat controversial to the fact that the ER membranes are indeed poor sources of cholesterol and sphingolipids (PRINZ, 2002; VAN MEER and LISMAN, 2002), they indicate to an important notion that distinct membrane environments with functional implications can occur already in ER membranes. This concept has been supported by the work of Alfalah et al. (2005), which has revealed that Tween-20 could be used as a suitable detergent to isolate the DRMs containing ER-associated forms of proteins and that these microdomains acts as
early sorting platforms for apical and basolateral proteins. Composed of different lipids (rich in phosphatidylinositol and phosphatidylglycerol and poor in sphingolipids) than that of Triton X-100 DRMs, Tween-20-DRMs have been shown to incorporate apically sorted proteins like SI, LPH and DPPIV whereas basolaterally targeted proteins like MHC II and VSV-G were excluded from them. In addition to this finding, the first part of my thesis has also demonstrated that lipid microdomains in the ER could operate as platforms, where proteins can efficiently be packed and sent out of this compartment (HEIN et al., 2009). This hypothesis is supported by the finding that the loss of the GPI anchor results in exclusion of MDP from the Tween-20-DRMs in membranes (or ER-derived vesicles containing Tween-20-DRMs). The decreased forward transport rate and its accumulation in the ER suggest that ER-exit is the rate-limiting step in the trafficking of MDP. Association of this protein with membrane microdomains is mediated by the GPI-anchor and it results in rapid transport of MDP out of this compartment. It is likely that the ER exit of GPI-APs is achieved by a signal-mediated mechanism. Since the GPI anchor is inserted in the outer leaflet of the bilayer and can not interact with components of the COPII vesicular coat, it has been suggested that adaptor proteins interacting with the GPI-APs could mediate this selective packaging (MUNIZ et al., 2001). In yeast, the Emp24p and Erv25p protein complex have been hypothesised to undertake this function (SCHIMMOLLER et al., 1995; BELDEN and BARLOWE, 1996), although deletions of the entire family of p24 proteins have shown no conclusive secretion phenotype (SPRINGER et al., 2000). The ability of this protein complex to partition into lipid microdomains and the existence of their mammalian analogues remain to be elucidated. Nevertheless, advances in techniques that allow isolation of the ER-derived carriers have shed light on the components and mechanisms regulating ER to Golgi transport and they have challenged the current model, which argues that all secretory proteins travel together to the Golgi and are then sorted into distinct vesicular carriers for different destinations (GRIFFITHS and SIMONS, 1986). It is necessary to mention that the possibility to isolate more and more vesicle populations (e.g. COPI or COPII subpopulations) would substantially contribute to our knowledge in vesicular trafficking in early stages of secretory pathway. Detergent insolubility can serve as a further discrimination method, which can then facilitate the characterization of transport carriers containing different cargo.
4.2 Contribution of DRMs to cell differentiation in the gut

