

# 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 immunoisolated 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 NH<sub>2</sub> 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.

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**T**HE 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)<sup>1</sup> and coatomers have been demonstrated to be required for budding of intra-Golgi derived vesicles (Orci et al., 1993; Ostermann et al., 1993). The generation of endoplasmic reticulum derived vesicles in yeast requires the GTP-binding protein, Sar1p, 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 synaptosomal-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 SNARE (SNAP receptor) hypothesis, families of syntaxin and synaptobrevin homologues provide the specificity in vesicle docking before fusion (Söllner et al., 1993; Rothman, 1994) and complexes of SNAREs 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 Ca<sup>2+</sup>-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)

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**1. Abbreviations used in this paper:** ARF, ADP-ribosylation factor; NSF, *N*-ethylmaleimide sensitive factor; SLO, Streptolysin O; SNAPs, soluble NSF attachment proteins; SNAP-25, synaptosomal associated protein of 25 kD; SNARE, SNAP receptor; VSV-G, vesicular stomatitis virus glycoprotein.

several models have been proposed for this event, ranging from proteinaceous, ion channel-like pores (Almers, 1990) to purely lipidic pores only requiring a surrounding scaffold of proteins (Monck and Fernandez, 1994). 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 MDCK cells apical and basolateral proteins and lipids are sorted into distinct classes of vesicles in the TGN which are delivered to the polarized cell surface (Griffiths and Simons, 1986; Rodriguez-Boulan and Nelson, 1989). To identify components involved in these events, we have previously immunisolated apical and basolateral exocytic carrier vesicles 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 both the apical and basolateral pathways while the small GTPase rab8 was shown to be specific for the basolateral route 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, which we refer to as 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 underlying punctate structures by immunofluorescence microscopy. Using antibodies specific for annexin XIIIb in a cytosol dependent *in vitro* transport assay with permeabilized MDCK cells, we show that transport to the apical but not basolateral plasma membrane is significantly inhibited.

## 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 FITC-conjugated antibodies were purchased from Dianova (Hamburg, Germany), the rabbit anti-mouse IgG and deoxycholate were from Sigma (Deisenhofen, Germany), 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 stained 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., 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  $\mu$ g 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  $\mu$ l 80% NP-40 and 12.5  $\mu$ l ampholytes 7-9 were added and the volume was made up to 250  $\mu$ l with H<sub>2</sub>O. 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

Coomassie blue-stained spots of annexin XIIIb, annexin II, or keratin (~2  $\mu$ g each) were excised from four 2-D gels and pooled. After washing with water, the gel pieces were lyophilized and rehydrated in 100  $\mu$ l of 100 mM NH<sub>4</sub>HCO<sub>3</sub>, 0.5 mM CaCl<sub>2</sub> containing 1  $\mu$ g trypsin. After digestion (37°C for 12 h) peptide fragments were extracted from the gel slice with 100  $\mu$ l of 70% trifluoroacetic acid/0.01% Tween 20 and 100  $\mu$ l of 50% trifluoroacetic 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  $\times$  250 mm). Automated Edman degradation of peptides was performed using a sequencer (model 477A) connected to an 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 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' CCGGGAATTCGAAAAATGGG(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  $\lambda$  ZAP II MDCK cDNA library (Chavrier et al., 1990). Duplicate nitrocellulose filters were pre-hybridized for 2 h at 42°C in 5  $\times$  SSC, 50% formamide, 5  $\times$  Denhardt's solution, 1% SDS (Sambrook et al., 1989). Hybridization was carried out in the same solution, supplemented with <sup>32</sup>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 (Wisconsin Package, Genetics Computer Group, 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](mailto: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 NH<sub>2</sub>-terminal domains. The tree was corrected for multiple substitutions, analyzed by bootstrapping and visualized with Phylip (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 (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). 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 immunisolated apical exocytic carrier vesicles (Wandinger-Ness et al., 1990) from metabolically labeled cells were used. 600  $\mu$ l 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 immunisolated 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 anx13b 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  $\mu$ g 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 4°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  $\text{NH}_4\text{Cl}$  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 Axiophot photomicroscope (Carl Zeiss, Oberkochen, Germany) or with the EMBL confocal mi-

croscope and photographed with a Polaroid Freeze frame directly from the monitor.

