Annexin XIIIb: A Novel Epithelial Specific Annexin Is Implicated in Vesicular Traffic to the Apical Plasma Membrane

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Abstract. The sorting of apical and basolateral proteins into vesicular carriers takes place in the trans-Golgi network (TGN) in MDCK cells. We have previously analyzed the protein composition of immunopurified apical and basolateral transport vesicles and have now identified a component that is highly enriched in apical vesicles. 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 J. I. Gordon. 1992. J. Cell Biol. 116:405-422). Annexin XIIIb differs from annexin XIIIa in that it contains a unique insert of 41 amino acids in the NH2 terminus and is exclusively expressed in dog intestine and kidney. Immunofluorescence microscopy demonstrated that annexin XIIIb was localized to the apical plasma membrane and underlying punctate structures. Since annexins have been suggested to play a role in membrane-membrane interactions in exocytosis and endocytosis, we investigated whether annexin XIIIb is involved in delivery to the apical cell surface. To this aim 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 vesicular transport to the apical plasma membrane in MDCK cells.

The cellular endomembrane system is connected by vesicular transport routes that shuttle cargo between donor and acceptor compartments. Most of the known traffic pathways are now well delineated (Simons and Zerial, 1993) as are some of the molecular mechanisms that are involved in vesicle budding and specificity of vesicle docking and fusion (Pryer et al., 1992; Bennett and Scheller, 1993; Rothman, 1994). The GTP-binding protein ADP-Ribosylation Factor (ARF) and coatamer have been demonstrated to be required for budding of intra-Golgi derived vesicles (Orch et al., 1993; Ostermann et al., 1993). The generation of endoplasmic reticulum derived vesicles in yeast requires the GTP-binding protein, Sarlp, the guanine nucleotide exchange factor SEC12, and a distinct set of coat proteins (Barlowe and Schekman, 1993; Barlowe et al., 1994). The mammalian rab proteins and their yeast homologues are implicated in conferring directionality of transport (Zerial and Stenmark, 1993; Nuoffer and Balch, 1994). N-ethylmaleimide sensitive factor (NSF) and soluble NSF attachment proteins (SNAPs) and their yeast counterparts have been shown to participate in the final fusion events (Rothman, 1994). Syntaxin, synaptobrevin, synaptotagmin, and synaptoosomal-associated protein of 25 kD (SNAP-25) are involved in the docking and fusion of synaptic vesicles with the nerve terminal (Bennett and Scheller, 1993; Südhof et al., 1993). According to the SNAF (SNAP receptor) hypothesis, families of syntaxin and synaptobrevin homologues provide the specificity in vesicle docking before fusion (Götzler et al., 1993; Rothman, 1994) and complexes of SNAFes may represent the targets of regulation by members of the rab family of GTPases (Sogaard et al., 1994; Brennwald et al., 1994). Another class of proteins, the annexin family members, have a Ca2+-dependent lipid-binding activity and have also been implicated in membrane-membrane interactions such as those involved in vesicle docking, budding, or fusion (Creutz, 1992; Gruenberg and Emans, 1993).

The mechanisms responsible for membrane bilayer fusion 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)
Materials 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 donkey anti-rabbit rhodamine-conjugated and donkey anti-mouse FHC-conjugated antibodies were purchased from Dianova (Hamburg, Germany), the rabbit anti-mouse IgG and deoxycholate were from Sigma (Deisenhofen, Germany), and protein A-Sepharose was from Pharmacia (Freiburg, Germany).

Cell Culture, Viral Infection, and Immunolocalization

Growth media compositions, MDCK II cells, viruses, cell culture protocols, and immunolocalization of exocytic carrier vesicles from perforated MDCK II cells were as described previously by Wandinger-Ness et al. (1990).

