Vesicular Traffic in Polarized Epithelial Cells:

Identification and Characterization of General and Epithelial Specific Factors

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Summary

Polarized epithelial cells are differentiated into apical and basolateral plasma membrane domains separated by tight junctions. The apical cell surface usually faces the external milieu, the basolateral domain provides cell-cell and cell-substratum contact and is surrounded by body fluids. In Madin-Darby canine kidney (MDCK) cells the unique protein and lipid composition of the two surface domains is generated by sorting in the *trans*-Golgi network (TGN) and selective delivery to the cell surface by a vesicular carrier mechanism. Sorting to the apical plasma membrane has been proposed to be mediated by a co-clustering of proteins and glycosphingolipids in the TGN.

In order to identify the molecular machinery involved in sorting and transport of apically destined cargo I have characterized glycolipid-enriched, detergent-insoluble complexes from MDCK cells. Several proteins of the complexes were found to be components of immunoisolated apical and basolateral exocytic carrier vesicles thus representing prime candidates for the sorting machinery. By using preparative two-dimensional (2-D) gel electrophoresis and the information of 2-D gel databases I was able to identify and to purify several of these proteins for peptide microsequencing. Subsequently, I isolated the cDNAs encoding VIP36 (Vesicular-Integral Membrane Protein of 36 kDa) and annexin XIIIb.

VIP36 is a glycolipid raft component present in apical and basolateral vesicular carriers. The protein had a significant sequence similarity to leguminous plant lectins. VIP36 was shown to be an integral membrane protein localized to the Golgi apparatus and the cell surface, presumably recycling between them. The protein might bind to sugar residues of glycoproteins, glycolipids or glycosylphosphatidyl inositol-anchored proteins and provide a link between the luminal face of glycolipid rafts and the cytosolic vesiculation machinery. A mammalian homologue of VIP36 is localized to the early secretory pathway. This suggests that a new family of hitherto unknown animal lectins may be involved in the sorting of saccharide bearing molecules throughout the biosynthetic pathway.

Annexin XIIIb is a component enriched in apical exocytic carrier vesicles and is a new member of an epithelial specific sub-family of annexins. Annexin XIIIb was localized to the apical cell surface and vesicular structures in MDCK cells. Annexins are implicated in membrane-membrane interactions and annexin XIIIb may be involved in delivery to the apical cell surface in MDCK cells.

With the identification of these new, general and epithelial specific factors it will be possible to gain access to additional components of the epithelial sorting and targeting machinery to elucidate the molecular mechanisms responsible for protein and lipid sorting and vesicular transport.

The biosynthesis of proteins is mediated by ribosomes localized to the cytoplasm. The majority of proteins have to be delivered to a precisely defined location within the cell in order to perform their function. This requires a molecular mailing system that decodes delivery addresses (sorting signals) carried by the proteins and transports the cargo to the correct destination. On their itinerary through the cell proteins may be directly imported into membrane bound organelles or, following translocation into the endoplasmatic reticulum (ER), travel along the secretory pathway to the Golgi apparatus, endosomes, lysosomes or the cell surface.

Sorting Signals

Within the last two decades a variety of sorting and targeting signals have been identified. They usually consist of a linear stretch of amino acids or a signal patch that is formed by non-adjacent residues. Signals described include those for the translocation of proteins across the membrane of bacteria and the ER, for the import of proteins into mitochondria, chloroplasts, peroxisomes, the nucleus and lysosomes (Verner and Schatz, 1988; von Heijne, 1990; Dice, 1992). For the sorting of proteins in the endomembrane system, signals reponsible for the retention in or the recycling to the ER, the Golgi apparatus and the TGN have been identified (Pelham and Munro, 1993). In addition, the signals for delivery of soluble and integral membrane proteins to lysosomes (von Figura, 1991; Kornfeld, 1992), for sorting/retention in endosomes (Bakke and Dobberstein, 1992) and for the clustering into coated pits and internalization have been elucidated (Trowbridge, 1993). In polarized epithelial cells signals for the sorting of membrane proteins to the basolateral cell surface (Mostov et al., 1992) and for the delivery of glycosylphosphatidyl inositol (GPI)-anchored proteins to the apical membrane domain have been described (Lisanti and Rodriguez-Boulan, 1990). The signals and mechanisms involved in secretion of proteins along a non-classical secretory pathway are beginning to be unraveled in bacteria and yeast but are so far unknown in mammalian cells (Kuchler, 1993).

Sorting and Transport Machinery

With the signals at hand the next task is to identify the molecular machinery that is involved in signal recognition, membrane translocation and vesicular transport processes. This is generally pursued by genetic approaches in bacteria and yeast, biochemical transport assays and biochemical dissection of vesicular carriers. A comprehensive description of components involved in protein translocation across membranes of bacteria (Pugsley, 1990), the ER (Rapoport, 1992; Simon, 1993; Dobberstein, 1994), mitochondria (Baker and Schatz, 1991; Pfanner et al., 1992; Hannavy et al., 1993), chloroplasts (Knight et al., 1993) and peroxisomes (Subramani, 1993) is now available. Receptors for the retention signal of soluble ER proteins (Pelham, 1991) and

for mannose-6-phosphate (Kornfeld, 1992), specifying delivery of lysosomal enzymes to lysosomes, have been identified. Among the cytosolic components involved, adaptins and clathrin have been shown to interact with endocytic signals and are implicated in endocytosis and transport from the TGN to endosomes/lysosomes (Pearse and Robinson, 1990; Kirchhausen, 1993).

In the recent years a flurry of *in vitro* vesicular transport assays (Rothman and Orci, 1992; Pryer et al., 1992) has allowed further dissection of the secretory pathway. In addition, studies on synaptic vesicle docking and fusion (Südhof et al., 1993; Bennett and Scheller, 1994) and the use of neurotoxins (Niemann et al., 1994) contributed to novel insights. These approaches led, for example, to the identification of factors involved in intra-Golgi transport such as NSF (NEM-Sensitive Factor), SNAPs (Soluble NSF Attachment Protein), ARFs (ADP-Ribosylation Factor) and COPs (coatomers) (Rothman and Orci, 1992; Kreis, 1992). A new family of SNAREs (SNAP Receptors) was identified (Söllner et al., 1993) and proposed to be generally involved in vesicular transport and membrane fusion reactions (Rothman and Warren, 1994). For several components a refined analysis of their interactions has been carried out and suggested that the pairing of a vesicle SNARE with its cognate target SNARE may contribute to the specificity of vesicular trafficking (Söllner et al., 1994; Calakos et al., 1994).

Annexins (lipocortins) are another class of proteins implicated in membrane-membrane interactions and secretion (Creutz, 1992). Members of the annexin family promoted the aggregation of secretory granules *in vitro* (Drust and Creutz, 1988) and were shown to be directly involved in Ca^{2+}-dependent exocytosis in chromaffin cells and in the budding of clathrin coated vesicles (Ali et al., 1989; Lin et al., 1992; for further discussion see *part IV*).

Low molecular weight GTP-binding proteins of the rab family are involved in the regulation of vesicular transport. They are proposed to use GTP hydrolysis as a kinetic proofreading device prior to membrane fusion to provide directionality to vesicular delivery (Bourne, 1988) and are implicated in a variety of transport steps within the endomembrane system including endosome-endosome fusion (Gruenberg and Clague, 1992; Zerial and Stenmark, 1993; Ferro-Novick and Novick, 1993). The specific subcellular localization of individual rab proteins to distinct compartments of the exocytic and endocytic pathway provided new organelle markers and served to outline the road maps of vesicular transport (Simons and Zerial, 1994).

Taken together, the outcome of the combination of biochemical and genetic approaches was highly rewarding. The majority of factors that were identified biochemically hold a bacterial (only for protein translocation; Dobberstein, 1994) or yeast counterpart identified by genetic means (Schekman, 1990; Pryer et al., 1992) suggesting that the majority of components involved in membrane translocation, sorting, vesicular transport and membrane fusion reactions are widely conserved (Bennett and Scheller, 1993; Dobberstein, 1994). While novel components or mammalian yeast homologues are being continously identified (e.g. Sec1/Munc18; O'Connor, 1994) the integration of all factors into a coherent view remains a challenge. On the molecular level, most sorting and transport processes are still poorly understood.

The elucidation of how vesicular transport is regulated is only at its start. Considering the profound changes within the endomembrane system during mitosis, e.g. the disassembly of the nuclear envelope and Golgi apparatus, it is evident that precise regulatory mechanisms must exist (Warren, 1993). In interphase cells, heterotrimeric G-proteins have been implicated in secretion, endosome-endosome fusion and protein sorting in polarized epithelial cells (Barr et al., 1991; Leyte et al. 1992; Aridor et al., 1993; Colombo et al., 1992; Pimplikar and Simons, 1993). A cascade of protein kinases are involved in modulating regulated secretion in adrenal chromaffin cells (Ely et al., 1990). Two cytosolic factors, Exo 1 and Exo2, have been purified and shown to stimulate regulated secretion in chromaffin cells (Morgan and Burgoyne, 1992; Morgan et al., 1993). Exo1 is a member of the 14-3-3 class of proteins which have been implicated in diverse cellular processes including the regulation of protein kinase C (PKC; Aitken et al., 1992). Exo2 was shown to be the catalytic subunit of protein kinase A (PKA). Constitutive secretion is also regulated by PKC (De Matteis et al., 1993). The control of ARF binding to the Golgi complex by PKC may provide the link to constitutive secretion and represent one step in a cascade of regulatory mechanisms involved (De Matteis et al., 1993). Dynamin (*Shibire* in *Drosophila melanogaster*) is a GTP-binding protein implicated in endocytosis (Trowbridge, 1993). The GTPase activity of dynamin was shown to be stimulated by binding to SH3 domains which would allow a coupling to other molecules involved in signal transduction (Gout et al., 1993).

Although it is obvious that the membrane bilayer is involved in vesicular transport this aspect has only recently been appreciated (Simons and van Meer, 1988; Glaser, 1993; Marsh, 1993). Evidence is accumulating that the sorting of proteins to the apical cell surface in epithelial cells involves glycosphingolipid-enriched microdomains (see *part I* and *part II*). The retention of Golgi enzymes has been proposed to be governed by the bilayer thickness, dependent on the cholesterol content (Bretscher and Munro, 1993). Phospholipid-transfer proteins were shown to be involved in secretion in yeast (Bankaitis et al., 1990; Dowhan, 1991) and mammals (Hay and Martin, 1993). A lipid kinase complex has been implicated in vacuolar protein sorting in yeast (Herman et al., 1992) and phospholipase D was shown to be a downstream effector of ARF (Brown et al., 1993; Cockcroft et al., 1994; Kahn et al., 1993). Thus, it seems that the local regulation of phospholipid metabolism is a critical aspect of membrane traffic. The modification of lipids by kinases and lipases may either generate second messengers for downstream effectors and/or directly affect the biophysical properties of the budding membrane bilayer.

Vesicular Transport and Protein Sorting in Epithelial Cells

Epithelial cells form a permeability barrier between the external and internal environment of the body and are involved in physiological processes such as secretion, absorption and ion transport. The cell surface of polarized epithelial cells is differentiated into an apical and basolateral domain with a unique protein and lipid composition (Simons and Fuller, 1985; Wandinger-

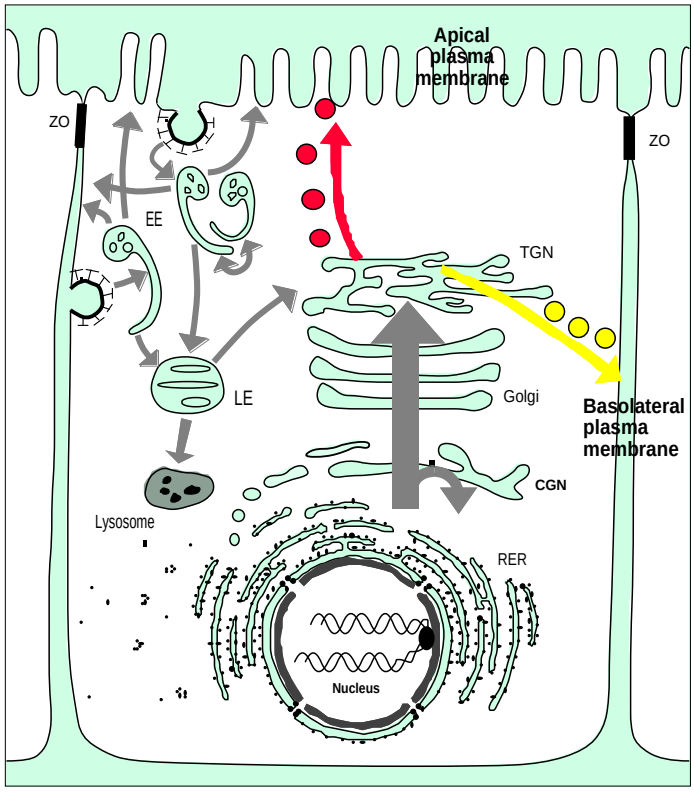

Figure 1: Schematic view of a polarized epithelial cell and the exocytic and endocytic pathways. RER, rough endoplasmatic reticulum; CGN, *cis*-Golgi network; TGN, *trans*-Golgi network; EE, early endosome; LE, late endosome; ZO; zonula occludens (tight junctions).

Ness and Simons, 1991; Rodriguez-Boulan and Powell, 1992). The intermixing of components between the two plasma membrane domains is prevented by tight junctions (*zonula occludens;* Gumbiner, 1993). Proteins and lipids in transit to the cell surface are sorted in the TGN (Griffiths and Simons, 1986) and delivered to their destinations by a vesicular carrier mechanism. The generation of the polarized phenotype of epithelial cells is a multistage process requiring extracellular cues. It involves a dynamic rearrangement of the cell surface, intracellular organelles and the cytoskeleton (Rodriguez-Boulan and Nelson, 1989). Microtubules and actin filaments are implicated in vesicular transport to the cell surface (Mays et al., 1994).

In simple renal epithelial cells, the delivery of vesicles to the plasma membrane follows a direct pathway from the TGN to the apical and basolateral cell surface (Simons and Wandinger-Ness, 1990). In hepatocytes all proteins are first transported to the basolateral membrane and apical cargo is then transcytosed to the bile canalicular membrane domain (Bartles and Hubbard, 1988). A combination of both mechanisms, exocytic and endocytic/transcytotic sorting and delivery, is encountered in intestinal epithelial cells (Louvard et al., 1992). Secreted proteins in different epithelial cell types behave similarly as their membrane protein counterparts. In hepatic cells, proteins are almost exclusively secreted from the basolateral cell surface whereas in renal and intestinal cells specific polarized secretion occurs from both, the basolateral and apical plasma membrane domain (Bartles and Hubbard, 1988; Rodriguez-Boulan and Powell, 1992; Louvard et al., 1992). Whether secretory proteins and membrane proteins are packaged together into identical vesicular carriers is a matter of debate (Boll et al., 1991) and remains to be solved.