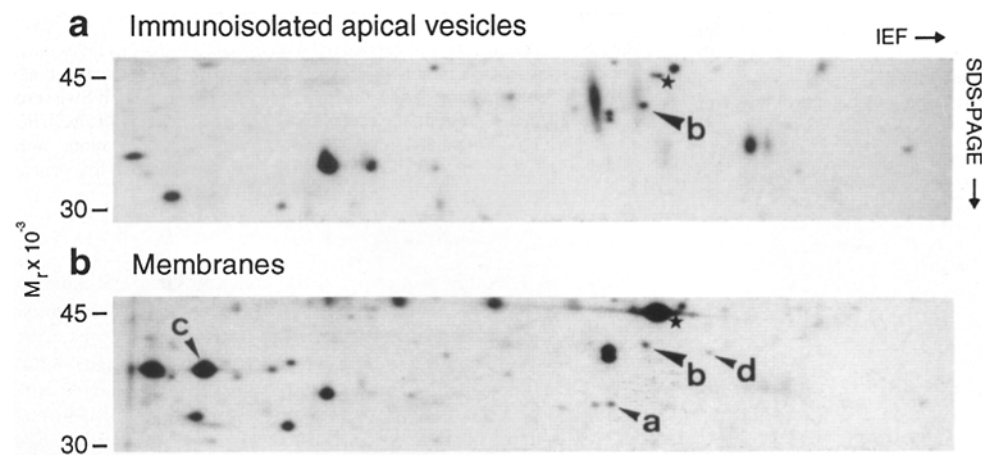
### In Vitro Transport of HA and Vesicular Stomatitis Virus Glycoprotein in Permeabilized MDCK Cells

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 (final concentration). The anx13b peptide was used at a concentration of 20  $\mu$ M. 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 immunisolated 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 immunisolated 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



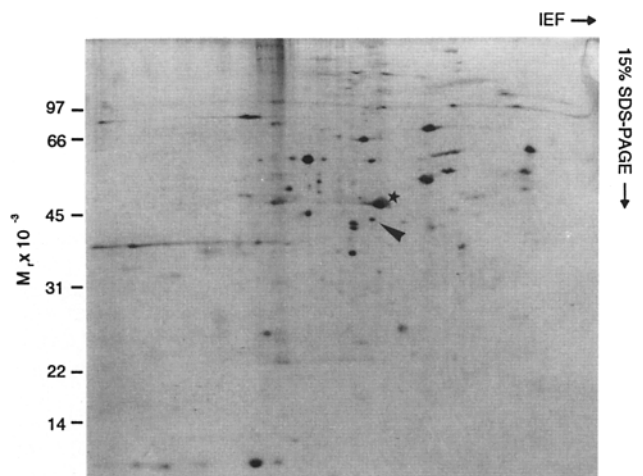
**Figure 1.** Comparison of the protein composition of immunisolated apical exocytic carrier vesicles with a membrane fraction from MDCK cells. (a) Immunisolated apical vesicles were obtained from metabolically labeled, influenza virus WSN ts61-infected MDCK cells. After mechanical cell perforation the released vesicles were purified by flotation to the interface of a 1.2-M/0.8 M sucrose gradient and immunisolated with an antibody directed against the cytoplasmic domain of WSN

hemagglutinin. (b) The membrane fraction was prepared from a postnuclear supernatant of metabolically labeled MDCK cells and collected from the interface of a 1.2 M/0.8 M sucrose gradient. The samples were resolved on large 2-D gels by IEF and 15% SDS-PAGE. The protein named A23 in Wandinger-Ness et al. (1990) is labeled with *b*. Protein *a* was identified as annexin XIIIa by Western blotting and proteins *c* and *d* were identified as annexin II and keratin 18, respectively, by microsequencing. An asterisk marks the position of actin.

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 NH<sub>2</sub>-terminal peptide of annexin XIIIa. They further showed that this component had a 41-amino acid insert five amino acids from the NH<sub>2</sub> terminus of annexin XIIIa (Gordon, J., personal communication). We therefore reasoned that the NH<sub>2</sub> 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 NH<sub>2</sub>-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 ob-



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

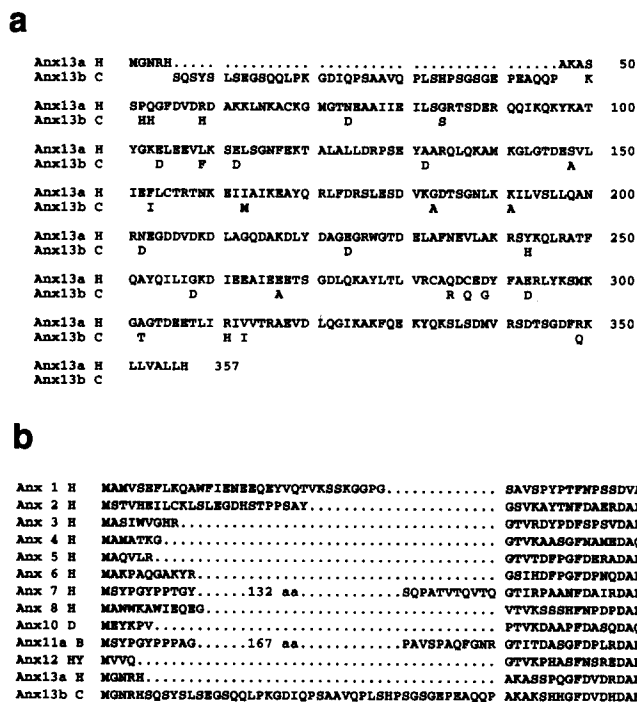
tained. Out of the 16 clones further analyzed, 12 gave rise to a 603-bp fragment by PCR using the aforementioned primers while four clones generated a 726-bp product. Two of each group were partially sequenced and differed only in the length of the 5' non-coding regions. The shorter cDNA corresponded 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