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). Whenever indicated, the BioRad (Hercules, CA) Mini-Protean II 2-D cell was used instead according to the manufacturer's recommendations, except for the IEF tube gel composition that corresponded to the gel mixture of Bravo (1984). Preparative amounts of proteins were resolved using the Millipore Investigator IEF first dimension. After electrophoresis the gels were fixed in 45% methanol and 7% acetic acid and stains with Coomassie blue or treated for fluorography using Entensify (Dupont, Brussels, Belgium).

Isolation of Annexins

A light cellular membrane fraction was prepared from metabolically labeled or nonlabeled MDCK cells as previously described (Fiedler et al., 1990) 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. After heating to 97°C for 4 min the sample was cooled to room temperature, 150 mg urea, 25 μl 80% NP-40 and 12.5 μl amphotericin B-79 were added and the volume was made up to 250 μl with H2O. The urea was dissolved at 37°C and the sample was spun for 5 min at 37°C in the Espendorf centrifuge before loading.

Amino Acid Sequence Analysis

Coomassie blue-stained spots of annexin XIIIb, annexin II, or keratin (<2 μg each) were excised from four 2-D gels and pooled. After washing with water, the gel pieces were lypophylized and rehydrated in 100 μl of 100 mM NH4HCO3, 0.5 mM CaCl2 containing 1 μg trypsin. After digestion (37°C for 12 h) peptide fragments were extracted from the gel slice with 100 μl of 70% trifluoroacetic acid/0.01% Tween 20 and 100 μl of 50% trifluoroacetic acid/0.01% Tween 20 (modified from Sturrock and Collins, 1993). The combined fractions were concentrated and subjected to reverse-phase-HPLC using Vydac 218TP (2.1 × 250 mm). Automated Edman degradation of peptides was performed using a sequencer (model 477A) and data set on-line PTH-analyzer (Appl. Biosystems, Inc., Foster City, CA; model 120).

Molecular Cloning of Annexin XIIIb

Total RNA was prepared from confluent MDCK cells using the guanidine hydrochloride procedure (Chirgvin et al., 1979). MDCK cDNA was 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′-CCGGGATTCTCGAATATTGGA(A/C/G/T)AA(C/T)AA(A/C/G/T) C(A/C/T)3′ and 5′-CGCCCTCGAG(A/G)TT(A/G)AAIGC(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)TG(A/G)TC 3′. Of the 726-bp and 603-bp fragments obtained, the 726-bp fragment was used for screening of a λZAP II MDCK cDNA library (Chavrier et al., 1990). Duplicate nitrocellulose filters were prehybridized for 2 h at 42°C in 5 × SSC, 0.5% formamide, 5 × Denhardt's solution, 1% SDS (Sambrook et al., 1989). Hybridization was carried out in the same solution, supplemented with 32P-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, Upplands, Sweden).

Computer Sequence Analysis

Basic sequence analysis was carried out with the GCG programs (Wisconsin Package, Genetics Computer Group, Madison, WI) (Devereux et al., 1984). MPetch (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 posttranslational 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 NH2-terminal domains. The tree was corrected for multiple substitutions, analyzed by bootstrapping and visualized with Phylop (Felsenstein, 1993).

Preparation of Antibodies, Immunoprecipitation, and Immunoblotting

Polyclonal sera were raised against synthetic peptides covalently coupled to keyhole limpet haemocyanin using residues 20-36 of annexin XIIIb and the mAbs sample 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). Mock affinity-purified antibodies from preimmune serum were prepared identically using the anx13b column.