Epithelial Sorting Signals

The expression of aminopeptidase N, dipeptidylpeptidase IV and neutral peptidase in heterologous cells showed that their sorting signals were generally recognized in different epithelial cell types (Rodriguez-Boulan and Powell, 1992). Yet, they were delivered to the analogous cell surface along the pathways displayed by endogenous proteins of the host cell. Contrary to these observations a tissue specific localization to the apical or basolateral plasma membrane has been described for the low density lipoprotein receptor (LDL-R; Pathak et al., 1990). The mannose-6-phosphate receptor, a basolateral protein in MDCK cells (Prydz et al., 1990), was found on the apical pole of osteoclasts (Baron et al., 1990). In addition, the transferrin receptor was localized to the basolateral membrane in MDCK cells (Fuller and Simons, 1986) but found on both, the basolateral and apical cell surface in a human trophoblast-like cell line (Cerneus and van der Ende, 1991), further illustrating the variable localization observed for individual proteins.

Considering all experimental data available it was proposed that the sorting machinery in epithelial cells might be generally conserved but differ in their efficiency and localization depending on the cell type (Simons and Wandinger-Ness, 1990). It was suggested that the delivery to the basolateral membrane domain may be the analogue of the secretory pathway to the cell surface in non-polarized cells (Pfeffer and Rothman, 1987) and occur by default whereas the delivery to the apical surface would be signal-mediated. However, recent studies have shown that the cytoplasmic domains of several membrane proteins contain a basolateral sorting determinant (BSD) suggesting that both, the apical and basolateral pathway, are signal-mediated. The BSD was initially located in the proximal 17 amino acids of the polymeric immunoglobulin receptor (pIgR; Casanova et al., 1991) and has been shown to reside in the cytoplasmic tail of several basolateral membrane proteins (Hopkins, 1991; Matlin, 1992; Mostov et al., 1992). A typical BSD usually consists of a 15-20 amino acid long segment normally located close to the membrane spanning domain. Some BSDs closely resemble the tyrosine-containing signals involved in rapid endocytosis, others are clearly different in sequence. BSDs are capable to divert genetically engineered apical proteins to the basolateral cell surface strongly suggesting that they may interact with a specific basolateral sorting receptor and mediate sorting of the protein into the basolateral vesicle. Considering the sequence diversity of BSDs it is not excluded that more than one basolateral sorting mechanism exists.

Sorting signals of secreted proteins have not been identified. For apically destined cargo the GPI-anchor (Low, 1989; McConville and Ferguson, 1993) was shown to mediate apical delivery (Brown et al., 1989; Lisanti et al., 1989). Despite numerous efforts the signals for the delivery of transmembrane proteins to the apical cell surface have not been determined. They are generally believed to be encoded within the exoplasmic domain of apical proteins (Rodriguez-Boulan and Powell, 1992) but since no primary amino acid sequence homology has been observed among apically secreted or targeted membrane proteins the sorting signal is

likely to be contained in three dimensional patches of the ectodomains. A possible involvement of the transmembrane domain has not been conclusively demonstrated (Rodriguez-Boulan and Powell, 1992). The apical sorting signal must be widespread and redundant in nature since many basolateral membrane proteins deprived of their cytoplasmic BSD are delivered to the apical cell surface as well (Hopkins, 1991). In addition, this indicates a hierarchical arrangement of basolateral and apical sorting signals. The BSD usually overrides the exoplasmic apical sorting determinant.

Epithelial Sorting Machinery and Regulation

Putative machinery components interacting with BSDs have not been characterized but the use of an *in vitro* transport system recently allowed to identify several proteins interacting with the cytoplasmic domain of a basolaterally targeted protein (Pimplikar et al., 1994). Polarized protein transport was reconstituted in streptolysin O (SLO) permeabilized MDCK cells (Gravotta et al., 1990, Kobayashi et al., 1992; Pimplikar and Simons, 1993). Following viral infection, the delivery to the two membrane domains was measured by the arrival of vesicular stomatitis virus glycoprotein (VSV-G) and influenza virus hemagglutinin (HA) at the basolateral and apical cell surface, respectively. The addition of a peptide corresponding to the cytoplasmic tail of VSV-G protein inhibited the delivery to the basolateral surface. In addition, a 200-230 kDa protein (Tin-2, tail interacting protein-2) could be cross-linked to the cytoplasmic tail of VSV-G. Tin-2 association did not occur in the ER or at the cell surface but was specific for a TGN localization of VSV-G suggesting that Tin-2 may be involved in the basolateral sorting process. Machinery components involved in sorting of apical proteins have not been identified.
The regulation of sorting and/or transport to the cell surface in MDCK cells involves heterotrimeric G-proteins (Pimplikar and Simons, 1993). The apical and basolateral pathways are controlled by G_s and G_i proteins, respectively. The activition of apical transport by stimulation of G_s might be mediated by PKA and PKC since apical transport can be stimulated by the addition of PKA and PKC activators (Pimplikar and Simons, submitted). In addition, the rab GTPase rab8 was implicated in basolateral delivery in MDCK cells and dendritic delivery in neurons (Huber et al., 1993a, 1993b).

Transcytosis

Transcytotic pathways connect the basolateral and the apical cell surface of epithelial cells (Rodman et al., 1990). A putative signal for transcytosis of the pIgR has been described (Casanova et al., 1990). The transport of the pIgR from the basolateral to the apical membrane domain in MDCK cells was shown to involve phosphorylation of a serine residue in the cytoplasmic domain. However, recent results indicate that serine phosphorylation is only required for pIgR transcytosis in the absence of dimeric IgA, its physiological ligand (Hirt et al., 1993).

This leaves the signal for basolateral to apical transcytosis undetermined. Previously, the involvement of the proximal and distal BSDs of the LDL-R (Matter et al., 1992) in endocytosis and transcytosis in MDCK cells has been analyzed. It was found that the distal BSD was involved in the recycling of a chimeric protein from basolateral endosomes back to the basolateral cell surface (Matter et al., 1993). A protein lacking the distal BSD was efficiently transcytosed to the apical membrane domain. In contrast, an intact proximal BSD was necessary for transcytosis from the apical to the basolateral cell surface. These results suggest that the cellular machinery involved in the delivery to the basolateral cell surface from both, the TGN and from basolateral and apical endosomes may involve similar mechanisms and share common components. Recently, evidence is accumulating that the delivery pathway from the TGN to the apical plasma membrane and the transcytotic route from the basolateral to the apical cell surface share common characteristics. Not only do they show similar sensitivity to the fungal metabolite brefeldin A and a similar dependence on microtubules (Rodriguez-Boulan and Powell, 1992) but both pathways seem also to be regulated by the G_s class of trimeric G-proteins (Pimplikar and Simons, 1993; Bomsel and Mostov, 1993). The similarity of both pathways is further supported by the finding that pIgR transcytosis from the basolateral to the apical membrane domain is stimulated by activators of PKC (Cardone et al., 1994).
Transcytotic vesicles have been purified from hepatocytes and a 108 kDa peripheral, putative coat protein was implicated in transcytotic vesicle fusion *in vitro* (Sztul et al., 1991; Sztul et al., 1993). Low molecular weight GTP-binding proteins are supposedly involved in transcytotic delivery as well. A candidate protein is rab17 which was localized to the basolateral cell surface and apical endosomes in renal cells (Lütcke et al., 1993).

Model: Glycosphingolipid Rafts as a Sorting-Platform for Apical Proteins

The exoplasmic leaflet of the apical cell surface of epithelial cells is enriched in glycosphingolipids (GSL), presumably providing protection against the external environment (van Meer et al., 1987; van Meer and Burger, 1992; van Meer, 1993). GSL can form dynamic, liquid-crystalline-like clusters within the surrounding fluid phospholipids in the membrane bilayer. This is supposedly due to the unique property of the frequently hydroxyl modified ceramide backbone and the sugar headgroup moieties to form intermolecular hydrogen bonds (Pascher, 1976; Thompson and Tillack, 1985). This led to the proposal that the sorting of proteins and lipids to the apical cell surface is intimately related (Simons and van Meer, 1988; Simons and Wandinger-Ness, 1990) and mediated by a co-clustering or raft formation of GSL and apically sorted proteins in the TGN (Fig.2). The inclusion of apical proteins would involve a direct interaction with GSL or an association with a putative sorting-receptor bearing an inherent affinity for GSL-enriched rafts. This would in turn lead to exclusion of basolaterally destined cargo from the sorting-platform.

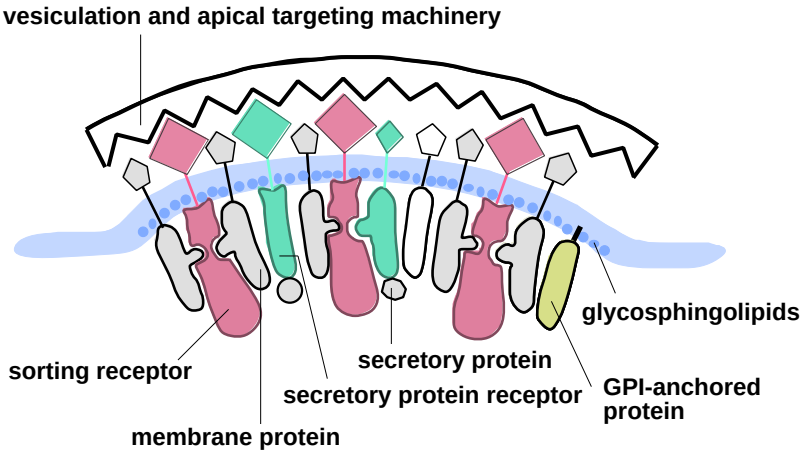

Figure 2: Hypothetical "apical" raft in the *trans*-Golgi network of MDCK cells. The patch will vesiculate into an apical transport vesicle.

Experimental evidence supporting this view has been provided by Brown and Rose (1992). They found that in MDCK cells GPI-anchored proteins associate with GSL during transit through the Golgi. Association was assayed by measuring the TX-100 solubility of placental alkaline phosphatase (PLAP) and was shown not to depend on protein-protein interactions or interaction with cytoskeletal elements but rather on the lipid environment encountered by PLAP on its itinerary to the cell surface. The detergent-insoluble residues isolated from whole cells were found to be highly enriched in GSL. The incorporation of apical integral membrane proteins into similar structures was implied by earlier studies of Skibbens et al. (1989) using MDCK cells where newly synthesized influenza hemagglutinin (HA) was observed to become TX-100 insoluble late in the Golgi but before arrival at the cell surface. More recently, GPI-linked proteins were found in detergent-insoluble residues in intestinal epithelial (Caco-2) cells as well (Garcia et al., 1993). Interestingly, of the apical integral membrane proteins analyzed, sucrase isomaltase (SI) was preferentially detergent-insoluble. In contrast to several other apical proteins, SI is exclusively delivered directly from the TGN to the apical cell surface in Caco-2 cells (Matter et al., 1990).

The process of inclusion into glycolipid rafts must be regulated and allow the release or mobilization of delivered proteins at the cell surface. In agreement with this it was found that the GPI-anchored fusion protein gD1-DAF (herpes simplex virus gD1 fused to decay accelerating factor) arrives at the cell surface as an immobilized cluster which equilibrates slowly with resident mobile gD1-DAF (Hannan et al., 1993).

The components involved in the sorting and delivery process to the apical plasma membrane may be specific for the direct apical pathway from the TGN or alternatively exert a similar function for the transcytotic route from the basolateral to the apical membrane domain. The latter scenario implies that machinery components involved in the sorting and transport of apically destined proteins would be delivered to the basolateral cell surface/endosomes prior to exerting

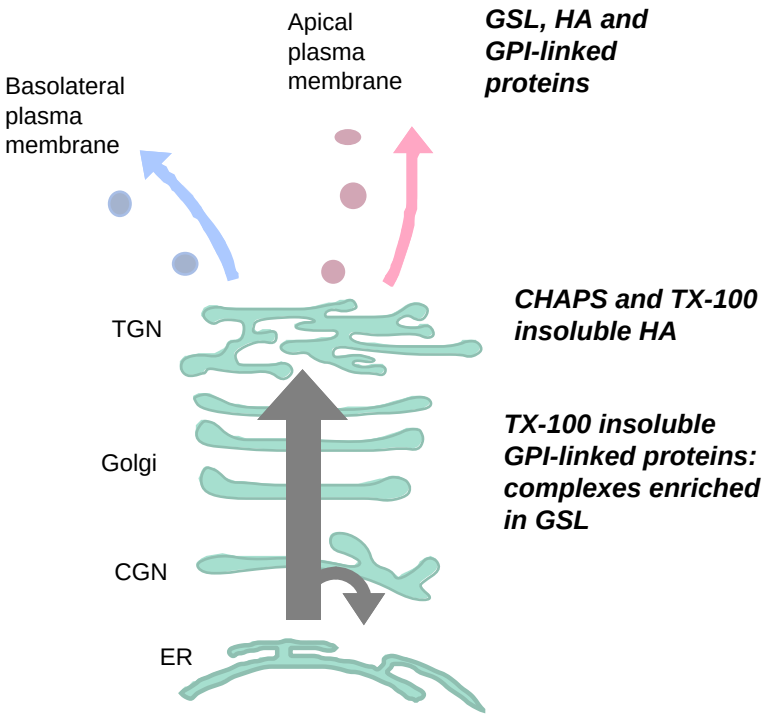

Figure 3: Schematic depiction of the development of detergent-insoluble complexes during passage through the secretory pathway in a polarized epithelial cell (Based on Skibbens et al., 1989; Brown and Rose, 1992; Kurzchalia et al., 1992; Fiedler et al., 1993; Garcia et al., 1993).

a function in transcytotic delivery. At present, it can also not be excluded that more than one sorting mechanism for apical membrane proteins exists in epithelial cells.

Identification and Characterization of General and Epithelial Specific Factors

The purpose of this dissertation was to identify components generally involved in protein sorting and vesicular delivery in epithelial cells with a focus on the apical transport machinery. In the examples of protein sorting and targeting discussed above (lysosomal delivery, ER-retention, endocytosis) sorting signals were usually identified first which then allowed to delineate the interacting cellular machinery. In contrast, the approach taken here was to biochemically dissect exocytic carrier vesicles delivering their cargo from the TGN to the apical and basolateral cell surface in order to gain access to the sorting and transport machinery.