1	TGGCCGTGAGGAGAAGTCTCTCTAATGAAATACAGAACAACTGTCTTAGAAAAACGAA	
61	AAATGGGCAATCGTCATAGCCAGCTTCTACTCCCTCTCAGAGGCGAGTCAACAGTGGCCCAA	20
	M G N R H <b>S</b> <b>O</b> <b>S</b> <b>V</b> <b>S</b> <b>L</b> <b>P</b> <b>S</b> <b>E</b> <b>F</b> <b>G</b> <b>S</b> <b>O</b> <b>Q</b> <b>T</b> <b>P</b> <b>E</b>	
121	AGGGGACATCCAACCCCTCAGCAGCCGTCGACGCTCTCAGCCACCCATCAGGGAGTGGAGA	40
	<b>G</b> <b>D</b> <b>N</b> <b>O</b> <b>P</b> <b>S</b> <b>A</b> <b>A</b> <b>V</b> <b>O</b> <b>P</b> <b>L</b> <b>S</b> <b>H</b> <b>P</b> <b>S</b> <b>G</b> <b>S</b> <b>G</b> <b>E</b>	
181	GCCAGAGGCCAACAGCCTGCCAAAGCGAAAGCCATCATGGTFTTGACGTGGATCACGA	60
	<b>R</b> <b>S</b> <b>P</b> <b>A</b> <b>Q</b> <b>Q</b> <b>P</b> <b>A</b> <b>K</b> <b>A</b> <b>K</b> <b>S</b> <b>H</b> <b>H</b> <b>G</b> <b>F</b> <b>D</b> <b>V</b> <b>D</b> <b>H</b> <b>D</b>	
241	TGCCAAAAGCTGAACAAAGCCTGCAAGGAAGTGGGAACCTGATGAAGCAGCCATCTTGA	80
	<b>A</b> <b>K</b> <b>K</b> <b>L</b> <b>N</b> <b>K</b> <b>A</b> <b>C</b> <b>K</b> <b>G</b> <b>M</b> <b>T</b> <b>D</b> <b>E</b> <b>A</b> <b>A</b> <b>I</b> <b>E</b>	
301	GATCTTATCAAGCAGGACTTCAGATGAGAGGCAACAAATCAAGCAGAACTACAAGGCCAAC	100
	<b>I</b> <b>L</b> <b>S</b> <b>S</b> <b>R</b> <b>T</b> <b>S</b> <b>D</b> <b>E</b> <b>R</b> <b>Q</b> <b>Q</b> <b>I</b> <b>K</b> <b>Q</b> <b>K</b> <b>Y</b> <b>K</b> <b>A</b> <b>T</b>	
361	GTATGGCAAGGACCTGGAGGAGTGTTCAGAGTGACCTGAGTGGGAACCTTCGAGAAGAC	120
	<b>Y</b> <b>G</b> <b>K</b> <b>D</b> <b>L</b> <b>E</b> <b>E</b> <b>V</b> <b>F</b> <b>K</b> <b>S</b> <b>D</b> <b>L</b> <b>S</b> <b>G</b> <b>N</b> <b>F</b> <b>E</b> <b>K</b> <b>T</b>	
421	AGCCTTGGCCCTACTGGACCCCGCCAGCGAGTACGATGCCCGCAGCTGCAAAAGGCCAT	140
	<b>A</b> <b>L</b> <b>A</b> <b>L</b> <b>L</b> <b>D</b> <b>R</b> <b>P</b> <b>S</b> <b>E</b> <b>Y</b> <b>D</b> <b>A</b> <b>R</b> <b>Q</b> <b>L</b> <b>K</b> <b>A</b> <b>K</b> <b>A</b> <b>M</b>	
481	GAAAGTCTGGCCACAGATGAGGCGAGTCTCATTGAGATCTGTGTACGAGAACCAATAA	160
	<b>K</b> <b>G</b> <b>L</b> <b>G</b> <b>T</b> <b>D</b> <b>E</b> <b>A</b> <b>V</b> <b>L</b> <b>I</b> <b>E</b> <b>I</b> <b>L</b> <b>C</b> <b>T</b> <b>R</b> <b>T</b> <b>N</b> <b>K</b>	
541	GGAGATCATGGCCATTAAGGAAGCCTACCAAGGTTATTTGACAGGAGCCCTGATATCAGA	180
	<b>E</b> <b>I</b> <b>M</b> <b>A</b> <b>I</b> <b>K</b> <b>E</b> <b>A</b> <b>Y</b> <b>Q</b> <b>R</b> <b>L</b> <b>F</b> <b>D</b> <b>R</b> <b>S</b> <b>L</b> <b>E</b> <b>S</b> <b>D</b>	
601	TGTCAAAGCTGATACAAGTGGAAACCTAAAAGCAATTTCTGTGTCCCTGTACAGGCTAA	200
	<b>V</b> <b>K</b> <b>A</b> <b>D</b> <b>T</b> <b>S</b> <b>G</b> <b>N</b> <b>L</b> <b>K</b> <b>A</b> <b>I</b> <b>L</b> <b>V</b> <b>L</b> <b>L</b> <b>Q</b> <b>A</b> <b>N</b>	
661	TCGTGATGAAGGAGATGACCTGGGACAAAGATCTAGCTGGTCAAGGATGCCAAAGATCTGTA	220