For immunoprecipitation a pelleted, light cellular membrane fraction (Kurzchalia et al., 1992; Fiedler et al., 1993) or immunosolated apical exocytic carrier vesicles (Wandinger-Ness et al., 1990) from metabolically labeled cells were used. 600 µl of 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 150 mM NaCl, 1% NP-40, 0.4% Deoxycholate, 0.4% SDS including 1 mg/ml BSA and protease inhibitors (buffer A) was added to the samples. The immunosolated membranes had previously been solubilized in 2-D gel lysis buffer and thus contained additional 150 mM urea, 0.06% NP-40, 0.03% ampholines, pH 7-9, and 1.5 mM DTT. Insoluble material was removed by centrifugation in the Eppendorf centrifuge. After overnight incubation at 4°C in the presence of affinity-purified annex13b antibodies, immune complexes were bound to protein A-Sepharose (preblocked with BSA and nonlabeled MDCK cell lysate) for 1 h at 4°C. The protein A-Sepharose was washed four times with buffer A (SDS increased to 0.8% final concentration) and once with PBS before addition of 2-D gel lysis buffer and resolution with the BioRad Mini 2-D cell.

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, Rockford, IL) 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 a 1:40 dilution of the affinity-purified anti-anx13b antibodies overnight at 40°C, and then in 1:3,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (BioRad) as the second antibody for 1 h at room temperature. As a blocking solution, 5% nonfat dried milk, 0.2% Tween-20 (Sigma) was used. Bands were detected using ECL (Amersham, Braunschweig, Germany).

**Immunofluorescence Microscopy**

Cells grown on coverslips and on polycarbonate filters were washed with 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 labeling 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 NH4Cl in PBS for 20 min. After an additional 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-anx13b antisera was used at a dilution of 1:5. The cells were incubated for 30 min at 37°C and washed with PBS. Primary antibodies were visualized with preadsorbed donkey anti-rabbit rhodamine-conjugated or anti-mouse FITC-conjugated antibodies. The coverslips were viewed and photographed with an Axioskop photomicroscope (Carl Zeiss, Oberkochen, Germany) or with the EMBL confocal microscope and photographed with a Polaroid Freeze frame directly from the monitor.

**In Vitro Transport of HA and Vesicular Stomatitis Virus Glycoprotein 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 exactly as previously outlined (Huber et al., 1993; Pimplikar et al., 1994). Streptolysin O (SLO) was a generous gift of S. Bhakdi, University of Mainz, Germany. The transport assay was performed in triplicates without or with exogenously added cytosol (HeLa cytosol, 8 mg/ml) in the absence or presence of 60 nM or 600 nM of affinity-purified anti-anx13b antibody (fixed concentration). The anxi13b peptide was used at a concentration of 20 µM. The amount of mock affinity-purified preimmune serum used was equivalent to a concentration of 600 nM of affinity-purified antibodies.

**Results**

**Purification of Annexin XIIIb**

The apical and basolateral transport vesicles immunosolated from MDCK cells contain both common and unique components (Wandinger-Ness et al., 1990). The component that was most enriched in the apical vesicles was A23 (Fig. 1, spot b; 38-fold enriched in apical vs basolateral carriers). This protein, as well as one other apical protein, was classified to be a membrane protein by Triton X-114 phase partitioning. Based on these criteria we selected A23 for further studies and first wanted 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. The identity of A23 was verified by comparison of this membrane fraction (Fig. 1 b) with immunosolated apical vesicles (Fig. 1 a) by analytical 2-D gels. This demonstrated that A23 and the protein marked b in the membrane fraction had an identical isoelectric point and apparent molecular weight. Moreover, no other protein was overlapping with or present in its immediate vicinity in the membrane fraction, suggesting that A23 and this protein were identical (see Fig. 6 for further confirmation). For protein isolation membranes
were solubilized with SDS and resolved by preparative two-dimensional (2-D) gel electrophoresis (Fig. 2). The identity of the Coomassie blue-stained protein with the protein identified on analytical gels was confirmed by comigration (not shown). Coomassie blue-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. 3, 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 found in annexin XIIIa (intestine-specific annexin) described by Wice and Gordon (1992). However, A23 had an apparent molecular mass of 40 kD in SDS-PAGE and not 36 kD, 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 protein with an apparent molecular mass of 42 kD that cross-reacted with an antibody raised against an NH₂-terminal peptide of annexin XIIIa. They further showed that this component had a 41-amino acid insert five amino acids from the NH₂ terminus of annexin XIIIa (Gordon, J., personal communication). We therefore reasoned that the NH₂ termini of annexin XIIIa and A23 would be conserved and simplified by our PCR-based approach to obtain a partial cDNA encoding A23. Two degenerate oligonucleotides encoding the NH₂-terminal peptide MGNRH of annexin XIIIa and part of the peptide WGTDELAFNEVLAK 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 16 clones further analyzed, 12 gave rise to a 603-bp fragment by PCR using the aforementioned primers while four 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 1-1800 (Fig. 3) but was lacking nucleotides 77-199 (shaded box). The longer cDNA only lacked nucleotides 1-40 at the 5' end but contained the insert.
of nucleotides 77-199 (Fig. 3, shaded box). We shall refer to these as annexin XIIIa and annexin XIIIb, respectively.