Carrier vesicles were isolated from perforated MDCK cells. In this system vesicular transport is reconstituted in an ATP- and temperature-dependent manner (Simons and Virta, 1987). Conditions were established that allowed the release and purification of vesicular carriers (Bennett et al., 1988). Viral infection was used as a tool to introduce abundant marker proteins into the apical and basolateral pathway. The use of antibodies raised against the cytoplasmic domains of influenza HA and VSV-G protein, which are efficiently sorted to the apical and basolateral cell surface in MDCK cells, respectively (Rodriguez-Boulan and Pendergast, 1980), made immunoisolation of apical and basolateral vesicular carriers possible. The resolution and characterization of these by two-dimensional (2-D) gel electrophoresis provided the basis for all further studies and showed that three classes of proteins could be identified (Wandinger-Ness

et al., 1990). The majority of vesicular components was found in apical as well as in basolateral exocytic carriers while a subset of proteins was specifically co-enriched with influenza HA in the apical vesicles and a third class of proteins was co-enriched with VSV-G in the basolateral carriers. All vesicular proteins were of potential interest but the complexity of the vesicle composition required the restriction of the analysis to a subset of the proteins.
For the apical route, machinery candidates included vesicular components specifically co-enriched in apical carriers as well as molecules physically associated with influenza HA or glycolipid rafts. Initial chemical crosslinking experiments of influenza HA to other vesicular proteins were not conclusive. Thus, another approach that was taken was to extract exocytic carrier vesicles with different detergents in an attempt to isolate detergent-insoluble residues that would specifically include the apical passenger protein influenza HA but not the basolateral VSV-G protein (Kurzchalia et al., 1992). VIP21 (Vesicular-Integral membrane Protein of 21 kDa)-caveolin was one of the vesicular components included into the detergent-insoluble residues and was purified and further characterized (Kurzchalia et al., 1992). The molecule was localized to the Golgi apparatus and the cell surface. At the plasma membrane, VIP21-caveolin was found to be highly clustered in glycolipid-enriched domains, caveolae (Rothberg et al., 1992; Anderson, 1993a, 1993b; for further discussion, see Dupree et al., 1993). The physiological function of VIP21 is a matter of debate. It was speculated to be a purely structural component of the caveolae coat (Rothberg et al., 1992) and the lack of expression of VIP21-caveolin in lymphocytes correlates with the absence of morphologically defined caveolae (Fra et al., in preparation). On the other hand, the high amount of VIP21-caveolin localized to the Golgi apparatus in steady state and its presence in immunoisolated exocytic carrier vesicles isolated from virally infected cells suggests that VIP21-caveolin may recycle between the Golgi and cell surface. Circumstantial evidence favors an involvement in protein sorting. In cultured Fischer rat thyroid (FRT) epithelial cells (Nitsch et al., 1985) the majority of GPI-anchored proteins as well as C6-NBD glucosylceramide are delivered to the basolateral cell surface. However, one basolaterally targeted GPI-anchored protein analyzed was completely detergent-soluble (Zurzolo et al., 1993, 1994). In FRT cells VIP21-caveolin is not expressed (Sargiacomo et al., 1993; Zurzolo et al., 1994). Thus, the absence of VIP21-caveolin may correlate with a missorting of GPI-anchored proteins. The expression of VIP21-caveolin in all epithelia and species has so far not been thoroughly surveyed.

The aim of *part I* of this work was to analyze the protein and lipid composition of detergent-insoluble residues isolated from crude membranes of MDCK cells by using Triton X-114 and CHAPS (3-[(3-cholamidopropyl-dimethylammonio]-2-hydroxy-propanesulfonate) extraction procedures (Fiedler et al., 1993a). The resolution of the complex proteins on 2-D gels led to a convergence with the earlier studies on TGN-derived exocytic carrier vesicles. Comparison revealed that a subset of the proteins present in the apical and basolateral vesicular carriers were present in the detergent-insoluble complexes. In addition, the CHAPS insoluble residues were

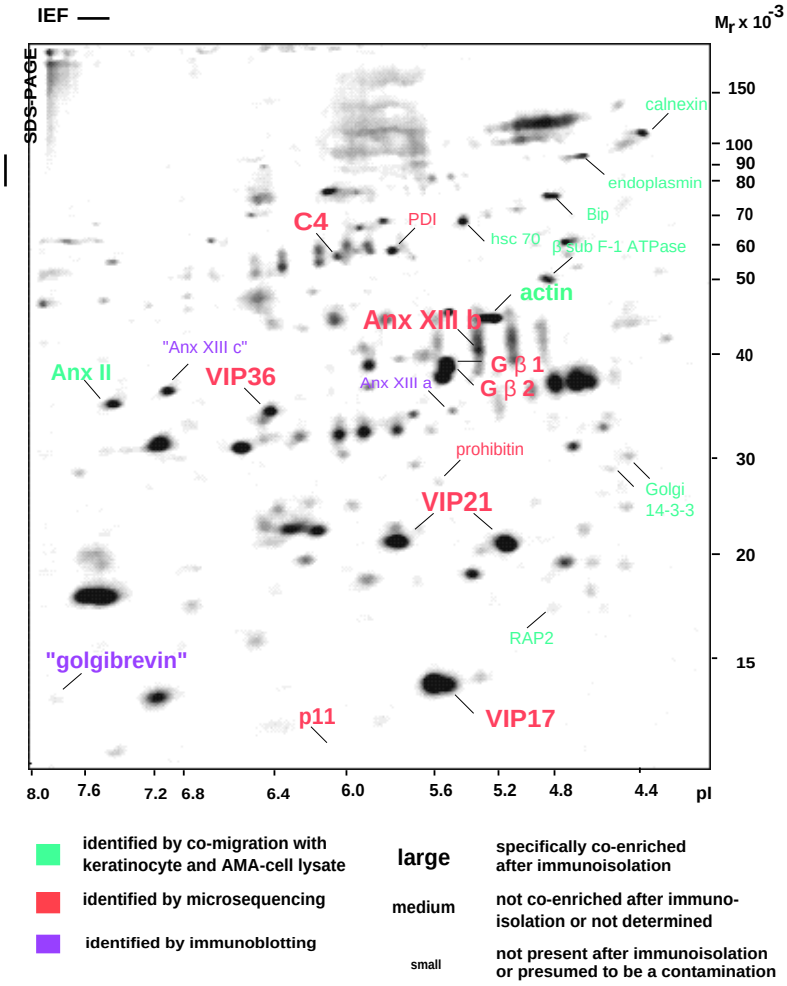

Figure 4: Analysis of the protein composition of crude, non-immunoisolated exocytic carrier vesicles of MDCK cells. Carrier vesicles were isolated from metabolically labeled, perforated MDCK cells. Following co-migration with cell lysates from metabolically labeled human AMA cells or non-labeled keratinocytes the gels were processed by autoradiography or autoradiography and silver staining. This allowed comparison of the vesicle composition with the comprehensive 2-D gel databases (Celis et al., 1991, 1992). Other proteins were isolated from preparative 2-D gels and subjected to microsequencing or identified by immunoblotting. Whenever possible, references for the human protein are given: VIP21 (Kurzchalia et al., 1992); VIP36 (Fiedler et al., 1994); VIP17 (Alonso and Weissman, 1987); Anx XIIIa (Wice and Gordon, 1992); Anx XIIIb, "Anx XIIIc" (Fiedler et al., in preparation); Gβ1 (Codina et al., 1986); Gβ2 (Fong et al., 1987); prohibitin (Nuell et al., 1991); C4 (unpublished results); PDI (2), previously thought to be a phospholipase C (Hempel and DeFranco, 1991); p11 (Kube et al., 1991); "golgibrevin" (immunologically related to synaptobrevin II; unpublished results); RAP2 (Pizon et al., 1988); Golgi 14-3-3 (Isobe et al., 1991; Celis et al., 1992); Anx II (Huang et al., 1986); β and γ actins (Nakajima-Ijima et al., 1988; Erba et al., 1988); β subunit F-1 ATPase (Ohta and Kagawa, 1986); hsc 70 (Dworniczak and Mirault, 1987); Bip (Ting and Lee, 1988); endoplasmin (Maki et al., 1990); calnexin (Galvin et al., 1992).

found to retain GSL and cholesterol, similarly as the Triton-insoluble complexes (Brown and Rose, 1992). The use of detergent-insolubility as a means for defining protein-protein and protein-lipid associations has obvious limitations. Associating proteins may be removed from the complexes during detergent extraction. In addition, artefactual aggregation could be induced during detergent treatment. Nevertheless, it was gratifying that the first component isolated, VIP21-caveolin, was localized to GSL-enriched domains in the cell in agreement with the original assumptions (Dupree et al., 1993). Thus, the vesicular, detergent-insoluble proteins represented prime candidates for further analysis.

In the second phase of this work several components of the detergent-insoluble complexes and exocytic carrier vesicles were identified. As a first step the composition of carrier vesicles was compared to comprehensive 2-D gel databases (Celis et al., 1991, 1992). This was performed by co-migration experiments with cell lysates from transformed human amnion (AMA) cells and human keratinocytes and allowed the assignment of several already identified proteins (Fig.

4, green). The next step was to use preparative 2-D gels to purify candidate proteins for microsequencing. Components co-enriched in apical vesicular carriers were of particular interest. In this way, various ER contaminations could be identified in crude exocytic carrier vesicles and excluded from further analysis (calnexin, endoplasmin, Bip, PDI). Some of these had originally been characterized as components specifically co-enriched together with influenza HA in apical exocytic carrier vesicles (Wandinger-Ness et al., 1990). In contrast to VSV ts045 G-protein, the temperature sensitive HA of the influenza virus strain used (WSN ts61) was not efficiently chased into the TGN and exocytic carrier vesicles at the permissive temperature. Thus, ER membranes released from the perforated MDCK cells also contributed to the specifically immunoisolated membrane fraction.

In *part II* of this work the identification and characterization of VIP36 (*V*esicular-*I*ntegral Membrane *P*rotein of *36* kDa), a component of detergent-insoluble complexes in MDCK cells, is described (Fiedler et al., 1994a). The cDNA encoding VIP36 was isolated. VIP36 was shown to be a type I integral membrane protein localized to the Golgi apparatus, endosomal and vesicular structures and the plasma membrane as predicted for a protein involved in transport between the Golgi and the cell surface. Interestingly, VIP36 showed a significant amino acid sequence similarity to leguminous plant lectins, suggesting that it might bind to sugar residues of glycolipids, GPI-anchors or glycoproteins. Most recent data indicate that VIP36 is a glycoprotein. Legume lectins require Ca^{2+} and Mn^{2+} for their sugar binding activity. Preliminary results suggest that the bacterially expressed exoplasmic domain of VIP36 binds Ca^{2+} in a saturable manner with a dissociation constant in the micromolar range (unpublished data). Experiments are in progress to analyze the potential sugar binding and function of VIP36 in detail.

Part III of this dissertation (Fiedler et al., 1994b) describes the homology of VIP36 to ERGIC-53, a protein localized to the intermediate region between the ER and Golgi apparatus and presumably recycling between them (Schweizer et al., 1988; Schindler et al., 1993). This finding suggests that a putative new family of VIP36-like animal lectins exists in the secretory pathway and may be generally involved in the secretion or retention of saccharide bearing molecules.

In *part IV* of this work a component enriched in apical exocytic carrier vesicles, annexin XIIIb, was purified, the cDNA encoding the protein was isolated and further analyzed (Fiedler et al., in preparation). This study revealed that annexin XIIIb is a new member of an epithelial specific sub-family of annexins. Annexin XIIIb was exclusively localized to the apical cell surface and vesicular structures in MDCK cells. Initial functional analysis of annexin XIIIb was carried out with an *in vitro* transport system and showed that annexin XIIIb might be involved in vesicular traffic to the apical plasma membrane.

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Part I

Glycosphingolipid-Enriched, Detergent-Insoluble Complexes in Protein Sorting in Epithelial Cells

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(1993) *Biochemistry* 32, 6365-6373.

Part II

VIP36, a Novel Component of Glycolipid Rafts and Exocytic Carrier Vesicles in Epithelial Cells

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Part III

A Putative Novel Class of Animal Lectins in the Secretory Pathway Homologous to Leguminous Lectins

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(1994) *Cell* (Letter, in press).

Lectins are a structurally diverse class of proteins which selectively bind to specific carbohydrate structures. They can be grouped together into families of leguminous and cereal lectins in plants (Lis and Sharon, 1986) and pentraxins and C-, S- and P-type lectins in animals (Drickamer and Taylor, 1993). Animal lectins are involved in e.g. the endocytosis of glycoproteins, the regulation of cell migration and adhesion and the sorting and delivery of lysosomal enzymes. Until recently animal and plant lectins appeared to be unrelated. With the elucidation of the three dimensional structure of the human serum amyloid P component (Emsley et al., 1994), a pentraxin family member, it has become obvious that the protein folds of pentraxins and leguminous plant lectins are closely related. Pentraxins and plant lectins do however not show any similarity in their primary amino acid sequences.

We have recently described a significant sequence similarity of VIP36 to leguminous plant lectins (Fiedler et al., 1994). VIP36 is a type I integral membrane protein which is localized to the *trans*-Golgi apparatus, apical and basolateral transport vesicles in epithelial cells and the cell

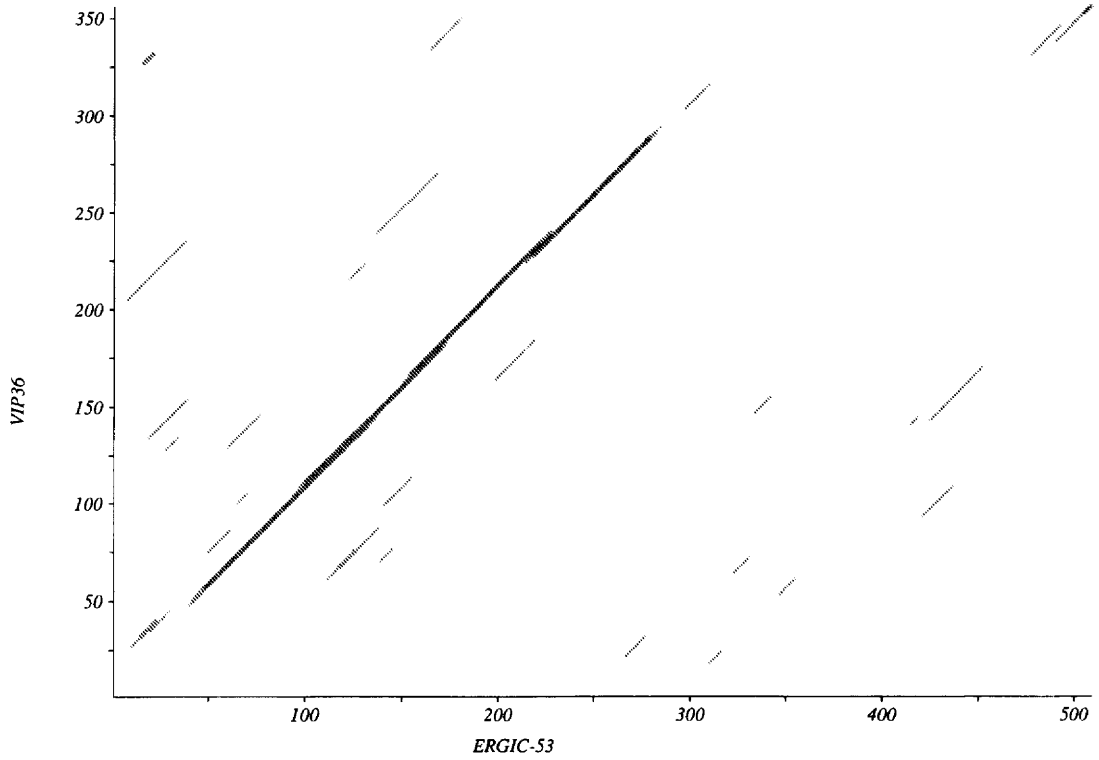

Figure 1: Homology of VIP36 with ERGIC-53. ISSC (Argos, 1987) was used to generate a homology search matrix between VIP36 and ERGIC-53. The search window lengths ranged from 5 to 35 in steps of 2. The peak values (S) are scored as a number of standard deviations (σ) above the matrix mean for each window length. For this comparison thick, long vertical bars indicate $S \geq 5\ \sigma$; lighter, long vertical bars refer to $4.5\ \sigma \leq S < 5.0\ \sigma$ and short bars signify $4.0\ \sigma \leq S < 4.5\ \sigma$. The string of high peak values along the main diagonal of the search matrix of the N-terminal domains is highly statistically significant. For the TFASTA search the k-tup was set to 1, the scores were init1 136, initn 324, opt 412 for VIP36 against ERGIC-53. According to the GCG program gap (using program default parameters) the alignment of VIP36 with ERGIC-53 scored more than 26 standard deviations above the average quality of 100 randomly shuffled sequences.