	<b>R</b> <b>D</b> <b>E</b> <b>G</b> <b>D</b> <b>D</b> <b>V</b> <b>D</b> <b>K</b> <b>D</b> <b>L</b> <b>A</b> <b>G</b> <b>Q</b> <b>D</b> <b>A</b> <b>K</b> <b>D</b> <b>L</b> <b>Y</b>	
721	CGATGACGGGGATGGCCGCTGGGGCCAGGATGAGCTTGCCTTCAATGAAGTCTTGGCCAA	240
	<b>D</b> <b>A</b> <b>G</b> <b>D</b> <b>G</b> <b>R</b> <b>W</b> <b>G</b> <b>T</b> <b>D</b> <b>E</b> <b>L</b> <b>A</b> <b>F</b> <b>N</b> <b>E</b> <b>V</b> <b>L</b> <b>A</b> <b>K</b>	
781	GAGGAGCCACAAGCAATACGTGCTACCTTCAAGCTTACCAAAATCTCATCGATAAAGD	260
	<b>R</b> <b>S</b> <b>H</b> <b>K</b> <b>Q</b> <b>L</b> <b>R</b> <b>A</b> <b>T</b> <b>F</b> <b>Q</b> <b>A</b> <b>Y</b> <b>Q</b> <b>I</b> <b>L</b> <b>I</b> <b>D</b> <b>K</b> <b>D</b>	
841	CATCGAGGAGCCATTGAAGCAGAAACATCAGGAGACCTGCAGAAAGCCCTACTTAACCTCT	280
	<b>I</b> <b>E</b> <b>E</b> <b>A</b> <b>I</b> <b>E</b> <b>A</b> <b>E</b> <b>T</b> <b>S</b> <b>G</b> <b>D</b> <b>L</b> <b>Q</b> <b>K</b> <b>A</b> <b>Y</b> <b>L</b> <b>T</b> <b>L</b>	
901	CGTGAGGTGTGCCCGGGACAGGAGGGCTATTTGCGCGACCGCTGTGACAAGTCCATGAA	300
	<b>V</b> <b>R</b> <b>C</b> <b>A</b> <b>R</b> <b>D</b> <b>Q</b> <b>E</b> <b>G</b> <b>Y</b> <b>F</b> <b>A</b> <b>D</b> <b>R</b> <b>L</b> <b>Y</b> <b>K</b> <b>S</b> <b>M</b> <b>K</b>	
961	GGGCACGGCCCGATGAGGAGACGCTAATTCACATCATCTGTACCAGGGCCGAGGTGGA	320
	<b>G</b> <b>T</b> <b>G</b> <b>T</b> <b>D</b> <b>E</b> <b>T</b> <b>L</b> <b>I</b> <b>H</b> <b>I</b> <b>I</b> <b>V</b> <b>T</b> <b>R</b> <b>A</b> <b>E</b> <b>V</b> <b>D</b>	
1021	CCTTCAGGATATAAAGCAAGTTTCCAAGAGAATGATCAGAAGTCCCTCTCAGACATGGT	340
	<b>L</b> <b>Q</b> <b>G</b> <b>I</b> <b>K</b> <b>A</b> <b>K</b> <b>F</b> <b>Q</b> <b>E</b> <b>K</b> <b>Y</b> <b>Q</b> <b>K</b> <b>S</b> <b>L</b> <b>S</b> <b>D</b> <b>M</b> <b>V</b>	
1081	TCGCTCAGACACCTCTGGGACTTCCAGAACTGCTGGTGGCCCTCTTGCATGAACCCAA	357
	<b>R</b> <b>S</b> <b>D</b> <b>T</b> <b>S</b> <b>G</b> <b>D</b> <b>F</b> <b>Q</b> <b>K</b> <b>L</b> <b>L</b> <b>V</b> <b>A</b> <b>L</b> <b>L</b> <b>H</b> *	
1141	GCCAGCATGGTGAAGCACATGGAGAAGCCACCTTTGTCAGAGTCCATTTCAACCCAGA	
1201	TTTGCAAAATGTGACCCAGCAGCAGCAAAAAGTATTGAAGAATCCTGGCTTCTTCTGGCAG	
1261	CCTGAATGCTGTGGCCAGGCCAAGCTGTTTAGAAGGGCAGCAGCTCAAAAACGCTTGGGC	
1321	AGATCACCCGTAATGCTGGCTTTCAGCAGTCCCTGAGCAGCCTCTCTGCTTGCCTTAGCA	
1381	TGGCAAACTGAATGCTTCTGAAACACAAATGAGATAGTGTGATGAAAGAACTGCAC	
1441	TTGTAATAAAACATTCAGAAGAATAGGTATCCGACAGAGTGGGTAGAGACTGAAATGGTA	
1501	AGGTACAGGGAAGACAAATTAATCAGTGTATCAATTTTCCTCTCTGGGGGGGCTGGAC	
1561	TGGCTCAGTGGTTAAGGTCTGGCTTTCAGCTCAAGTCATGATCTTGGGTCTGGGACC	
1621	AAGCCCATGTCCGGCTCCCTGCTCAGCAGGGAATCTGCTTCTCTCTCTCTCTCTCTCT	
1681	CTCTCCACTGGCACTCCACTTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT	
1741	CTCTCTCCAAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAA	