Almost a decade ago, using two model apical proteins (SI and LPH), Jacob and Naim (2001) have demonstrated that at least two distinct vesicle populations existed among apically targeted carriers that originate from the TGN. This discovery was followed by the development that these distinct vesicles utilized different cytoskeletal tracks from TGN to apical cell surface (JACOB et al., 2003). Lactase associated vesicles (vesicles containing LPH, LAVs) have been shown to be delivered to the apical surface in an actin-independent fashion whereas sucrase associated vesicles (SAVs) have been found to associate with actin cytoskeleton along the pathway from the TGN to the apical cell surface. In pursuit of the identification of the components of these vesicles, myosin Ia, annexin II, alpha-kinase, and galectin-3 have been discovered to exclusively associate with SAVs (JACOB and NAIM, 2001; JACOB et al., 2004; HEINE et al., 2005; DELACOUR et al., 2006). Different from LAVs, SAVs have been detected to contain cholesterol and sphingolipid-rich membrane microdomains (Triton X-100-DRMs) which, all the abovementioned protein components have been shown to associate with. Consequently, the Naim laboratory has been engaged in elucidating the functional attributes of these proteins. In separate studies, through transient downregulation of myosin Ia, annexin II, and galectin-3, it could be shown that these proteins were involved in a machinery that not only provided the delivery of SAVs to apical membrane but might also be contributing to the establishment of cell polarity (see references above). Given the ability of these proteins to associate with DRMs, it has become an intriguing question how DRMs contributed to sorting and delivery of apical transport vesicles and more importantly, how and to which extent they were involved in mechanisms initiating and regulating cell polarity. As a phospholipid binding protein that has been shown to interact with DRM lipids (AYALA-SANMARTIN et al., 2001; RESCHER et al., 2004), annexin II has been a promising candidate to help reveal the role of DRMs in the abovementioned processes. In order to investigate this, I set out to establish an annexin II knock-down cell line, which expressed SI, LPH and other intestinal hydrolases intrinsically and possessed the physiological and morphological features of an enterocyte. I therefore decided to use the colon adenocarcinoma derived Caco-2 cells, which can undergo spontaneous enterocytic differentiation in cell culture and start expressing increasing amounts of hydrolases of the small intestine as a function of time (PINTO et al., 1983; HAURI et al., 1985). Following the
isolation of the clone (Caco-2-A4) that expressed only 10-15% of normal annexin II levels, I first examined the DRM-association properties of several hydrolases and other marker proteins that are known to partition into Triton X-100-DRMs. Contrary to expectation, the ability of SI, DPPIV, ACE-2, and flotillin-2 to interact with Triton X-100-DRMs was not significantly altered (Fig.5). Apparently, annexin II is not critical neither for the formation of DRMs or for the association of transmembrane proteins with the DRMs. Nevertheless, this information was not sufficient to exclude a possible regulatory function for annexin II. Since annexin II has been found to be involved in dynamic membrane internalization events at cholesterol-, sphingolipid-, and PI(4,5)P2-rich membrane sites such as macropinocytosis, bacterial uptake and early endosome formation (MERRIFIELD et al., 2001; ZOBIACK et al., 2002; PITTIS et al., 2003) I set out to test this function in a virus uptake assay. In cooperation with Dr. Jörg Glende from the Department of Virology and the University of Veterinary Medicine in Hannover, we have established an assay in which we subjected wild type and A4 Caco-2 cells to infection with pseudo types of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) according to the established protocol (REN et al., 2006). ACE-2 is the receptor for SARS-CoV (LI et al., 2003) and virus attachment and entry into host cells have been shown to be mediated by lipid microdomains on plasma membrane (LI et al., 2007). Caco-2 is a suitable model to study the infection in vitro since these cells express it intrinsically. We have hypothesised that in the absence of annexin II, the surface stability or DRM-clustering of ACE-2 (through annexin II and engagement of actin at virus attachment sites, which would initiate virus uptake) upon virus attachment would be disturbed, and that would impair the infection process. Indeed, we observed a 75% reduction in the infectibility of the Caco-2-A4 with the SARS pseudo type (Fig.6). We also tested the susceptibility of the infection process to external cholesterol addition since we believed that addition of cholesterol would rescue the A4-phenotype simply by facilitating DRM formation. This phenomenon is observed in Caco-2 cells where addition of cholesterol increases the infection rate to 200% although number of receptor molecules does not change (Fig.7). To our surprise, cholesterol addition did not improve infection in Caco-2-A4 (Fig.7, see grey bars), suggesting that this process does not depend on the formation or maintenance of lipid microdomains at the plasma membrane. In fact, the reduction in the expression levels of ACE-2 (Fig.8) seems
Figure 5. DRM-protein interaction profiles in wild type Caco-2 cells vs. Caco-2-A4. Caco-2 cells were separated into Triton X-100 resistant (P, pellet) and soluble (S, supernatant) fractions by ultracentrifugation (HEIN et al., 2009). Equal amount of protein samples were loaded on separate gels and proteins of interest were detected following western blotting. The ratio of proteins found in P fraction versus S did not show any significant differences among wild type Caco-2 (WT) and clone Caco-2-A4 (A4).

Figure 6. SARS-CoVpp infection. Equal numbers of Caco-2-WT and Caco-2-A4 cells were seeded one day prior to infection. Confluent monolayers were infected with SARS-CoV pseudotype (SARS-CoVpp) VSV-S (VSV carrying S protein from SARS-CoV and a GFP reporter) and VSV-G (VSV carrying GFP instead of its G protein) as the control virus. Infected cells were counted and depicted as a relative percentage in this graph. Almost 75% reduction in the number of Caco-2-A4 cells is seen in the infection with SARS-CoVpp whereas the infection with the control virus (VSV-G) results only in an insignificant reduction in Caco-2-A4.