The calculated molecular weights and isoelectric points of the encoded proteins were 39,606 Da and pI 5.2 for annexin XIIIb which was in good agreement with the values observed for A23 on 2-D gels, and 35,479 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. 4a). 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 NH2-terminal domain which might be generated by alternative splicing of the mRNA. A comparison of the variable NH2-terminal domains of annexins I-XI is shown in Fig. 4b. The NH2 terminus of annexin XIIIb does not show any significant similarity to the other family members. A search in the Prosite library (Bairoch, 1991) revealed that annexin XIIIb contains several potential phosphorylation sites for the terminal domain which might be generated by alternative splicing of the mRNA. A comparison of the variable NH2-terminal domains and the beginning of the first conserved repeating domain is shown in Fig. 4b. For the alignment the sequences of human annexins I-VIII (H), annexins XIa and XIb (B), Drosophila melanogaster annexin (D), Hydra vulgaris annexin (HY), and canine annexin XIII (C) were used. The 5'-end of the Drosophila melanogaster annexin IX CDNA has not been isolated.

**Annexin XIIIb Is Present in Apical Carrier Vesicles**

To further analyze annexin XIIIb, we raised polyclonal antiserum against an NH2-terminal peptide common to annexins XIII and annexin X (Anx13) and antiserum against an NH2-terminal peptide unique to annexin XIII (Anx13b). The affinity-purified anti-annexin antibodies were used to immunoprecipitate annexin XIIIb. The antibody was specific and reacted only with one protein of the correct isoelectric point and apparent molecular weight (Fig. 6a). For the immunoprecipitation, the MDCK membrane fraction served as a starting material (Fig. 6b). The antibody was specific and reacted only with one protein of the correct isoelectric point and apparent molecular weight (Fig. 6c). To identify annexin XIIIb in the membrane fraction the immunoprecipitate was mixed with the membranes (Fig. 6d). This demonstrated that the antibody recognizes the originally purified protein A23 (Figs. 1 and 2). The slightly different mobilities of some proteins in the second dimension of the 2-D gels in Fig. 6 compared to Figs. 1 and 2 is due to the different gel system used.

To further confirm the presence of annexin XIIIb in apical carrier vesicles, we used immunospecific apical vesicles (Wandinger-Ness et al., 1990) for the immunoprecipitation.

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**Figure 4. 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 NH2-terminal domains of representatives of all known annexins. Gaps are represented by dots that are inserted between the variable NH2-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 (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.