Entry[a]	**Score Profile_2**[b]	**Score Profile_5**[c]
VIP36		25.29[d]
ERGIC-53		23.58[d]
Lec_Eryco	6.17	36.91[d]
Lec_Latsp	6.15	39.23[d]
Lec1_Latoc	6.02	17.71
Leca_Latar	5.84	18.82
Leca_Latod	5.79	18.91
Leca_Latti	5.78	18.70
Leca_Latci	5.52	18.94
Lec_Vicfa	5.31	39.78[d]
Top false hit	5.31	6.09

Table I: Scoring characteristics of profiles with the Swissprot database. **a** Entries are from Swissprot, VIP36 and ERGIC-53 were translated from the EMBL database. **b** The profile was generated with the GCG program profilemake using the 'stringent' option from an alignment of amino acids 45-286 of VIP36 and amino acids 31-281 of ERGIC-53 generated with the GCG program pileup using program default parameters. Scores were normalized to sequence length. The alignments of the profile in the search output with Lec_Eryco, Lec_Latsp and Lec_Vicfa showed the highest absolute score. **c** The profile was generated with GCG pileup and profilemake using program default parameters from an alignment of VIP36, ERGIC-53, Lec_Eryco, Lec_Latsp and Lec_Vicfa spanning residues 60-281 of VIP36 and corresponding residues of the aligned sequences. Scores were normalized to sequence length. **d** Sequence was included in the profile.

surface, presumably recycling between the plasma membrane and the Golgi. By searching the EMBL/Genbank DNA databases (Rel. 38/79) with TFASTA (Pearson, 1990) we have now noticed that the new database entry ERGIC-53 is homologous to VIP36 (Fig.1). ERGIC-53 is a type I integral membrane protein of unknown function (Schindler et al., 1993) which is probably identical to gp58 (Saraste et al., 1987) and is localized in the intermediate region between the endoplasmatic reticulum and the Golgi (Schweizer et al., 1988), presumably recycling between them. The most interesting finding of our search is that the sequence similarity between VIP36 and ERGIC-53 is exactly in the region which shows homology to legume lectins (Fig.2 and Table I).

A homology search matrix of the VIP36 and ERGIC-53 sequences was generated with the ISSC program (Argos, 1987). The complete exoplasmic domain of VIP36 could be aligned with ~290 N-terminal amino acids of ERGIC-53 (Fig.1), sharing 33% identical and 55% similar positions. The quality of the alignment scored more than 26 standard deviations above the average quality of 100 randomly shuffled sequences which is considered highly statistically significant (see legend Fig.1). To test whether amino acids conserved in VIP36 and ERGIC-53 would lead to the retrieval of leguminous plant lectins or additional new family members a profile derived from the alignment of the N-terminal domains of VIP36 and ERGIC-53 was used for a profile search (Gribskov et al., 1990) of the Swissprot protein database (Rel. 27). Legume lectins were scoring exclusively in the first eight positions and in 17 out of the top 20 positions of the search output suggesting that ERGIC-53 is also homologous to leguminous lectins (Table I). VIP36, ERGIC-53 and the three most related lectins were analyzed with the

Figure 2: Alignment of legume lectins, VIP36 and ERGIC-53. Sequences were aligned with the GCG program pileup as described in the text using a gap weight of 2.7 and length weight of 0.1. Amino acid sequences are shown in single-letter code, dots indicate gaps, amino acid positions are indicated on the right side of the alignment. Consensus residues that are present in at least 3 of the 5 sequences are boxed. Conserved changes are indicated by shaded boxes. The conserved Asp131, Gly149 and Asn166 of VIP36 and corresponding residues of the other proteins are labelled with a star. Accession numbers Genbank/EMBL (X), Swissprot (P) and references: Lec_Latsp, P16349 (Richardson et al., 1987); Lec_Vicfa, P02871 (Hopp et al., 1982); Lec_Eryco, P16404 (Adar et al., 1989); ERGIC-53, X71661 (Schindler et al., 1993); VIP36, X76392 (Fiedler et al., 1994).

GCG program gap and aligned with pileup (Devereux et al., 1984). The two sequences of highest similarity were aligned first and the sequences of lower similarity were successively aligned with the aligned family of sequences (Fig.2). Although VIP36 and ERGIC-53 share only 19-24% amino acid sequence identity with legume lectins, domains and residues functionally important for metal and sugar binding in leguminous lectins (Sharon, 1993) are particularly conserved in VIP36 (Fiedler et al., 1994) and ERGIC-53 as well, further strengthening the homology. These residues include Asp131, Gly149 and Asn166 of VIP36 (Fig.2) and the corresponding amino acids of the aligned proteins. No additional proteins with these conserved features were retrieved with a profile search from the EMBL/Genbank DNA databases. The predicted secondary structure of the N-terminal domain of ERGIC-53 is abundant in β-sheet characteristics, very similar to VIP36, and can be aligned with the known secondary structure of leguminous lectins (not shown) which may indicate a common main chain fold. In contrast, the C-terminal part of the exoplasmic domain of ERGIC-53 is hydrophilic and abundant in predicted α-helical structures, suggesting that it may form a stalk that separates the lectin domain from the membrane.

These findings establish VIP36 and ERGIC-53 as members of a new family of plant lectin homologues in the secretory pathway of animal cells. It is tempting to speculate that VIP36 and ERGIC-53 recognize sugar residues of glycoproteins, glycolipids or glycosylphosphatidyl inositol anchors and are involved in the sorting or recycling of proteins and/or lipids. It may be

that oligosaccharide sidechains play a more important role in biosynthetic traffic than hitherto recognized.

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Part IV

Annexin XIIIb - a New Epithelial Specific Annexin in Vesicular Traffic to the Apical Plasma Membrane

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(in preparation)

Abstract. The selective delivery of proteins to the apical and basolateral plasma membrane in simple epithelial cells is mediated by sorting in the *trans*-Golgi network (TGN). We have previously analyzed the protein composition of immunoisolated apical and basolateral exocytic carrier vesicles from Madin-Darby canine kidney (MDCK) cells and now purified a component highly enriched in apical vesicles for peptide microsequencing. Isolation of the encoding cDNA revealed that this protein, annexin XIIIb, is a new isoform of the epithelial specific annexin XIII sub-family which includes the previously described intestine-specific annexin (annexin XIIIa; Wice, B.M. and Gordon, J.I., 1992. *J. Cell Biol*. 116:405-422). Annexin XIIIb differed from annexin XIIIa by containing a unique insert of 41 amino acids in the N-terminus and was exclusively expressed in dog intestine and kidney. Only annexin XIIIb, but not annexin XIIIa or the immunologically related proteins c and d were enriched in apical exocytic carrier vesicles. The subcellular localization of annexin XIII was analyzed by immunofluorescence and immunoelectron microscopy and demonstrated that annexin XIII isoforms were localized to the TGN, endosomes and the apical and lateral plasma membrane. Annexin XIIIb was restricted to the apical plasma membrane and vesicular structures beneath. Annexins are implicated in membrane-membrane interactions in exocytosis and endocytosis. To test the involvement of annexin XIIIb in delivery to the apical cell surface we used permeabilized MDCK cells and a cytosol dependent *in vitro* transport assay. Antibodies specific for annexin XIIIb significantly inhibited the transport of influenza virus hemagglutinin from the TGN to the apical plasma membrane while the transport of vesicular stomatitis virus-glycoprotein to the basolateral cell surface was unaffected. We propose that annexin XIIIb plays a role in delivery to the apical plasma membrane in MDCK cells.

Introduction

The cellular endomembrane system is connected by vesicular transport routes that shuttle cargo between donor and acceptor compartments. The traffic pathways are now well delineated (Simons and Zerial, 1993) as are some of the mechanisms that are involved in vesicle budding and specificity of vesicle docking and fusion (Pryer et al., 1992; Gruenberg and Clague, 1992). The GTP-binding protein ARF (ADP-Ribosylation Factor) and coat proteins are required for budding of intra-Golgi derived vesicles (Rothman and Orci, 1992; Ferro-Novick and Novick, 1993), the mammalian rab proteins and their yeast homologues are implicated in conferring directionality of transport (Bourne, 1988; Zerial and Stenmark, 1993) and NSF (NEM Sensitive Factor) and SNAPs (Soluble NSF Attachment Proteins) and their yeast counterparts have been shown to be involved in a number of fusion steps (Rothman and Orci, 1992). Syntaxin, synaptobrevin, synaptotagmin and SNAP-25 (SyNaptosomal Associated Protein of 25 kDa) are involved in the fusion of synaptic vesicles with the nerve terminal (Bennett and Scheller, 1993; Südhof et al., 1993; DeBello et al., 1993) and according to the SNARE (SNAP receptor) hypothesis families of syntaxin and synaptobrevin homologues may

generally provide the specificity in vesicle docking prior to fusion (Söllner et al. , 1993; Rothman and Warren, 1994).

The components and mechanisms involved in the membrane fusion itself are still unclear. Based on morphological studies on exocytic fusion pores in mast cells (Chandler and Heuser, 1980) and electrophysiological data (Breckenridge and Almers, 1987) several models have been proposed, ranging from proteinaceous, ion channel-like pores (Almers, 1990) to purely lipidic pores only requiring a surrounding scaffold of proteins (Monck and Fernandez, 1992). Studies on viral membrane fusion suggest that a collar of proteins may provide close contact between membranes and expose a hydrophobic bridge that allows the flow of lipids between bilayers (Bentz et al., 1990; White, 1992).

In polarized epithelial cells proteins and lipids destined for the apical or basolateral cell surface are sorted in the TGN which requires factors to mediate the sorting process and to distinguish the apical from the basolateral pathway (Simons and Fuller, 1985; Griffiths and Simons, 1986; Rodriguez-Boulan and Nelson, 1989). In order to identify components involved in this process we have previously immunoisolated apical and basolateral exocytic carrier vesicles from the simple epithelial cell line MDCK (McRoberts et al., 1981) and analyzed their protein composition by two dimensional (2-D) gels (Bennett et al., 1988; Wandinger-Ness et al., 1990). VIP21 (Kurzchalia et al., 1992; Dupree et al., 1993) and VIP36 (Fiedler et al., 1994) were identified as components of the apical and basolateral pathway and the small GTPase rab8 was shown to be specific for the basolateral pathway and to be involved in transport to the basolateral cell surface (Huber et al., 1993). To date, no factors of the putative sorting and targeting machinery that are specific for the apical secretory route have been identified.

Here we describe the purification of a component that was highly enriched in apical exocytic carrier vesicles. Isolation of the encoding cDNA showed that this protein, annexin XIIIb, is a new member of the annexin XIII sub-family. Annexin XIIIb was exclusively expressed in dog intestine and kidney and localized to the apical cell surface and vesicular structures by immunofluorescence and immunoelectron microscopy. Among other proposed physiological functions annexins have been implicated in membrane-membrane interactions such as those involved in vesicle docking, budding or fusion (Creutz, 1992; Gruenberg and Emans, 1993). Using antibodies specific for annexin XIIIb in a cytosol dependent *in vitro* transport assay with permeabilized MDCK cells only transport to the apical but not basolateral plasma membrane was significantly inhibited. Annexin XIIIb is the first putative machinery component identified that is specific for the apical pathway in MDCK cells and may play a role in vesicular transport to the apical cell surface.

Material and Methods

Materials

Unless otherwise indicated, all chemicals were obtained from the sources described previously (Bennett et al., 1988; Wandinger-Ness et al., 1990; Kurzchalia et al., 1992). The mouse monoclonal 17L10 anti-WSN HA antibody was a kind gift of T. Yewdell, NIH. The donkey anti-rabbit rhodamine-conjugated and donkey anti-mouse FITC-conjugated antibodies were from Dianova (Hamburg, Germany).

Cell Culture and Viral Infection

Growth media compositions, MDCK II cells, viruses and cell culture protocols were described previously by Wandinger-Ness et al. (1990). Viral infection with WSN ts61 and accumulation of HA in the TGN by a temperature block at 20 °C were as described by Matlin and Simons (1983).

SDS-PAGE and 2-D Gel Electrophoresis

SDS-PAGE on 12 % gels was performed as described (Bennett et al., 1988). Resolution of proteins in two dimensions by IEF and SDS-PAGE, based on the method of Bravo (1984), was performed according to Wandinger-Ness et al. (1990), except for preparative amounts of proteins, that were resolved using the Millipore Investigator IEF first dimension. After electrophoresis the gels were fixed in 45 % methanol and 7 % acetic acid and stained with Coomassie blue or treated for fluorography using Entensify (Dupont).

Isolation of Annexin XIIIb

A total cellular membrane fraction was prepared from metabolically labelled or non-labelled MDCK cells as previously described (Kurzchalia et al., 1992; Fiedler et al., 1993) and was pelleted in the ultracentrifuge at 100 000 g for 1 h. The sample was prepared for the IEF first dimension according to Ames and Nikaido (1976) with slight modifications. Approximately 50 µg of protein were directly solubilized in 1.7 % SDS, 170 mM DTT. Following heating to 97 °C for 4 min the sample was cooled to room temperature, 150 mg urea, 25 µl 80 % Nonidet P-40 and 12.5 µl ampholytes 7-9 were added and the volume was made up to 250 µl with H_2O. The urea was dissolved at 37 °C and the sample was spun for 5 min at 37 °C in the eppendorf centrifuge before loading.