**Figure 3.** 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 1-1800 without nucleotides 77-199 (enclosed amino acids are in a shaded box), the annexin XIIIb cDNA lacked nucleotides 1-40 but contained the insert of nucleotides 77-199. Two in frame stop codons are preceding the 5' ATG in position 8 and 17. These sequence data for canine annexin XIIIa and annexin XIIIb are available from EMBL/GenBank/DBJ under accession number X80208 and X80209, respectively.

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 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. 4 a). 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 NH<sub>2</sub>-terminal domain which might be generated by alternative splicing of the mRNA. A comparison of the variable NH<sub>2</sub>-terminal domains of annexins I-XIII is shown in Fig. 4 b. The NH<sub>2</sub> 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 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



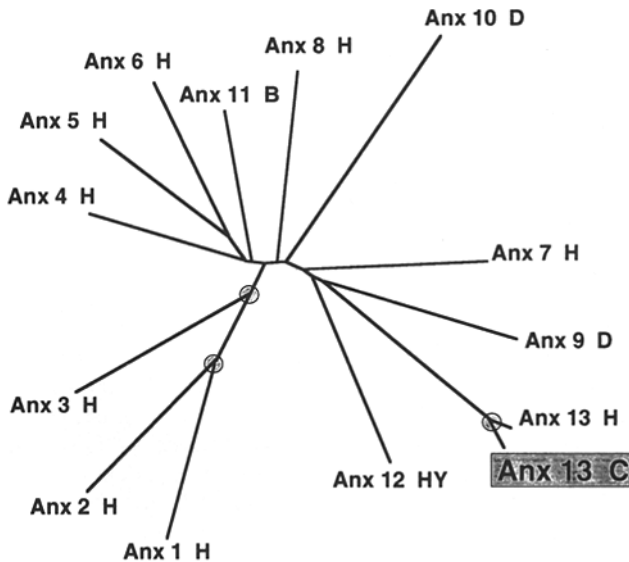
**Figure 4.** Alignment of annexin XIIIa, XIIIb, and other annexins. (a) Alignment of human annexin XIIIa (intestine-specific annexin) with canine annexin XIIIb using the GCG program "gap." Gaps are indicated by dots. For annexin XIIIb only amino acids different from annexin XIIIa are indicated. The overall identity and similarity is 90% and 96%, respectively. (b) Alignment of the NH<sub>2</sub>-terminal domains of representatives of all known annexins. Gaps are represented by dots that are inserted between the variable NH<sub>2</sub>-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.

is located within the unique 41 NH<sub>2</sub>-terminal amino acids. Three of the four conserved annexin segments (endonexin folds) are retrieved by the program (some of the repeats are also not recognized in annexin IX, X, XI, and XII). Interestingly, annexin XIIIb contains a potential NH<sub>2</sub>-terminal myristoylation site. Analysis of the evolutionary distances of representatives of all known annexins (see Materials and Methods) showed that only annexins I, II, and III but not annexin XIII could be subgrouped into a distinct branch of the annexin family (Fig. 5).