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**Figure 5. 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. 4b) spanning residues 47-357 of annexin XIIIb and the corresponding residues of the aligned sequences. The comparison excluded the variable NH2-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 labeled with a circle.
Annexin XIIIb is present in immunosolated apical carrier vesicles. (a) An affinity-purified anti-peptide (anx13b) antibody directed against annexin XIIIb was used to immunoprecipitate annexin XIIIb from a membrane fraction from metabolically labeled MDCK cells (obtained as in Fig. 1 b). (b) Membrane fraction. (c) Mixture of immunoprecipitated annexin XIIIb (a) with the membrane fraction (b). (d) Annexin XIIIb immunoprecipitated from immunosolated apical carrier vesicles (obtained as in Fig. 1 a). Due to the limited amount of immunosolated carrier vesicles available, all samples were resolved with the BioRad Mini 2-D cell by IEF and 12% SDS-PAGE. This resulted in slightly different mobilities of some proteins in the second dimension compared to Figs. 1 and 2. Annexin XIIIb is labeled with an arrowhead in b and c.

The immunoprecipitated protein (Fig. 6 d) can be perfectly aligned with annexin XIIIb from the MDCK membrane fraction. Since no other protein is present in the immediate vicinity of A23 in apical vesicles, this unequivocally demonstrates the presence of annexin XIIIb in apical exocytic carrier vesicles.

We next used the affinity-purified anti-anx13 antibodies for Western blotting of the MDCK membrane fraction (data not shown). In addition to annexin XIIIa and annexin XIIIb (Fig. 1, spots a and b) the antibody cross-reacted with two further proteins (Fig. 1, spots c and d). To determine their identity they were purified from Coomassie blue-stained 2-D gels, enzymatically digested in the gel slice, and analyzed by microsequencing. Protein c gave rise to a fragment corresponding to residues 178-195 of bovine annexin II, protein d gave rise to a fragment corresponding to residues 149-157 of human keratin 18. Neither annexin XIIIa nor annexin II, a very abundant MDCK protein localized to endosomes as well as to the apical and basolateral plasma membrane (Harder and Gerke, 1993; Parton, R. G., unpublished), was present in the immunosolated apical vesicles (Fig. 1). This further illustrates the high specificity of the immunosolation and excludes the possibility that the immunosolated annexin XIIIb is derived from a plasma membrane contamination.

Annexin XIIIb Is Expressed in Intestine and Kidney

We next analyzed the tissue distribution of annexin XIIIb by Western blotting of dog tissue homogenates. This showed that annexin XIIIb was exclusively expressed in dog intestine and kidney (Fig. 7 a). The reactions were specific since they could be inhibited by the addition of 100 μg/ml of the respective peptide (Fig. 7 b). In addition, the anti-anx13b antibodies reacted with proteins of an apparent molecular mass of ∼20 kD in pancreas and ∼55 kD in kidney and liver, the identity of which remains unknown. Neither of these proteins was detected in MDCK cells (not shown) and they may thus represent abundant cross-reacting proteins only present in the tissue homogenates.

Figure 7. Western blot surveys of dog tissue homogenates for the presence of annexin XIIIb. Total cell lysates (10 μg/lane) were resolved by 13% SDS-PAGE, transferred to nitrocellulose and probed with affinity-purified anti-anx13b antibodies in the absence (a) or presence (b) of 100 μg/ml of anx13b peptide.
Figure 8. Immunofluorescence localization of annexin XIIIb. Subconfluent MDCK cells were permeabilized with 0.1% Triton X-100 after fixation with 4% paraformaldehyde. Immunostaining with affinity-purified anti-annx13b antibodies in the absence (a) or presence (b) of 50 μg/ml of anxl3b peptide. Bars, 8 μm.

Cellular Localization of Annexin XIIIb

Since annexin XIIIb was biochemically identified as a component of apical exocytic carrier vesicles, we determined its subcellular localization in MDCK cells by immunofluorescence microscopy with affinity-purified antisera. Annexin XIIIb was labeled on punctate structures above the nucleus and throughout the cells in subconfluent, non-polarized MDCK cells (Fig. 8 a). This labeling could be inhibited by addition of 50 μg/ml of the anxl3b peptide (Fig. 8 b). To analyze the localization of annexin XIIIb in fully polarized MDCK cells, we performed confocal microscopy. Annexin XIIIb was localized almost exclusively to the apical pole and restricted to the upper quarter of the cells. Strong labeling was detected in X-Y views taken along the apical membrane (Fig. 9 a). Punctate labeling was also detected in focal planes through the upper quarter of the cells (Fig. 9 b) but was strongly decreased or absent in the cell middle (Fig. 9 c), in the lower quarter of the cells (Fig. 9 d), and on the basolateral side (Fig. 9 e).