Amino-acid Sequence Analysis

Four Coomassie blue stained spots of annexin XIIIb (~2 µg) were excised from the 2-D gels and pooled. After washing with water, the gel pieces were lyophilized and rehydrated in 100 µl of 100 mM NH_4HCO_3, 0.5 mM $CaCl_2$ containing 1 µg trypsin. After digestion (37 ˚C for 12 h) peptide fragments were extracted from the gel slice with 2 x 100 µl of 70 % trifluoracetic acid/ 0.01 % Tween 20 and 50 % trifluoracetic acid/ acetonitrile/ 0.01 % Tween 20 (modified from Eckerskorn and Lottspeich, 1989). The combined fractions were concentrated and subjected to reverse-phase-HPLC using Vydac 218TP (2.1 x 250 mm). Automated Edman degradation of peptides was performed using an Applied Biosystems sequencer (Model 477A) connected to an on-line PTH-analyzer (Applied Biosystems, Model 120).

Molecular Cloning of Annexin XIIIb

Total RNA was prepared from confluent MDCK cells using the guanidinium hydrochloride procedure (Chirgvin et al., 1979). MDCK cDNAs were synthesized with the first-strand synthesis kit of Stratagene (La Jolla, CA) with oligo dT as the primer. PCR was carried out according to Chavrier et al. (1992). The specific degenerate oligonucleotides were 5' CCGGGAATTCGGAAAATGGG(A/C/G/T)AA(C/T)(A/C)G(A/C/G/T)CA(C/T) 3' and 5' CGCCCTCGAG(A/G)TT(A/G)AAIGC(A/C/G/T)A(A/G)(C/T)TC(G/A)TC 3'. Of the 726 bp and 603 bp fragments obtained, the 726 bp fragment was used for screening of a λ ZAP II MDCK cDNA library (Chavrier et al., 1990). Duplicate nitrocellulose filters were prehybridized for 2 h at 42˚C in 5 x SSC, 50 % formamide, 5 x Denhardts solution, 1 % SDS (Sambrook et al., 1989). Hybridization was carried out in the same solution, supplemented with ^{32}P-labeled probe overnight at 55˚C. Positive recombinants were screened for the presence and length of the 5' cDNA end by PCR using the Bluescript SK primer and the primers shown above. Nucleotide sequences of both cDNA strands were determined using the T7 sequencing kit (Pharmacia, Uppsala, Sweden).

Computer Sequence Analysis

Basic sequence analysis was carried out with the GCG programs (Madison, WI) (Devereux et al., 1984). MPsrch (Sturrock and Collins, 1993) was used to search Swissprot release 28. This program is accessible by e-mail under *Blitz@EMBL-Heidelberg.DE*. Potential sites for post-translational modifications were identified by search in the Prosite library (Bairoch, 1991). The phylogenetic tree was calculated as described by Higgins et al. (1992) over a multiple sequence alignment of the annexins shown in Fig. 4, spanning residues 47-357 of annexin XIIIb and the corresponding residues of the aligned annexins, thus excluding the hypervariable N-terminal domains.

The tree was corrected for multiple substitutions, analyzed by bootstrapping and visualized with Phylip (Felsenstein, 1993).

Preparation of Antibodies and Immunoblotting

Polyclonal sera were raised against synthetic peptides covalently coupled to keyhole limpet haemocyanin using residues 20-36 (KGDIQPSAAVQPLSHPSK; anx13b), including an additional K, and residues 47-64 (AKAKSHHGFDVDHDAKKL; anx13) of annexin XIIIb according to Kreis (1986). Sera were collected after the sixth injection of antigen. For affinity purification, the peptides were linked directly to CNBr-activated Sepharose 4B according to the manufacturer (Pharmacia). Serum (1.4 ml) was passed continuously over the matrix overnight at 4°C. Bound antibody was eluted with 0.2 M glycine pH 2.8 and the fractions neutralized with 1 M Tris-HCl (pH 8.0). For immunoblotting dog tissues were homogenized with a Dounce homogenizer in modified SDS-sample buffer. The protein concentrations were determined with the Micro BCA Protein Assay (Pierce) and 10 µg of each were resolved by SDS-PAGE and transferred to nitrocellulose in a blotting buffer consisting of 25 mM Tris, 190 mM glycine and 20 % methanol. Blots were incubated with 1:40 dilutions of the affinity-purified sera overnight at 4 °C and then in 1:3000 dilution of horseradish preoxidase conjugated goat anti-rabbit IgG (BioRad) as the second antibody for 1 h at room temperature. As a blocking solution, 5 % non-fat dried milk, 0.2 % Tween-20 (Sigma) was used. Transfer and blotting of 2-D gels was carried out identically. Bands were detected using ECL (Amersham).

Immunofluorescence Microscopy

Cells grown on coverslips and on polycarbonate filters were washed with phosphate-buffered saline (PBS), fixed in 4 % paraformaldehyde in PBS for 20 min and permeabilized with 0.1 % Triton X-100 in PBS for 4 and 6 min, respectively, at room temperature. To reduce unspecific labelling of filter grown cells they were denatured with 6 M Guanidine-HCl in 50 mM Tris-HCl (pH 7.5) for 10 min at room temperature (Peränen et al., 1993). All cells were rinsed in PBS and the free aldehyde groups quenched with 50 mM NH_4Cl in PBS for 20 min. After a further rinse in PBS the cells were incubated in 0.2 % gelatin in PBS for 30 min and the first antibody diluted in 0.2 % gelatin-PBS was added. The affinity-purified anti anx13 and anti anx13b antisera were used at a dilution of 1:20 and 1:5, respectively. The cells were incubated for 30 min at 37 °C and washed with PBS. Primary antibodies were visualized with pre-adsorbed donkey anti-rabbit rhodamine-conjugated or anti-mouse FITC-conjugated antibodies. The coverslips were viewed and photographed with an Axiophot photomicroscope (Carl Zeiss, Oberkochen, Germany) or with the EMBL confocal microscope and photographed with a Polaroid Freeze frame directly from the monitor.

Immunoelectron Microscopy

Fully polarized, filter grown cells were fixed with 8 % paraformaldehyde in 250 mM Hepes (pH 7.35). Pieces of filter were sandwiched together with 10 % gelatin PBS, infiltrated with sucrose, and frozen in liquid nitrogen. After sectioning, grids were incubated for 1 h at 37 °C on PBS to dissolve and remove the gelatin. Labelling with antibodies and protein A-gold was performed as described previously (Griffiths et al., 1984; Griffiths et al., 1985).

In Vitro Transport of HA and VSV-G in Permeabilized MDCK Cells

The *in vitro* transport assay was based on the protocols earlier described (Kobayashi et al., 1992; Pimplikar and Simons, 1993) and carried out and quantitated exactly as previously outlined (Huber et al., 1993; Pimplikar et

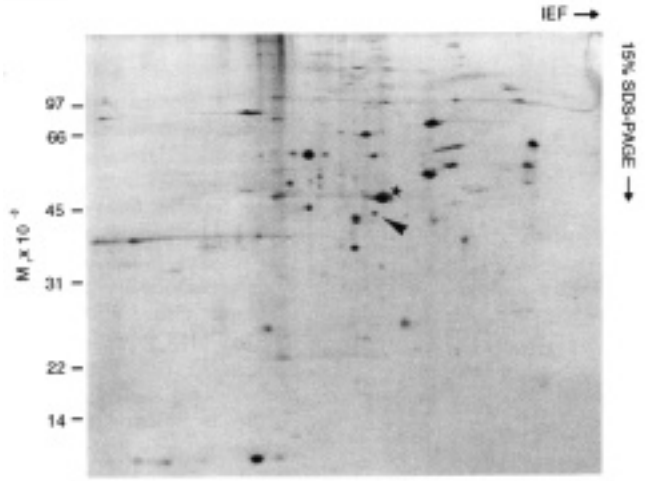

Figure 1: Isolation of annexin XIIIb. MDCK membranes were solubilized with SDS and resolved by preparative 2-D gel electrophoresis. The gels were stained with Coomassie blue. Annexin XIIIb [named A23 in Wandinger-Ness et al. (1990)], labelled with an arrowhead, was excised and analyzed by microsequencing. An asterisk marks the position of actin.

al., 1994). Streptolysin O was a generous gift of S. Bhakdi, University of Mainz, Germany. The transport assay was performed in duplicates with or without exogenously added cytosol (HeLa cytosol, 8 mg/ml) in the presence or absence of the indicated concentrations of affinity-purified anti anx13b antibody.

Results

Purification of Annexin XIIIb

The characterization of immunoisolated exocytic vesicles previously established a class of proteins common to both, apical and basolateral carriers and components unique to apical or basolateral transport vesicles (Wand–inger-Ness et al., 1990). The component named A23 was 38 fold enriched in apical versus basolateral carriers and, according to Triton X-114 phase partitioning, was classified as one of only two apical specific membrane proteins in total. Therefore it was of particular interest to determine its identity. We used a total cellular membrane fraction from MDCK cells prepared as previously described (Kurzchalia et al., 1992; Fiedler et al., 1993) for the purification of A23. Membranes were solubilized with SDS and resolved by preparative two-dimensional (2-D) gel electrophoresis (Fig. 1). The identity of A23 was verified by comparison of the total cellular membrane fraction with purified exocytic carrier vesicles by 2-D gels (not shown). Coomassie-stained spots of A23 were excised from four gels and pooled. Enzymatic digestion in the gel slice and chromatographic separation of the peptides allowed the determination of the amino acid sequence of three fragments (Fig. 2, open box). Comparison of the peptides obtained with the Swissprot protein database by using MPsrch (Sturrock and Collins, 1993) revealed that all three (with the exception of one single amino acid position) matched peptides

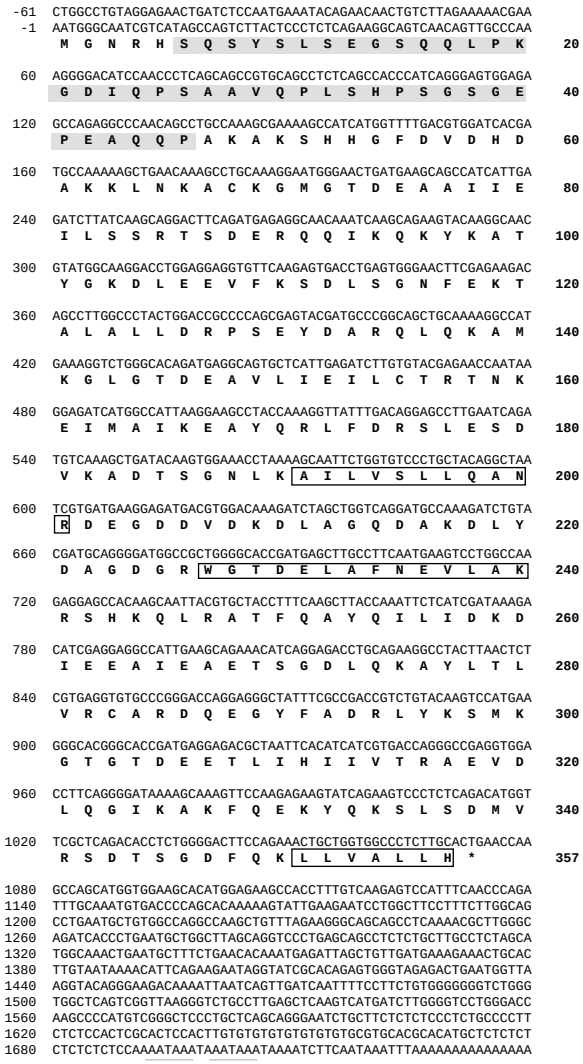

Figure 2: Nucleotide sequences of the annexin XIIIa and annexin XIIIb cDNAs and amino acid sequences of the encoded proteins. Peptides identified by microsequencing are boxed. The asterisk denotes the stop codon, the putative polyadenylation signals at the 3' end are underlined. The isolated annexin XIIIa cDNA corresponded to nucleotides -61 to 15 and 139 to 1739, the annexin XIIIb cDNA lacked nucleotides -61 to -22 but contained an insert of nucleotides 16 to 138 (encoded amino acids are in a shaded box). Two in frame stop codons are preceding the 5' ATG in position -51 and -44.

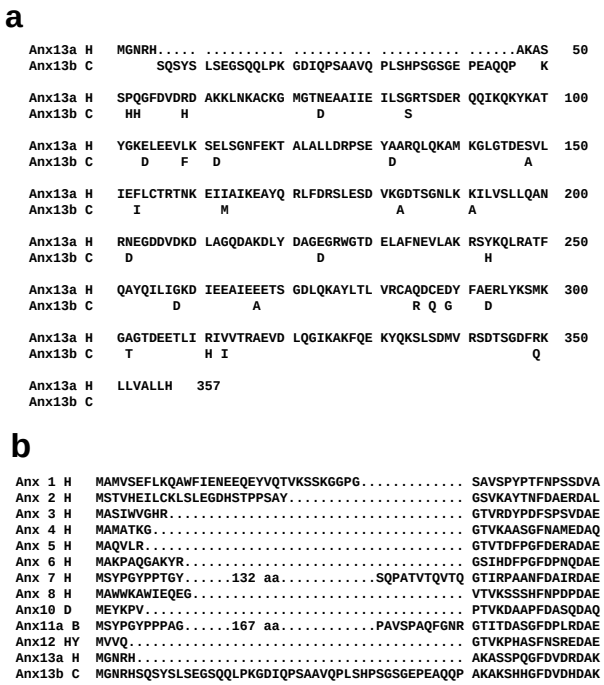

Figure 3: Alignment of annexin XIIIa, XIIIb and other annexins. (*a*) Alignment of human annexin XIIIa (intestine-specific annexin) with canine annexin XIIIb using the GCG program gap. Gaps are indicated by dots. For annexin XIIIb only amino acids different from annexin XIIIa are indicated. The overall identity and similarity is 90 % and 96 %, respectively. (*b*) Alignment of the N-terminal domains of representatives of all known annexins. Gaps are represented by dots that are inserted between the variable N-terminal domains and the beginning of the first conserved repeating domain. For the alignment the sequences of human annexins I - VIII (H), *Drosophila melanogaster* annexin X (D), bovine annexin XIa (B), *Hydra vulgaris* annexin XII (HY), human annexin XIIIa (H) and canine annexin XIIIb (C) were used. The 5' end of the *Drosophila melanogaster* annexin IX cDNA has not been isolated.

found in annexin XIIIa (intestine-specific annexin) described by Wice and Gordon (1992). However, A23 had an apparent molecular weight of 40 kDa in SDS-polyacrylamide gel electrophoresis (PAGE) and not 36 kDa as reported for annexin XIIIa which suggested that A23 might be a variant of the latter.