Figure 7. Cholesterol does not enhance SARS-CoVpp infection in Caco-2-A4. The infection assay was repeated in with or without the addition of cholesterol to the cells. In WT cells the infection rate was enhanced up to two-fold whereas in Caco-2-A4 addition of cholesterol did not greatly alter the infection rate. In control infection with VSV-G, extrinsic cholesterol did not influence the infection rates in both of the cells either.
to be the reason behind the reduced rate of infection with the SARS-CoVpp. This has also been one of the key observations that led to the detection of impaired cell development and differentiation in Caco-2-A4. Combined with the morphological data, it was possible to conclude that the downregulation of annexin II solely was sufficient to perturb the differentiation processes in Caco-2 cells. The effects observed in Caco-2-A4 seem to be specific to loss of this particular protein despite the possibility that some compensatory action could be undertaken by other members of the family present in Caco-2 cells. I investigated this by using a polyclonal antibody raised against annexin VI, which was a kind gift from Prof. Annette Draeger from the University of Bern, Switzerland. I separated total protein samples from Caco-2 cells on an SDS gel and subjected this gel to immunoblotting. Western blot analysis has revealed no apparent changes in expression rates of the annexins, which could have been upregulated to compensate the lack of annexin II (Fig.9). This way, I could reassure that the effects observed in Caco-2-A4 could be related exclusively to the loss of annexin II function.

This study has revealed that loss of annexin II causes a disruption and a delay in the differentiation of enterocytes; however the mechanism by which annexin II exerts its function to promote cell differentiation is not clear. Previous studies have suggested that annexin II

![Figure 8. ACE-2 levels are reduced in Caco-2-A4.](image)

**Figure 8. ACE-2 levels are reduced in Caco-2-A4.** Equal amount of total protein from lysates of WT and A4 Caco-2 cells were loaded on a 10% SDS-gel and immunoblotted with polyclonal ACE-2 antibody. Steady-state levels of ACE-2 are reduced in clone A4 approximately 3 times compared to the WT levels.

![Figure 9. Expression analysis of annexins in Caco-2 cells.](image)

**Figure 9. Expression analysis of annexins in Caco-2 cells.** 25µg total protein from the lysates of Caco-2 cells (wt and A4) was loaded on a 12% SDS gel and proteins were blotted on a membrane. Using the polyclonal anti-annexin VI antibody, several bands were detected after increased exposure time. Judging by their intensity and apparent molecular weight, strongest bands were denoted as annexins I, II, IV, V, VI, and XI. Except for the remarkable difference in the intensity of the bands representing annexin II (italic), no other strong differences in the expression levels of annexins could be detected.
could recruit RacI, Cdc42, and other Rho GTPases to certain membrane sites to conduct cellular functions involving actin polymerization like cell migration, formation of lamellipodia and cell polarization (HANSEN et al., 2002; BABBIN et al., 2007; MARTIN-BELMONTE et al., 2007). Acquisition of the polarized state requires major remodelling of the cell architecture and Cdc42 has been shown to interact with protein complexes that can initiate actin polymerization (ROHATGI et al., 1999). Analysis of Cdc42 mRNA levels in Caco-2-A4 did not reveal any differences in the levels of this protein in the absence of annexin II (result not shown), but this does not exclude a possible interaction between these proteins that orchestrates the initial phases of cell differentiation.