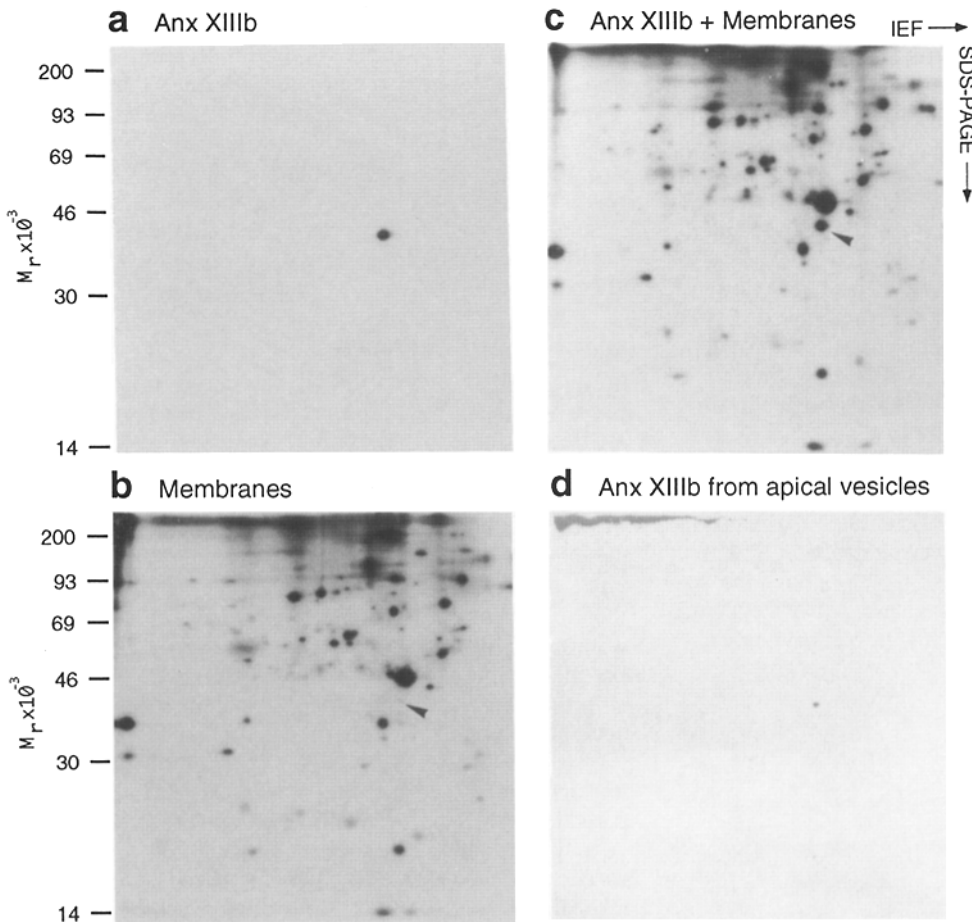
### Annexin XIIIb Is Present in Apical Carrier Vesicles

To further analyze annexin XIIIb, we raised polyclonal antisera against an NH<sub>2</sub>-terminal peptide common to annexin XIIIa and annexin XIIIb (anx13) and antisera against an NH<sub>2</sub>-terminal peptide unique to annexin XIIIb (anx13b). The affinity-purified anti-anx13b 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. 6 a). For the immunoprecipitation, the MDCK membrane fraction served as a starting material (Fig. 6 b). To identify annexin XIIIb in the membrane fraction the immunoprecipitate was mixed with the membranes (Fig. 6 c). 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 immunisolated apical vesicles (Wandinger-Ness et al., 1990) for the immunoprecipitation



**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. 4 b) spanning residues 47-357 of annexin XIIIb and the corresponding residues of the aligned sequences. The comparison excluded the variable NH<sub>2</sub>-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.



**Figure 6.** Annexin XIIIb is present in immunisolated 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 immunisolated apical carrier vesicles (obtained as in Fig. 1 a). Due to the limited amount of immunisolated 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.

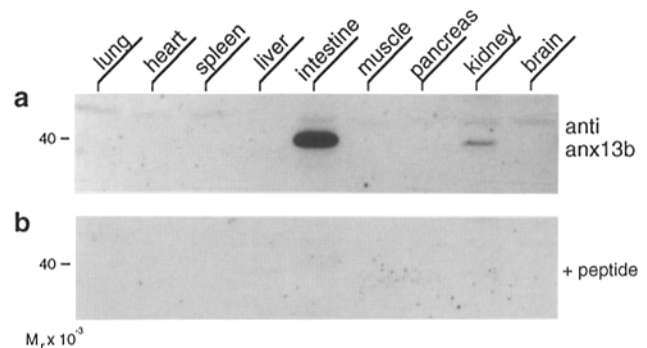
(Fig. 6 d). The immunoprecipitated protein (Fig. 6 d) can be perfectly aligned with annexin XIIIb from the MDCK membrane fraction. Since no other protein is present in the immediate vicinity of A23 in apical vesicles, this unequivocally demonstrates the presence of annexin XIIIb in apical exocytic carrier 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 immunisolated apical vesicles (Fig. 1). This further illustrates the high specificity of the immunisolation and excludes the possibility that the immunisolated annexin XIIIb is derived from a plasma membrane contamination.