Molecular Cloning of Annexin XIIIb

Wice and Gordon (1992) had reported an unknown component with an apparent molecular weight of 42 kDa that crossreacted with an antibody raised against an N-terminal peptide of annexin XIIIa. We therefore reasoned that the N-termini of annexin XIIIa and A23 would be conserved and simplified our polymerase chain reaction (PCR)-based approach to obtain a partial cDNA encoding A23. Two degenerate oligonucleotides encoding the N-terminal peptide MGNRH of annexin XIIIa and part of the peptide WGT**DELAFNE**VLAK obtained by microsequencing (bold) were used to amplify a 603 bp product, corresponding to the length of the annexin XIIIa cDNA fragment, and a novel 726 bp product. The 726 bp fragment was used to screen ~200 000 plaques of a λ ZAP II MDCK II cDNA library (Chavrier et al., 1990) and 30 hybridizing clones (corresponding to 0.015 % of the recombinants) were obtained. Out of the 18 clones further analyzed, 13 gave rise to a 603 bp fragment by PCR using the aforementioned primers while 5 clones generated a 726 bp product. Two of each group were partially sequenced and differed only in the length of the 5' non-coding regions. The shorter cDNA corresponded to nucleotides -61 to 1739 (Fig. 2) but was lacking nucleotides 16 to 138 (shaded box). The longer cDNA only lacked nucleotides -61 to -22 at the 5' end but contained the insert of nucleotides 16 to 138 (Fig. 2, shaded box). We named them annexin XIIIa and annexin XIIIb, respectively.

The calculated molecular weights and isoelectric points of the encoded proteins were 39606 Da and pI 5.2 for annexin XIIIb which was in good agreement with the values observed for A23 on 2-D gels, and 35479 Da and pI 5.4 for annexin XIIIa. Computer sequence analysis demonstrated that the amino acid sequence of canine annexin XIIIb was 90 % identical and 96 % similar to human annexin XIIIa (Fig. 3a). The identity to 31 other annexin family members ranged between 38 % and 47 %. Annexin XIIIb differed from annexin XIIIa by having a unique 41 amino acid insert in the N-terminal domain. A comparison of the variable N-terminal domains of annexins I-XIII, preceding the first 70 amino acid repeated domain, is shown in Fig. 3b. The N-terminus of annexin XIIIb does not show any significant similarity to the other family members or other proteins in the Swissprot protein database. A search in the Prosite library (Bairoch, 1991) revealed that annexin XIIIb contains several potential phosphorylation sites for casein kinase II and protein kinase C throughout the sequence and one potential tyrosine kinase phosphorylation site. Notably, a putative casein kinase II phosphorylation site is located within the unique 41 N-terminal amino acids. Three of the four conserved annexin segments (endonexin folds) are retrieved by the progamme (some of the repeats are also not recognized in annexin IX, X, XI and XII). Interestingly, annexin XIIIb contains a potential N-terminal myristoylation site. Analysis of the evolutionary distances of representatives of all known annexins (for details see Materials and Methods) showed that only annexins I,II and III but not annexin XIII could clearly be subgrouped into a distinct branch of the annexin family (Fig. 4).

Annexin XIII Isoforms in Exocytic Carrier Vesicles

To further analyze annexin XIII we raised polyclonal antisera against an N-terminal peptide common to annexin XIIIa and annexin XIIIb (anx13) and antisera against an N-terminal peptide unique to annexin XIIIb (anx13b). The specificity of the affinity purified antisera was demonstrated by Western blotting of a total cellular membrane fraction from MDCK cells (Fig.

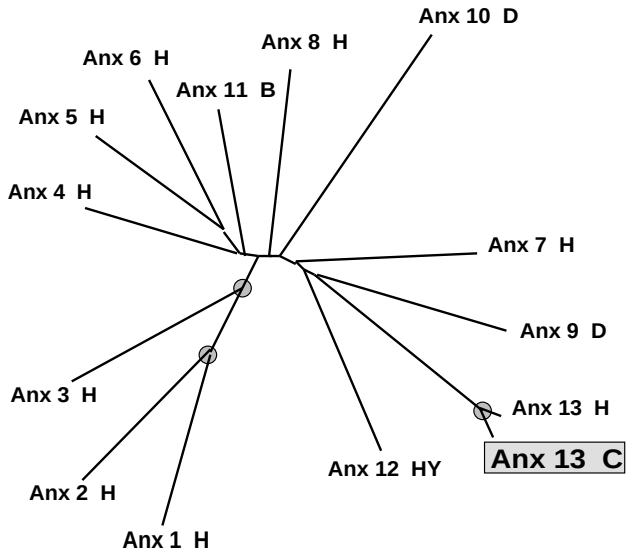

Figure 4: Phylogenetic tree representing the evolutionary distances between canine annexin XIII and representatives of all known annexin protein sequences. The tree was calculated as described by Higgins et al. (1992) over a multiple sequence alignment of annexins (see Fig. 3b) spanning residues 47 to 357 of annexin XIII b and the corresponding residues of the aligned sequences. The comparison excluded the variable N-terminal domains. The length of the branches is proportional to the actual distances between the sequences. Branchpoints of the tree that are above 88 % statistically significant are labelled with a circle.

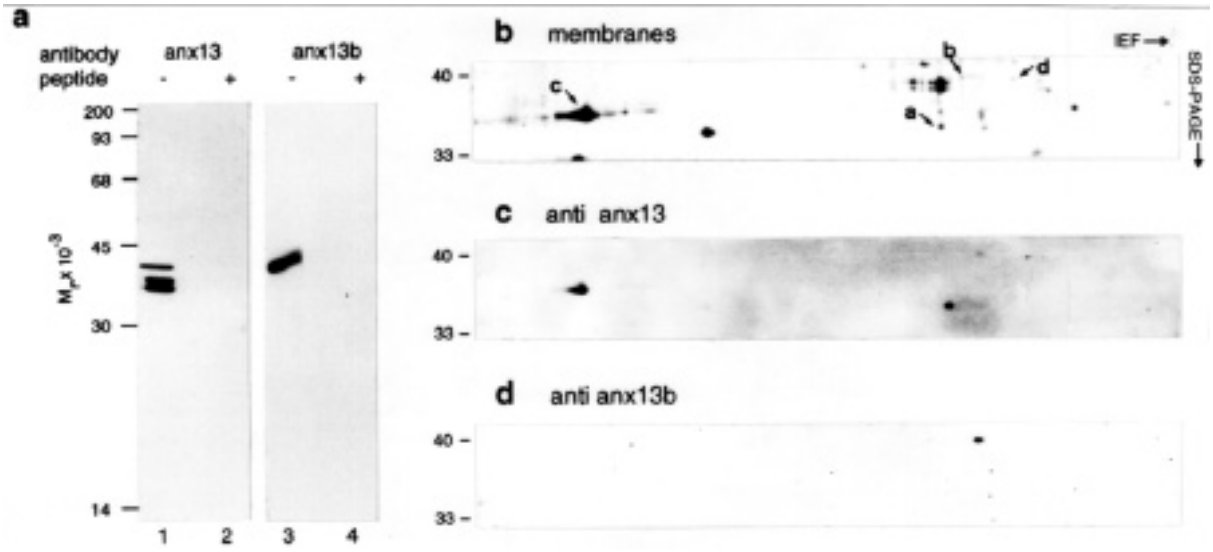

Figure 5: Annexin XIII isoforms in exocytotic carrier vesicles. (*a*) Specificity of the anti anx13 and anti anx13b antibodies. A Western blot of MDCK cell membranes was probed with affinity-purified anx13 (lanes 1, 2) and anx13b (lanes 3, 4) antisera in the absence (lanes 1, 3) or presence (lanes 2, 4) of 100 µg/ml of the respective peptide. (*b*) Autoradiogram of a Western blot of a mixture of MDCK cell membranes isolated from metabolically labelled and non-labelled cells. The Western blot was probed with affinity-purifed anx13 antisera (*c*), stripped and probed with affinity-purified anx13b antisera (*d*). The anx13b antibodies are specific for annexin XIIIb (spot b in *b*), the anx13 antibodies react with 4 proteins (spots a-d in *b*). Comparison with immunoisolated exocytic carrier vesicles [cf. Fig. 7 & 9 in Wandinger-Ness et al. (1990)] showed that only annexin XIIIb was enriched in apical exocytic carrier vesicles.

5). The anti anx13 antibody reacted with bands of 36 kDa, 37 kDa and 40 kDa apparent molecular weight (Fig. 5a, lane 1), whereas the anti anx13b antibody only recognized the 40 kDa band (Fig. 5a, lane 3). The reaction was specific since it could be inhibited by the addition of 100 µg/ml of the respective peptide (Fig. 5a, lane 2 and 4).

We then analyzed which of the proteins was present in exocytic carrier vesicles by im-

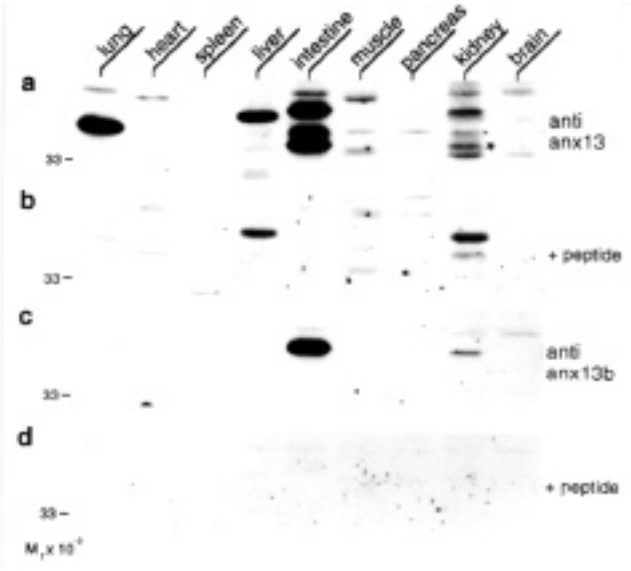

Figure 6: Western blot surveys of dog tissue homogenates for the presence of annexin XIII isoforms. Total cell lysates (10 µg/lane) were resolved by 13 % SDS-PAGE, transferred to nitrocellulose and probed with affinity-purified anx13 (*a* and *b*) or affinity-purified anx13b (*c* and *d*) antisera in the absence (*a* and *c*) or presence (*b* and *d*) of 100 µg/ml of the respective peptide. For clarity, the specifically reacting band of an apparent molecular weight of 36 kDa in kidney homogenate is labelled with a star in *a*.

munoblotting of a total cellular membrane fraction resolved on 2-D gels (Fig. 5b). The blot was reacted with anti anx13 antibodies (Fig. 5c), stripped and then incubated with anti anx13b antisera (Fig. 5d). Comparison with 2-D gels of immunoisolated exocytic carrier vesicles (cf. Fig. 7 & 9, Wandinger-Ness et al., 1990) demonstrated, that only annexin XIIIb was enriched in apical carrier vesicles. Proteins a and c were present in the crude vesicular fraction used as a starting material but were not specifically co-enriched with the viral glycoproteins after immunoisolation, protein d was not visible. According to the calculated molecular weight and isoelectric point, protein a most likely represents annexin XIIIa and corresponds to the 36 kDa band observed in SDS-PAGE (Fig. 5a), protein c corresponds to the 37 kDa band.
These data confirm that the isolated annexin XIIIb cDNA encodes the originally purified protein A23 and suggest that in addition to annexin XIIIa and XIIIb two further, immunologically related proteins c and d are present in MDCK cells. It cannot be excluded that proteins c and/or d are post-translationally modified forms of annexin XIIIa and/or annexin XIIIb.

Annexin XIIIb is Expressed in Intestine and Kidney

We analyzed the tissue distribution of annexin XIIIb *in vivo*. Annexin XIIIb was expressed in dog intestine and kidney (Fig. 6c). As expected, annexin XIIIa and also protein c were expressed in intestine (Fig. 6a). In contrast to the results reported by Wice and Gordon (1992)

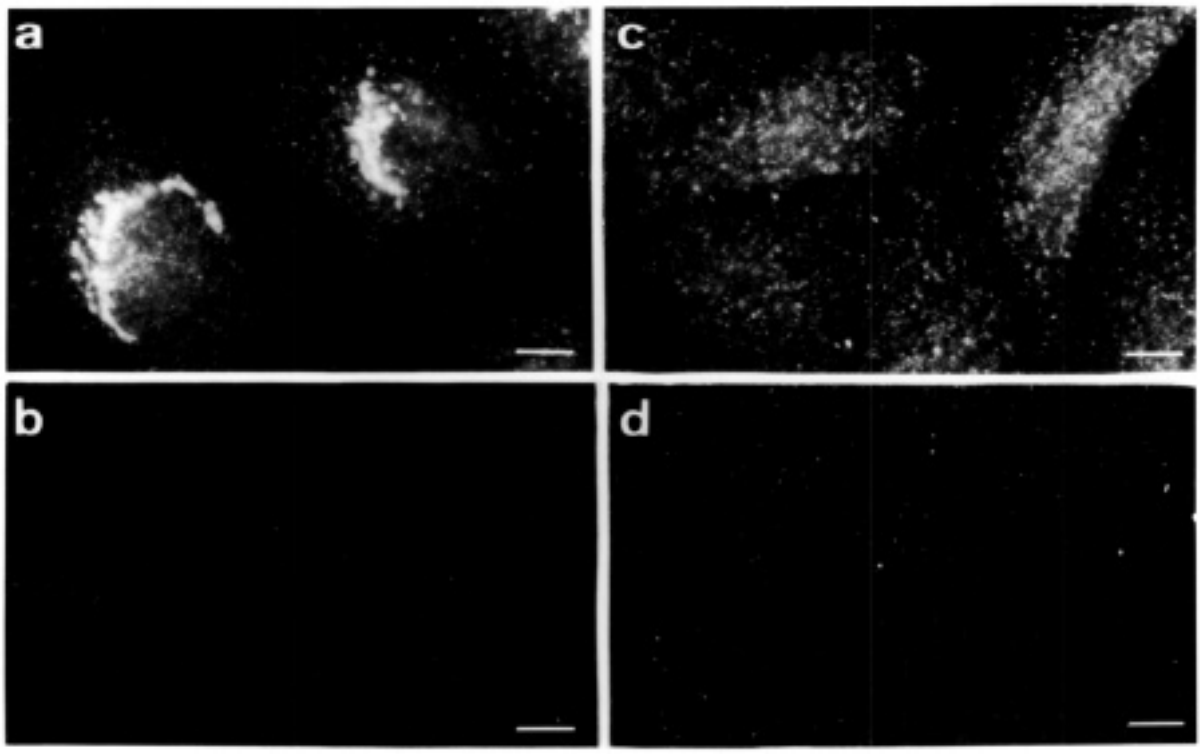

Figure 7: Immunofluorescence localization of annexin XIII isoforms. Subconfluent MDCK cells were permeabilized with 0.1 % Triton X-100 after fixation with 4 % paraformaldehyde. Immunostaining with affinity-purified anx13 (*a* and *b*) or affinity-purifed anx13b antisera (*c* and *d*) in the absence (*a* and *c*) of presence (*b* and *d*) of 50 µg/ml of the respective peptide. Bars, 8 µm.