Figure 9. Localization of annexin XIIIb in filter-grown MDCK cells by confocal microscopy. Cells were grown on Transwell filters for 4 d, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and denatured with 6 M Guanidine-HCl to reduce unspecific background staining (Peränen et al., 1993). X-Y views (a–e) of cells labeled with affinity-purified anti-annx13b antibodies. The focal planes are (a) apical, (b) upper cell quarter, (c) cell middle, (d) lower cell quarter, and (e) basolateral. Bars, 5 μm.
Annexin XIIib and Transport to the Apical Plasma Membrane

To test the possible involvement of annexin XIIib in the delivery from the TGN to the plasma membrane we used an in vitro transport assay that reconstitutes vesicular transport in SLO permeabilized MDCK cells (Kobayashi et al., 1992; Pimplikar and Simons, 1993; Pimplikar et al., 1994). The transport of both, the vesicular stomatitis virus glycoprotein (VSV-G protein) from the TGN to the basolateral cell surface as well as of influenza hemagglutinin (HA) to the apical cell surface, has been shown to be temperature-, ATP-, and cytosol-dependent. Addition of the affinity-purified anti-anx13b antibodies to the exogenously added cytosol preparation significantly inhibited the apical transport of influenza HA in a dose-dependent fashion (Fig. 10 a). This result was reproducibly obtained with different preparations of affinity-purified antisera. The reduction was specific since the delivery of VSV-G protein to the basolateral plasma membrane was not, or only slightly affected. Moreover, the inhibition of apical transport was abolished by the addition of anti-anx13b antibodies together with anx13b peptide (Fig. 10 b). Neither the addition of preimmune serum (Fig. 10 b), nor the addition of unspecific IgGs (not shown) at equivalent concentrations showed any effect on apical transport. These data implicate a role for annexin XIIib in vesicular delivery from the TGN to the apical cell surface.

Discussion

The cell surface of simple epithelial cells is differentiated into an apical and basolateral plasma membrane domain that are separated by tight junctions (Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989; Rodriguez-Boulan and Powell, 1992). The different protein and lipid composition of each domain is generated by the sorting of components into distinct classes of vesicles in the TGN (Wandinger-Ness et al., 1990). Both the apical pathway and also the transcytotic pathway connecting the apical and basolateral cell surface are likely to involve factors that are 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). Immunolocalization 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 almost forty times enriched in apical, as compared to basolateral 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 NH2-terminal domain. Annexins are a large family of proteins that are characterized by 4 or 8 repeats of an ~70-amino acid domain with 17 highly conserved amino acids, termed the endonexin fold. A hallmark of the annexin family is their Ca2+-dependent lipid-binding activity which may be directly related to their...
function (Crompton, 1988; Creutz, 1992; Moss, 1992). Annexins have been implicated in a number of processes including the metabolism of lipid-derived inositol-phosphates, the formation or modulation of ion channels, the membrane attachment of the cytoskeleton, protein kinase C inhibition and membrane–membrane interactions. Surprisingly, annexins have also been proposed to have extracellular functions as anti-inflammatory agents, phospholipase A2 inhibitors, and inhibitors of blood coagulation and to be involved in cell–matrix interactions (Raynal and Pollard, 1994). Whether all of these activities are physiological functions of annexins remains to be investigated.