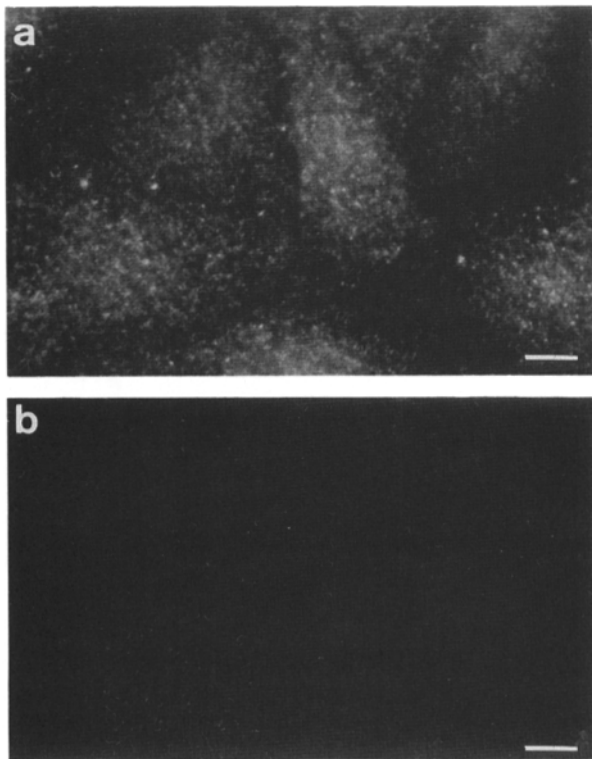
#### **Annexin XIIIb Is Expressed in Intestine and Kidney**

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  $\mu\text{g/ml}$  of the respective peptide (Fig. 7 b). In addition, the anti-anx13b antibodies reacted with proteins of an apparent molecular mass of  $\sim 20$  kD in pancreas and  $\sim 55$  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  $\mu\text{g/lane}$ ) 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  $\mu\text{g/ml}$  of anx13b peptide.

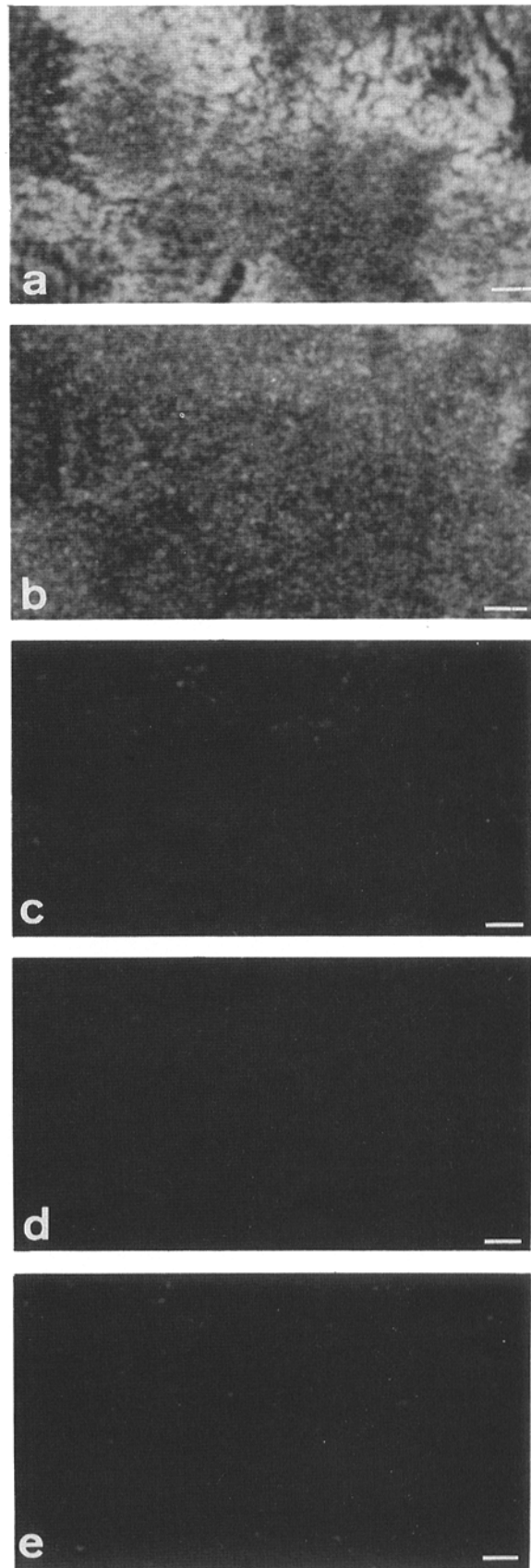


**Figure 8.** Immunofluorescence localization of annexin XIIIb. Subconfluent MDCK cells were permeabilized with 0.1% Triton X-100 after fixation with 4% paraformaldehyde. Immunostaining with affinity-purified anti-anx13b antibodies in the absence (a) or presence (b) of 50 µg/ml of anx13b peptide. Bars, 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 anx13b 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-anx13b antibodies. The focal planes are (a) apical, (b) upper cell quarter, (c) cell middle, (d) lower cell quarter, and (e) basolateral. Bars, 5 µm.