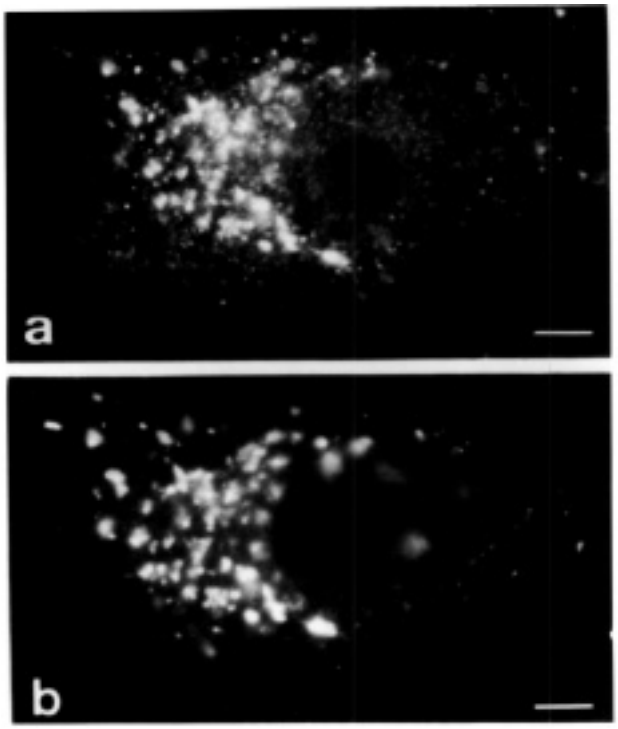

Figure 8: TGN localization of annexin XIII isoforms in MDCK cells. Labelling of the TGN with affinity-purifed anx13 antisera (*a*) co-localizes with influenza virus hemagglutinin (*b*) blocked in transport by incubation of the cells at 20 °C. Bars, 4 µm.

we found a low level of expression of annexin XIIIa in kidney as well (Fig. 6a, star). High quantities of protein c were expressed in lung (Fig. 6a). The reactions were specific since they could be inhibited by the addition of 100 µg/ml of the respective peptide (Fig. 6, b and d). In addition, the anti anx13b antibodies specifically reacted with proteins of an apparent molecular weight of ~20 kDa in pancreas and ~55 kDa in kidney and liver, and a protein of an apparent molecular weight of ~50 kDa was recognized by the anti anx13 antibodies in brain (not shown). This demonstrated that annexins XIIIa and XIIIb are exclusively expressed in intestine and kidney whereas the immunologically related proteins are restricted to tissues abundant in epithelial or polarized cells.

Cellular Localization of Annexin XIII

Annexin XIIIb was biochemically identified as a component of apical exocytic carrier vesicles. We determined its subcellular localization in MDCK cells by immunofluorescence and immunoelectron microscopy with affinity-purified antisera. Annexin XIIIb was labelled on punctate structures above the nucleus and throughout the cells in subconfluent, non-polarized MDCK cells (Fig. 7c). In addition to the punctate structures the anti anx13 antiserum strongly stained tubulo-vesicular structures close to the nucleus (Fig. 7a). The staining could be inhibited by addition of 50 µg/ml of the respective peptide (Fig. 7, b and d). We next analyzed the perinuclear structures in more detail. After infection of MDCK cells with the influenza virus WSN ts061, hemagglutinin (HA) transport was blocked in the TGN by incubation at 20 °C (Matlin

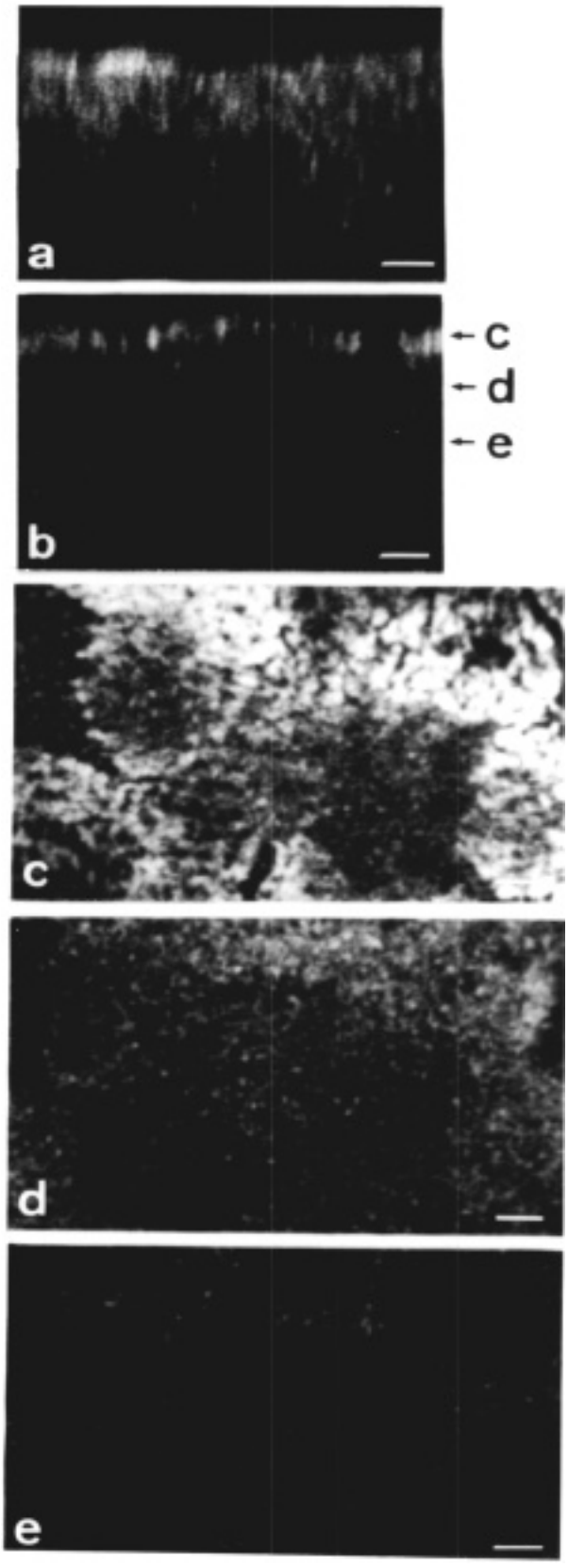

Figure 9: Confocal laser scan immunofluorescence of annexin XIII isoforms in filter-grown MDCK cells. Cells were grown on Transwell filters for 4 days, fixed with 4 % paraformaldehyde, permeabilized with 0.1 % Triton X-100 and denatured with 6 M Guanidine-HCl to reduce unspecific background staining. (*a*) X-Z view of cells stained with affinity-purified anx13 antiserum. (*b*) X-Z view and representative X-Y views (*c* - *e*) of cells stained with affinity-purified anx13b antibodies. The focal planes for *c, d* and *e* are indicated in *b*. Bars, 5 µm.

and Simons, 1983). In these cells, the perinuclear structures labelled with the anti anx13 antibody (Fig. 8a) almost entirely co-localized with HA (Fig. 8b).

To analyze the localization of annexin XIIIb in fully polarized MDCK cells we perfomed confocal laser scan microscopy. Lateral and apical structures were labelled with the anti anx13 antiserum as shown in an X-Z view in Fig. 9a. In contrast, annexin XIIIb was localized almost exclusively to the apical pole (X-Z view, Fig. 9b) and restricted to the uppper quarter of the cells. To reduce the background signal in immunofluorescence with filter-grown MDCK cells we used a guanidine-HCl denaturation procedure (Peränen et al., 1993). This might have influenced the overall resolution. Strong labelling was detected in X-Y views taken along the apical membrane (Fig. 9c). Punctate labelling was also detected in a focal plane from the upper quarter of the cells (Fig. 9d) but was strongly decreased or absent in the cell middle (Fig. 9e) and on the basolateral side (not shown).

The subcellular localization was confirmed by electron microscopy with polarized MDCK cells. Annexin XIIIb was exclu-

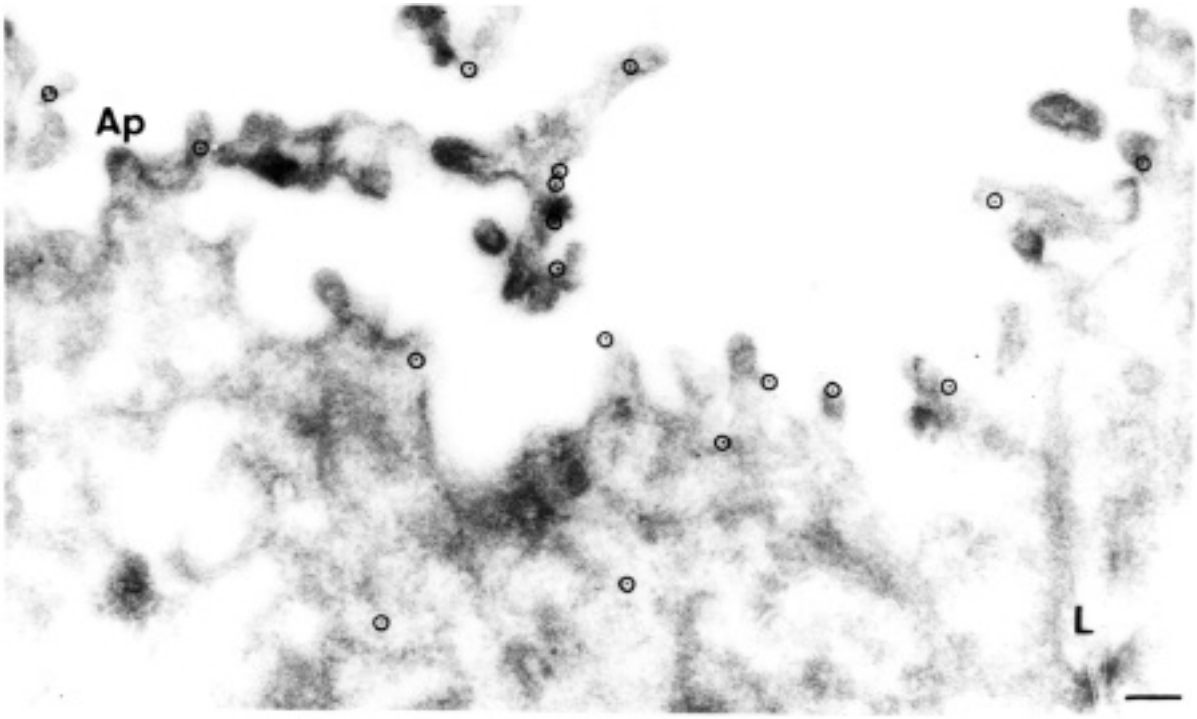

Figure 10: Immunoelectron microscopic localisation of annexin XIIIb in polarized MDCK cells. Thin frozen sections of MDCK cells grown on polycarbonate filters were incubated with affinity-purified anti-peptide antibodies to anx13b followed by protein A-gold. Some gold particles are circled. Significant labelling is associated with the apical plasma membrane (Ap). Lower labelling was found associated with the lateral membrane (L) and intracellular compartments. Bar, 200 nm.

sively localized to the apical plasma membrane and vesicular structures beneath (Fig. 10). In addition to these structures, the anti anx13 antibody labelled tubular/vesicular profiles beneath the apical surface, one side of the Golgi complex and the lateral plasma membrane (Fig. 11). Little cytosolic and filamentous staining was detected.

Annexin XIIIb and Transport to the Apical Plasma Membrane

To test the possible involvement of annexin XIIIb in delivery from the TGN to the plasma membrane we used an *in vitro* transport assay that reconstitutes vesicular transport in streptolysin O (SLO) permeabilized MDCK cells (Kobayashi et al., 1992; Pimplikar and Simons, 1993; Pimplikar et al., 1994).

The transport of vesicular stomatitis virus glycoprotein (VSV G-protein) and influenza virus hemagglutinin (HA) from the TGN to the basolateral and apical cell surface, respectively, was temperature, ATP and cytosol-dependent which allowed manipulation of the composition of exogenously added cytosol. Addition of the affinity-purified anti anx13b antibodies significantly inhibited the apical transport of influenza HA. The reduction was specific since the delivery of VSV-G protein to the basolateral plasma membrane was not affected (Fig. 12a). The dose dependence of the inhibition is shown in Fig. 12b. Even at very high antibody concentra-