Investigation of the functional importance of annexin II in Caco-2 cells has revealed a novel interaction partner of annexin II, cytokeratin 19 (K19). K19 belongs to the family of “soft” type of keratins (type I), which have for a long time been thought not to play any other roles except for providing mechanical stability. Unlike other cytoskeletal systems, keratins lack polar ends and molecular motors, which are regarded as fundamental properties of a scaffold that supports the directional transport of the vesicular carriers (RODRIGUEZ-BOULAN et al., 2005). This has also been the main reason why keratins have not been thought to contribute to the establishment of the morphological asymmetry in polarizing cells. Nevertheless, several keratins including K19 have already been found to be distributed in a polarized fashion in epithelia (FATH et al., 1993). K19 is located beneath the apical membrane (associated with the terminal web) (SALAS et al., 1997), where the formation of microvilli is promoted through interactions between proteins such as annexin II, K19, ezrin and actin. My results indicate that the lack of annexin II results in a K19 pool in the cells that is not or under phosphorylated. This can be explained in several ways. It is most likely that annexin II can per se recruit the kinase that regulates K19 to the interaction site. Annexin II, p112 heterotetramer can bind F-actin non-erythroid spectrins in the submembranous protein network (GERKE and WEBER, 1984) and can mediate interactions between several proteins within the cortical cytoskeleton. It is also possible that the interaction between K19 and its kinase can meet at membrane sites rich in cholesterol and sphingolipids and when annexin II is missing it is possible that these membrane microdomains harbouring the kinase and its substrate fail to coalesce. There is no direct evidence that keratins can interact with such membrane microdomains however, a few studies have shown that the lack of intermediate
filaments results in defective cholesterol exit from that late endosomes and impaired maturation of glycosphingolipids due to a defect in the recycling of these lipid between Golgi and the endosomal system (SARRIA et al., 1992; GILLARD et al., 1994; GILLARD et al., 1996). Reduced levels of the rafts lipids in the absence of vimentin have later been shown to impair the activity of the apical Na\(^+\) glucose transporter in DRMs (RUNEMBERT et al., 2002). It has also been postulated that the missorting of apical proteins in K8-null mice could be a result of such an impairment of the lipid microdomains although this has not been investigated in detail (AMEEN et al., 2001; STYERS et al., 2005). A third possibility is that annexin II can recruit a set of proteins (not only one kinase), which can initiate the polarization process like in the case of Cdc42 → Par6/aPKC or Cdc42 → N-Wasp → Arp2/3. It is very likely that regulators of K19 are recruited to the subapical region by annexin II as well. This awaits further proof and identification of the regulatory proteins would facilitate the search process. The possible set of interactions and events related to annexin II during formation of the microvilli are summarized in Figure 10.

Another important observation I made in Caco-2-A4 cells was the depletion of K19 from the BBM. Among all of the BBM located proteins, distribution of K19 to the apical compartment was mostly affected. Given the altered phosphorylation state, this indicates a link between the phosphorylation of K19 and its apical localization. It is possible that the imbalance in K19 phosphorylation can lead to its depolymerization and redistribution of K19 to the cytoplasm. Mutation of the major phosphorylation site (Ser 35) of K19 results in the production of short cytoplasmic filaments and perinuclear collapse in NIH-3T3 cells (ZHOU et al., 1999). K19 has higher basal phosphorylation rates compared to K8, K18, and K20 and this might render it much more susceptible to dephosphorylation and can result in severe polymerization defects (ZHOU et al., 1999). K19 has been shown to anchor γ-tubulin to subapical membrane was suggested by Pedro Salas (1999) to act as a potential microtubule organizing centre (MTOC) that functions as an intrinsic polarization cue after mitosis. A recent study has also supported the importance of K19 in epithelial polarization by demonstrating its ability to interact with the terminal web protein plastin I (fimbrin) (GRIMM-GUNTER et al., 2009). As a non-redundant actin bundling component of the terminal web, plastin I has been suggested to play a crucial role in the formation of microvilli by anchoring K19 to the actin network at base of the BBM. Here, once again, the apical
localization and linkage of K19 to the terminal web has been shown to be crucial for the reorganization of apical cytoskeleton and BBM. Defects in K19 polymerization per se can therefore lead to disorganization of the apical pole.

In this work, information from the Caco-2 cells adds to the many discovered functions of annexin II in and outside mammalian cells. Although it seems confusing and hard-to-believe that one protein can do so much in cells, I believe that defining its environment, interaction partners and its functions in detail will lead to the bigger picture and offer new alternatives in understanding pathologies of the intestinal tract.