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
dependent fashion (Creutz et al., 1978). Granule fusion required arachidonic acid or other cis-unsaturated fatty acids as cofactors (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
dependent exocytosis in chromaffin cells (Ali et al., 1989). Annexin II was also found to be a major component of endosomes as well as of plasma membrane (Emans et al., 1993) and to regulate endosome distribution in MDCK cells (Harder and Gerke, 1993). A role for annexin II in basolateral to canalicular transcytosis in hepatocytes was suggested based on the finding that its subcellular distribution relocates from basolateral to perinuclear and finally to apical concomitant with the transcytosis of cholestatic bile salts (Wilton et al., 1994). Annexin I has been localized to late endosomes and was postulated to be involved in multivesicular body formation (Putter et al., 1993). Evidence has also been provided suggesting that annexin VI is involved in the budding of clathrin-coated vesicles (Lin et al., 1992) but the significance of this observation has been questioned (Smythe et al., 1994).

One interesting feature of annexin XIIIB is its localization. By immunofluorescence microscopy annexin XIIIB was exclusively localized to the apical cell surface and to underlying punctate structures in MDCK cells. It also has a very restricted tissue expression being exclusively found in intestine and kidney. To further confirm the presence of annexin XIIIB in apical carrier vesicles, we used affinity-purified anti-annexin XIIIB antibodies to immunoprecipitate annexin XIIIB from an immunosolated apical vesicle preparation. The possibility that the immunoprecipitated protein was derived from a potential contamination of apical plasma membrane present in the vesicle fraction can be excluded since annexin XIIIA and annexin II, a very abundant MDCK cell protein localized to endosomes as well as to the apical and basolateral plasma membrane (Harder and Gerke, 1993; Parton, R. G., unpublished), were not present in immunosolated apical vesicles. It seems likely that the feature distinguishing annexin XIIIB from annexin XIIIA, the additional NH3-terminal 41 amino acids, are responsible for the specificity of the association with apical exocytic carrier vesicles.

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,b). Since annexin IV shows a similar tissue distribution to annexin XIIIB but contains only a very short NH3-terminal domain, we analyzed whether the COOH-terminal domains of annexins IV and XIII would show any unique features that would distinguish them from other annexins. However, this was not the case as judged by the sequence comparison of representatives of all known annexin family members. The analysis showed that annexins IV and XIII could not be subgrouped into a distinct phylogenetic branch of the annexin family.

Annexin XIIIB behaved as a membrane protein in phase partitioning in Triton X-114 (in the absence of added Ca2+; Wandinger-Ness et al., 1990) which is not usually observed for members of the annexin family. In this respect it is interesting to note that a feature unique to annexin XIIIA is its NH3-terminal myristoylation (Wice and Gordon, 1992). Wice and Gordon reported a protein immunologically related to annexin XIIIA with an apparent molecular mass of 42 kD which was also myristoylated and is now known to represent human annexin XIIIB (Gordon, J., personal communication). Since canine annexin XIIIB contains a potential NH3-terminal myristoylation site, it is likely to be myristoylated in MDCK cells as well. 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 might involve a cycle of dissociation and association with the membrane of 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 in the TGN. The antibodies, specifically binding to part of the 41 NH3-terminal amino acids unique to annexin XIIIB, presumably 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 (Söllner et al., 1993; Rothman, 1994) or rab GTPases (Zerial and Stenmark, 1993; Novick and Brenwald, 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 major challenge. Although NSF has been implicated in a number of membrane fusion events, it is possible that NSF- or SNAP-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. To the contrary, evidence is now accumulating that the apical trans-
port pathway, unlike the basolateral route, does not entail the general factors NSF and α-SNAP (Ikonen, E., M. Tagaya, C. Montecucco, O. Ullrich, and K. Simons, manuscript submitted for publication) and may thus involve isoforms of these molecules or a different mechanism for vesicle docking and membrane fusion. The involvement of annexin XIIIb in transport to the apical plasma membrane in MDCK cells may now facilitate the identification of other epithelial or apical specific components of the vesicular transport machinery.

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