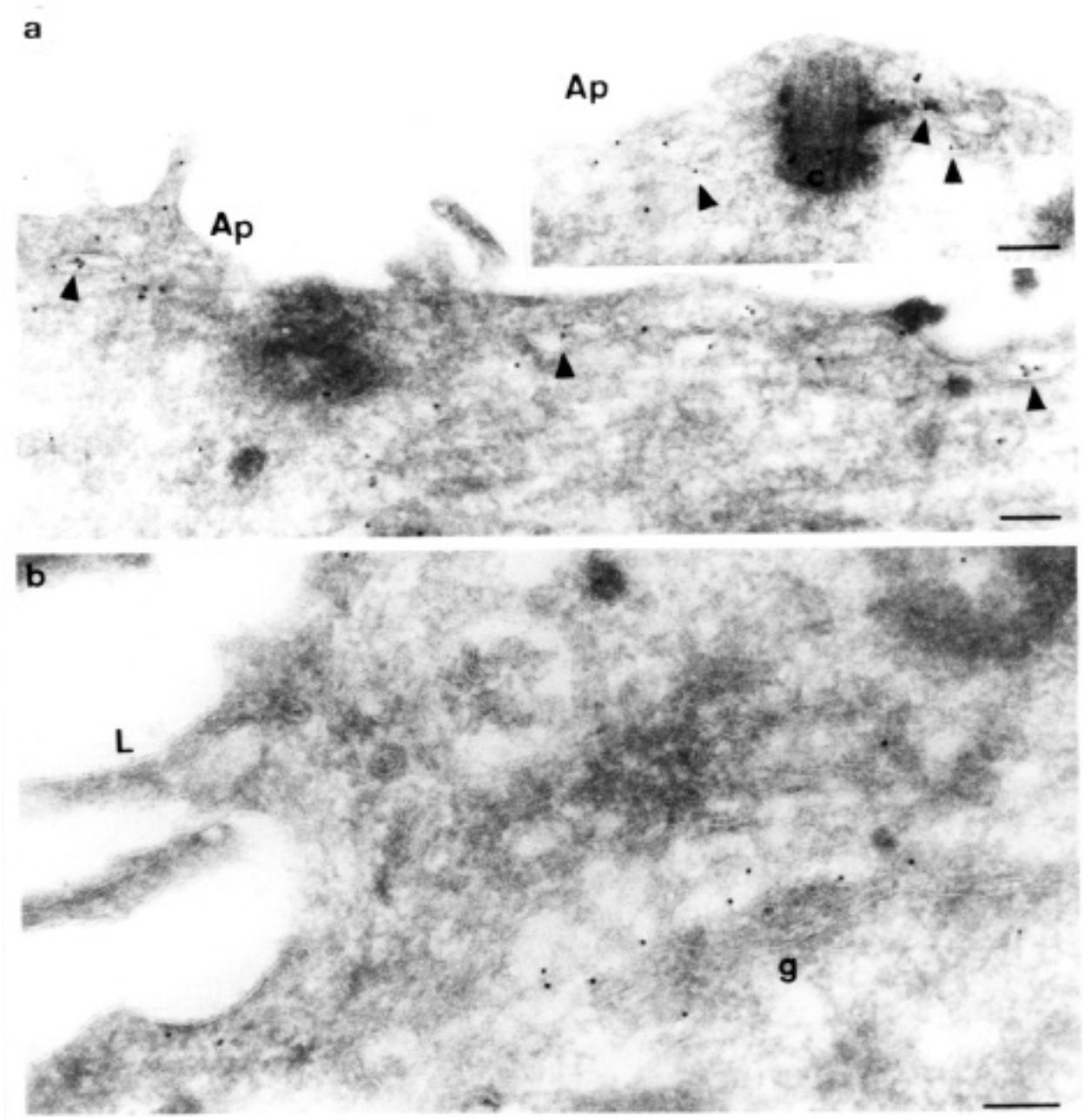

Figure 11: Immunoelectron microscopic localization of annexin XIII in polarized MDCK cells. Thin frozen sections of MDCK cells grown on polycarbonate filters were incubated with affinity-purified anti-peptide antibodies to anx13 followed by protein A-gold. In the apical region of the cell (panel *a*) labelling is evident on tubular/vesicular structures (arrowheads) underlying the apical plasma membrane (Ap). The inset shows a cluster of labelled structures close to a centriole (c). Panel *b* shows an area close to the lateral border (L). Labelling is evident close to the lateral plasma membrane and associated with one side of the Golgi complex (g). Bars, 200 nm.

tions basolateral delivery was only slightly affected. These data implicate annexin XIIIb in transport from the TGN to the apical cell surface.

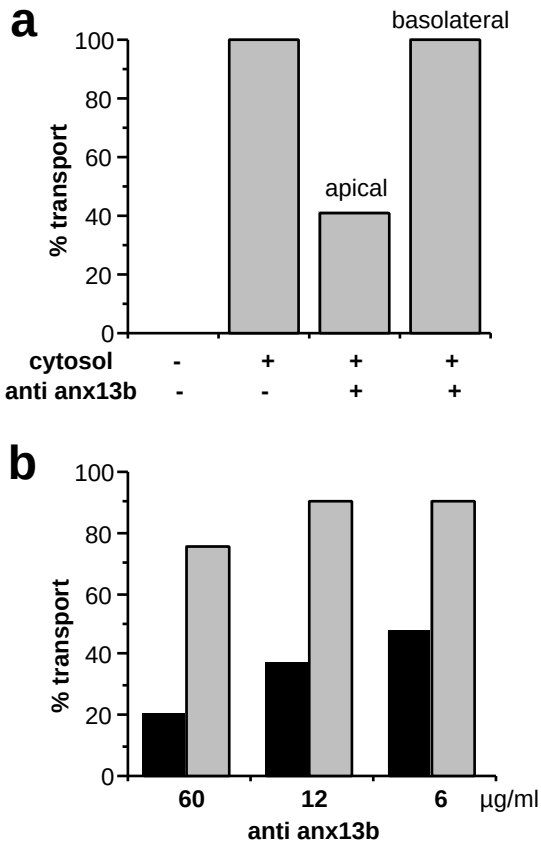

Figure 12: *In vitro* transport assay for annexin XIIIb. (*a*) MDCK cells grown on Transwell filters were permeabilized with streptolysin O from the apical or basolateral side and cytosol was added back with or without affinity-purified anti anx13b antibodies (~19 µg/ml final conc.). All samples were analyzed by 10 % SDS-PAGE, autoradiographed, scanned with a Phosphorimager (Molecular Dynamics) and the band intensities were calculated with Image Quant software. The values are means from two experiments. *In vitro* transport of influenza HA was performed on basolaterally permeabilized and cytosol-depleted MDCK cells. Arrival of HA at the plasma membrane was measured by its sensitivity to cleavage with trypsin. The % transport of HA was calculated as 2 x HA2 / (HA + 2 x HA2) x 100. The *in vitro* transport of VSV-G was performed on apically permeabilized, cytosol-depleted MDCK cells. The % of VSV-G transported to the basolateral surface was calculated as the fraction of VSV-G bound to protein A-agarose after surface immunoprecipitation with VSV-G specific antibodies (VSV-G bound / (VSV-G unbound + VSV-G bound) x 100). Cytosol dependent transport is presented as 100 % (transport in the presence of cytosol - transport in the absence of added cytosol). (*b*) Dose response to variable anti anx13b antibody concentrations. Apical transport (*filled columns*) and basolateral transport (*shaded columns*) was determined as described above.

Discussion

The cell surface of simple epithelial cells is differentiated into an apical and basolateral plasma membrane domain separated by tight junctions (Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989; Rodriguez-Boulan and Powell, 1992). The distinct protein and lipid composition of each domain is generated by sorting in the TGN (Griffiths and Simons, 1986). Not only the secretory pathways emerging from the Golgi apparatus but also the transcytotic pathways connecting the apical and basolateral cell surface might involve factors unique to epithelial cells to mediate sorting and to provide specificity in delivery and membrane fusion (Simons and Wandinger-Ness, 1990).

In our approach to characterize and identify proteins involved in the transport process we have previously used perforated MDCK cells to isolate apical and basolateral exocytic carrier vesicles (Bennett et al., 1988). Immunoisolation and separation by 2-D gels permitted the identification of components common to apical and basolateral carriers and proteins unique to the apical or basolateral pathway (Wandinger-Ness et al., 1990). Among putative factors that distinguish the apical and basolateral direction, to date, only the small GTPase rab8 has been found to be highly enriched in the basolateral pathway and to be involved in transport to the basolateral cell surface (Huber et al., 1993). No apical specific factors have been identified. We now report the purification of the component A23 which was 38-fold enriched in apical versus basolateral exo-

cytic carrier vesicles (Wandinger-Ness et al., 1990). Peptide microsequencing and the isolation of the encoding cDNA demonstrated that this protein, annexin XIIIb, is homologous to the previously described annexin XIIIa (intestine-specific annexin; Wice and Gordon, 1992) but contains a unique insert of 41 amino acids in the N-terminal domain.

Annexins are a large family of proteins that are characterized by 4 or 8 repeats of a ~70 amino acids domain with 17 highly conserved amino acids, termed the endonexin fold. A hallmark of the annexin family is their Ca^{2+}- and Ca^{2+}-dependent lipid-binding activity which may be directly related to their function (Crompton, 1988; Klee, 1988; Creutz, 1992; Moss, 1992). Annexins have been implicated in a number of processes including the metabolism of lipid-derived inositol-phosphates (Ross, 1990), the formation or modulation of ion channels (Pollard and Rojas, 1988; Diaz-Munoz et al., 1990; Kaetzel et al., 1994), the membrane attachment of the cytoskeleton (Gerke and Weber, 1984; Glenney et al., 1987), protein kinase C inhibition (Schlaepfer et al., 1992) and membrane-membrane interactions (Creutz et al., 1978; Drust and Creutz, 1988; Ali et al., 1989). In addition, in the extracellular medium annexins are proposed to function as anti-inflammatory agents and phospholipase A2 inhibitors (Goulding and Guyre, 1993; Flower and Rothwell, 1994) and as inhibitors of blood coagulation (Funakoshi et al., 1987). Whether all of these activities are physiological functions of annexins is still a matter of debate.

The involvement of annexins in membrane-membrane interactions has been well documented and originally stems from studies on annexin VII (synexin). Annexin VII was found to promote aggregation of secretory granules in a Ca^{2+}-dependent fashion (Creutz et al., 1978). Granule fusion required arachidonic acid or other cis-unsaturated fatty acids as co-factors (Creutz, 1981) and was suggested to be mediated by annexin VII providing a hydrophobic bridge for the flow of lipids between membrane bilayers in the fusion process (Pollard et al., 1992). More recently annexin II was shown to be directly involved in Ca^{2+}-dependent exocytosis in chromaffin cells (Ali et al., 1989). In addition, annexin II was found to be a major component of endosomes (Emans et al., 1993) and to regulate endosome distribution in MDCK cells (Harder and Gerke, 1993). Annexin I is a substrate for the EGF-receptor kinase (Fava and Cohen, 1984), was localized to late endosomes and might be involved in multivesicular body formation (Futter et al., 1993). Finally, annexin VI was demonstrated to be involved in the budding of clathrin-coated vesicles (Lin et al., 1992) but is not generally required in all cell types (Smythe et al., 1994).

Using antisera raised against an N-terminal peptide unique to annexin XIIIb and an N-terminal peptide common to annexin XIIIa and XIIIb, expression of annexins XIIIa and XIIIb was exclusively found in dog intestine and kidney. Furthermore, the immunologically related protein c was expressed in MDCK cells, intestine and lung confirming that annexin XIII isoforms are only expressed in tissues abundant in epithelial or polarized cells. We have no evidence for the presence of cDNAs in addition to those encoding annexin XIIIa and XIIIb in MDCK cells.

Annexins have been localized to a large number of membranes in the endomembrane system (Burgoyne and Geisow, 1989). Using an antibody raised against a consensus annexin peptide (Gerke, 1989) members of the annexin family were recently localized to the nuclear envelope, early and late endosomes, lysosomes, the Golgi and the plasma membrane in BHK cells (Gruenberg and Emans, 1993). By immunofluorescence and electron microscopy annexin XIIIb was exclusively localized to the apical cell surface and vesicular structures in MDCK cells. Annexin IV is also preferentially expressed in tissues abundant in epithelial cells (Kaetzel et al., 1989) and was localized to the apical cell surface in renal cells and epithelial cells of the uterus (Kojima et al., 1994; Kaetzel et al., 1994) but found at the basolateral cell surface in enterocytes and hepatocytes (Massey et al., 1991a, 1991b). Since annexin IV shows a similar tissue distribution to the annexin XIII isoforms and immunologically related proteins but contains only a very short N-terminal domain we wondered whether the C-terminal domains of annexins IV and XIII would show any unique features that would distinguish them from other annexins. This was, however, not indicated by the comparison of representatives of all known annexin family members which showed that annexins IV and XIII could not be subgrouped into a distinct phylogenetic branch of the annexin family, i.e. they do not show any closer primary amino acid sequence similarity to each other as compared to other annexins.

Annexin XIIIa or the immunologically related proteins were localized to the TGN, endosomes, the plasma membrane, and tubular/vesicular structures beneath the apical surface in MDCK cells. Only annexin XIIIb but not the other annexin XIII isoforms were found to be relatively enriched in apical exocytic carrier vesicles. Therefore it seems likely that the N-terminal 41 amino acids of annexin XIIIb provide the specificity for the localization to the apical plasma membrane and association with apical exocytic carrier vesicles by interaction with other components and/or modulation of the C-terminal domain common to annexin XIIIb and XIIIa. Our results are fully consistent with the earlier localization of annexin XIII by immunofluorescence which did, however, not allow discrimination between annexin XIIIb and the other annexin XIII isoforms (Wice and Gordon, 1992). A possible interaction of annexin XIIIb with VIP21 (Kurzchalia et al., 1992) and VIP36 (Fiedler et al., 1994), previously characterized as components common to apical and basolateral exocytic carrier vesicles, has so far not been investigated.

Annexin XIIIb behaved as a membrane protein in phase partitioning in Triton X-114 (in the absence of added Ca^{2+}; Wandinger-Ness et al., 1990) which is not usually observed for annexins. In this respect it is interesting to note that a feature unique to annexin XIIIa among other annexin family members is its N-terminal myristoylation (Wice and Gordon, 1992). Wice and Gordon reported a protein immunologically related to annexin XIIIa with an apparent molecular weight of 42 kDa which was also myristoylated. Since annexin XIIIb contains a potential N-terminal myristoylation site the 42 kDa protein most likely represents annexin XIIIb.

What is the function of annexin XIIIb? The specific inhibition of transport of influenza HA from the TGN to the apical plasma membrane by the addition of antibodies against annexin

XIIIb to the *in vitro* transport assay, suggests that annexin XIIIb is involved in this delivery process. For annexin XIIIb this could principally involve a dissociation-association cycle with apical exocytic carrier vesicles. Alternatively, a recycling from the apical cell surface back to the TGN (Brändli and Simons, 1989) and a rapid inclusion into newly formed carrier vesicles would explain the negligible steady-state level of annexin XIIIb observed on the TGN. The antibodies, specifically binding to part of the 41 N-terminal amino acids unique to annexin XIIIb, probably exert their effect by preventing the interaction of annexin XIIIb with a putative receptor on the vesicular surface and hence the binding to the vesicles. Alternatively, they may sterically block the interaction of annexin XIIIb with other essential components involved in membrane-membrane interactions in vesicle budding, docking or fusion such as NSF, SNAPs and SNAREs (Rothman and Orci, 1992; Söllner et al., 1993; Rothman and Warren, 1994) or rab GTPases (Bourne, 1988; Zerial and Stenmark, 1993; Novick and Brennwald, 1993). The observed lack of complete inhibition might be due to the inaccessibility of annexin XIIIb already bound to the carrier vesicles or could result from part of the delivered HA having already passed the site of action of annexin XIIIb in the transport process. Clearly, more work is necessary to demonstrate the exact function of annexin XIIIb and it will be interesting to see how annexin-mediated membrane-membrane interactions play a role in apical transport. The integration of all vesicular components into a coherent mechanistic scheme remains a challenge. Although NSF has been implicated in a number of membrane fusion events it is possible that NSF- or SNARE-independent membrane docking and fusion processes exist that might be mediated by annexins. To date, no apical specific SNAREs or rab proteins have been identified in epithelial cells.

Intriguingly, annexin XIII isoforms were localized to all compartments connected by vesicular transport pathways potentially unique to epithelial cells, namely the apical cell surface, endosomes, the TGN and lateral plasma membrane. Whether annexin XIIIa and the immunologically related proteins perform functions in recycling from the apical cell surface to the TGN and transcytosis between the apical and basolateral plasma membranes remains to be shown in the future. For now, the involvement of annexin XIIIb in transport to the apical plasma membrane in MDCK cells will allow us to gain access to other epithelial or apical specific components of the vesicular transport machinery.

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