**Figure 10. Model of annexin II and its interaction partners involved in epithelial differentiation.** After segregation of PI(3,4,5)P₃ and PI(4,5)P₂ to basolateral and apical membranes, annexin II localizes to the apical membrane and can recruit Cdc42, which in turn activates several signalling pathways (N-Wasp and Par6/aPKC that initiate actin remodelling at the membrane sites (see interrupted circle, up left). At the same time, annexin II maintains the interaction between K19 and its kinase (possibly a phosphatase as well) and K19 polymerization occurs. K19 network is anchored to the terminal web by plastin I and this network stabilizes the microfilaments and actin binding proteins like ezrin and spectrin. Microtubuli linked to K19 filaments serve as transport tracts and serve as an orientation guide for vesicle trafficking.
5. SUMMARY

Zeynep Hein - Implication of lipid microdomains in regulation of protein trafficking and epithelial cell morphology

The discovery of the lipid microdomains has provided a new insight to our understanding of cellular membranes and introduced us to a whole new dimension full of answers that can elicit many cellular processes from signalling to protein sorting and infection. The retrieval of these answers requires isolation and biochemical analyses of these microdomains and their components. To date, a variety of non-ionic detergents like Triton X-100, Lubrol WX, and Tween-20 have been successfully used to isolate such microdomains from in biological samples. Growing number of studies employing model proteins and several detergents in different cellular systems have conveyed a great deal of information about the functional characteristics of lipid microdomains. Nevertheless, further information is necessary to produce concrete explanations for the scope of the functions of lipid microdomains in vivo. This study has aimed to elucidate the implications of lipid microdomains in epithelial cells, especially in early protein sorting events and cell differentiation. Employing detergent insolubility as a discrimination method in Madin Darby Canine Kidney (MDCK) cells, it was revealed that GPI-anchor acts as a sorting determinant for a protein’s inclusion into Tween-20 resistant microdomains (Tween-20-DRMs). This interaction between the GPI-anchored protein membrane dipeptidase (MDP) and DRMs was found to be responsible for the rapid and efficient exit of MDP from the endoplasmic reticulum (ER). The removal of the GPI-anchor resulted in the exclusion of MDP from DRMs and an accumulation in the ER. The rate of Golgi to cell surface transport was similar for wild type and anchorless mutant and this has led to the conclusion that ER-exit was the bottleneck in the anterograde transport of MDP and possibly for other DRM-associated proteins. These finding support the theory that proteins are selectively packed in different carriers in the ER and lipid microdomains in the ER membranes contribute to the diversity of these specialized carriers and assure further transport of competent proteins. Such diversity is also present among trans-Golgi network (TGN)-derived vesicles. Detergent insolubility is a determinant for a subgroup of such vesicles that exploit actin based cytoskeletal tracts to reach apical cell membrane. Annexin II is a component of this vesicle species and it has been shown to be essential for the arrival of the contents of these carriers at the apical membrane. Annexin II is a lipid binding protein, which
has been shown to interact with lipids from membrane microdomains (cholesterol, PI(4,5)P2). It also can bind F-actin at membrane sites rich in cholesterol and sphingolipids and thereby participate dynamic membrane events such as vesicle fusion and fission. The mechanism how annexin II modulates membrane microdomains and its contribution to processes involving DRMs were investigated in intestinal cell model Caco-2. This was accomplished by stable downregulation of annexin II by RNAi. Caco-2-A4 clone was found to express only 10-15% of normal annexin II levels in wild type cells. Analyses revealed a decrease in expression of several intestinal hydrolases (sucrase isomaltase, angiotensin converting enzyme 2) and microvillus associated protein ezrin as well. Clone Caco-2-A4 also failed to display an increasing pattern of apical hydrolase levels with time. Morphological examination of ultrastructural features of the cells has supported these findings by reduced number and length of microvilli in Caco-2-A4. Additional to the impaired apical transport of brush border enzymes, it was discovered that apical distribution of cytokeratin 19 (K19) was severely disrupted in Caco-2-A4. This could be explained by the appearance of a non-phosphorylated pool of K19, which probably results in depolymerization and subsequent displacement of K19 from the apical cytoskeleton in the clone A4. I suggest that regulation of K19 by annexin II is one of the initial events taking place in cell differentiation. Annexin II is responsible for the apical localization of K19 and this supports the apical cytoskeletal scaffold on which the microvilli are formed.
6. ZUSAMMENFASSUNG

Zeynep Hein - Die Bedeutung von Lipid-Mikrodomänen in der Pathobiologie der intestinalen Mukosa


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Ich habe die Dissertation an folgender Institution angefertigt:
Institut für Physiologische Chemie, Stiftung Tierärztliche Hochschule Hannover

Die Dissertation wurde bisher nicht für eine Prüfung oder Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht.

Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.

Hannover, den 20.05.2009
Sina Zeynep Hein
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