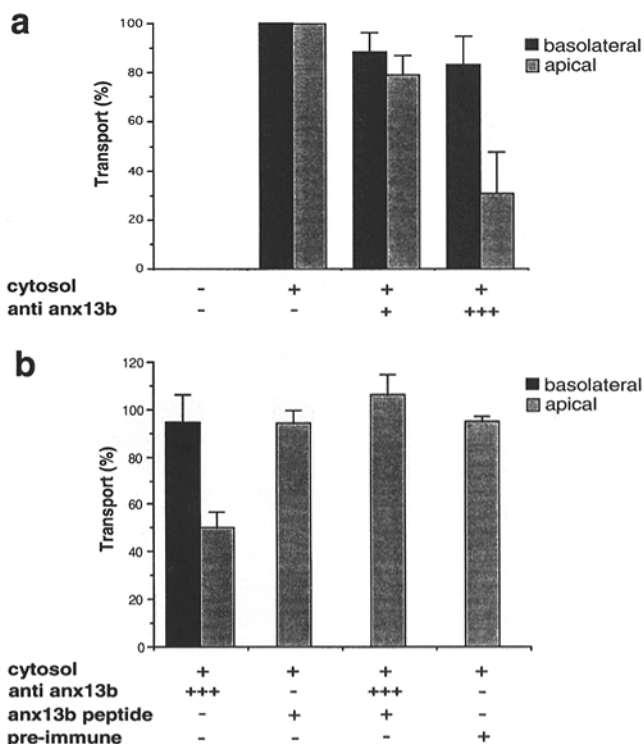
### Annexin XIIIb and Transport to the Apical Plasma Membrane

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-Boulant and Nelson, 1989; Rodriguez-Boulant 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). Immunoprecipitation 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 NH<sub>2</sub>-terminal do-



**Figure 10.** *In vitro* transport assay. (a) MDCK cells grown on Transwell filters were permeabilized with streptolysin O from the apical or basolateral side and cytosol was added back without (-) or with 60 nM (+) and 600 nM (+++) of affinity-purified anti-anx13b antibodies (final conc.). (b) Control experiments. Cytosol was added back to the cells without (-) or with (+++) 600 nM of affinity-purified anti-anx13b antibodies. In the apical transport assay anx13b peptide was used at a concentration of 20  $\mu$ M and the amount of mock affinity-purified preimmune serum added was equivalent to a concentration of 600 nM of affinity-purified anti-anx13b antibodies. All samples were analyzed by 10% SDS-PAGE, autoradiographed, scanned with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and the band intensities were calculated with Image Quant software. The values are mean  $\pm$  standard errors from three experiments performed with duplicate filters. *In vitro* transport of influenza HA was done 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  $2 \times \text{HA}_2 / (\text{HA} + 2 \times \text{HA}_2) \times 100$ . The *in vitro* transport of VSV-G was performed on apically permeabilized, cytosol-depleted MDCK cells. The amount of VSV-G transported to the basolateral surface was determined from the fraction of VSV-G bound to protein A-Sepharose after surface immunoprecipitation with VSV-G specific antibodies. The % transport of VSV-G was calculated as  $\text{VSV-G bound} / \text{VSV-G total} \times 100$ . Cytosol-dependent transport is presented as 100% (transport in the presence of cytosol-transport in the absence of added cytosol). For the experiments shown in a, the result was reproducibly obtained with two and three preparations of affinity-purified anti-anx13b antibodies for the basolateral and apical transport, respectively, and for the experiments shown in b with two different batches of affinity-purified antibodies.

main. Annexins are a large family of proteins that are characterized by 4 or 8 repeats of an  $\sim$ 70-amino acid domain with 17 highly conserved amino acids, termed the endonexin fold. A hallmark of the annexin family is their Ca<sup>2+</sup>-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  $Ca^{2+}$ -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  $Ca^{2+}$ -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 (Futter 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 immunoisolated 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 immunoisolated apical vesicles. It seems likely that the feature distinguishing annexin XIIIb from annexin XIIIa, the additional  $NH_2$ -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  $NH_2$ -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  $Ca^{2+}$ ; 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  $NH_2$ -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  $NH_2$ -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 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  $NH_2$ -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 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 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  $\alpha$ -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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