The Association of Annexin I with Early Endosomes Is Regulated by Ca^{2+} and Requires an Intact N-Terminal Domain

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Annexin I is a member of a multigene family of Ca^{2+}/phospholipid-binding proteins and a major substrate for the epidermal growth factor (EGF) receptor kinase, which has been implicated in membrane-related events along the endocytotic pathway, in particular in the sorting of internalized EGF receptors occurring in the multivesicular body. We analyzed in detail the intracellular distribution of this annexin by cell fractionation and immunoelectron microscopy. These studies used polyclonal as well as a set of species-specific monoclonal antibodies, whose epitopes were mapped to the lateral surface of the molecule next to a region thought to be involved in vesicle aggregation. Unexpectedly, the majority of annexin I was identified on early and not on multivesicular endosomes in a form that required micromolar levels of Ca^{2+} for the association. The specific cofractionation with early endosomes was also observed in transfected baby hamster kidney cells when the intracellular fate of ectopically expressed porcine annexin I was analyzed by using the species-specific monoclonal antibodies in Western blots of subcellular fractions. Interestingly, a truncation of the N-terminal 26, but not the N-terminal 13 residues of annexin I altered its intracellular distribution, shifting it from fractions containing early to those containing late and multivesicular endosomes. These findings underscore the regulatory importance of the N-terminal domain and provide evidence for an involvement of annexin I in early endocytotic processes.

INTRODUCTION

Intracellular compartments of eukaryotic cells are known to communicate with one another through the exchange of membrane vesicles. This membrane traffic involves vesicle budding from the donor membrane, vesicle translocation, vesicle docking at the acceptor membrane, and the actual membrane fusion event. Some of the principle mechanistic aspects underlying the individual steps are well-established, and a num-

ber of membrane-bound, cytoskeleton-associated proteins and cytosolic proteins have been shown to participate in membrane transport processes (for review, Pryer et al., 1992; Simons and Zerial, 1993; Rothman, 1994). These include certain coat proteins and the guanosine 5'-triphosphate binding protein ADP ribosylation factor (ARF) known to be required for at least some vesicle budding steps of the biosynthetic pathway (for review, Kreis and Pepperkok, 1994; Rothman, 1994). In most and possibly all membrane traffic events, so-called SNAP receptors (SNAREs) are then responsible for mediating the actual vesicle docking, a process in which specificity is conferred via certain vesicle (v) and target membrane (t) SNAREs found on

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Note: The sequence of the porcine annexin I cDNA is listed in the European Molecular Biology Laboratory (EMBL) data library under accession number X95108.

the respective membranes. The SNARE complexes interact with soluble NSF attachment proteins (SNAPs), which are involved in initiating, in conjunction with N-ethylmaleimide–sensitive fusion protein (NSF), the actual membrane fusion process (Rothman, 1994; Bennett, 1995). Moreover, SNAREs are thought to represent targets for small GTPases of the rab family, which seem to participate through a catalyzed GTPase cycle in specifying the correct membrane–membrane interactions (Simons and Zerial, 1993).

Annexins are members of a multigene family of proteins recently reported to participate in membrane transport steps along the biosynthetic and endocytotic pathway (for review, Gruenberg and Emans, 1993; Burgoyne and Clague, 1994; Gerke, 1996). All annexins are characterized by their Ca^{2+}-dependent interaction with negatively charged phospholipids and cellular membranes. This biochemical property is mediated through a well-conserved structural element, which is 70–80 amino acids in length and repeated either four or eight times within a given member of the family (for review, Creutz, 1992). The sum of these so-called annexin repeats forms a stable protein core, whose structure in the crystallized annexins I and V resembles that of a compact, slightly curved disk (Huber *et al.*, 1990; for recent review, Swairjo and Seaton, 1994). The Ca^{2+} (and most likely also phospholipid) binding sites of the annexins are found on the convex surface of the disk, whereas the concave side is most likely in close proximity to an N-terminal domain that precedes in the linear sequence the conserved annexin repeats. The N-terminal domains are susceptible to mild proteolytic attack and are probably less structured then the compact protein cores. They vary in length and in sequence between the different members of the family and are thought to confer specificity to the individual annexins with respect to subcellular localization and physiological function. Moreover, the N-terminal domains are of regulatory importance as they harbor phosphorylation sites for signal transducing protein kinases and, at least in some cases, binding sites for intracellular protein ligands (for review, Creutz, 1992).

An involvement of annexins in membrane–membrane and/or membrane–cytoskeleton interactions has been well-documented. Annexin II, for example, aggregates chromaffin granules at micromolar levels of Ca^{2+} when present in a heterotetrameric complex with its intracellular protein ligand p11 (Drust and Creutz, 1988), and the complex stimulates Ca^{2+}-evoked exocytosis in permeabilized chromaffin cells (Ali *et al.*, 1989; Sarafian *et al.*, 1991). The same annexin was also implicated in basolateral to canicular transcytosis in hepatocytes as it relocalizes from the basolateral to the perinuclear and finally the apical region of the cell concomitant with the transcytosis of cholestatic bile salts (Wilton *et al.*, 1994). Moreover, annexin

II seems to be involved in endocytotic events as it is present on purified early endosomes and is transferred from a donor to an acceptor endosomal membrane in an in vitro fusion assay (Emans *et al.*, 1993). It seems to be a central component of a Ca^{2+}-dependent endosomal fusion machinery (Mayorga *et al.*, 1994) and regulates the distribution of early endosomes in vivo (Harder and Gerke, 1993). The vesicular traffic to the apical plasma membrane of polarized epithelial cells seems to depend on annexin XIIIb because this annexin is highly enriched on purified apical transport vesicles and because an annexin XIIIb antibody significantly inhibits the apical transport in permeabilized Madin–Darby canine kidney cells (MDCK) (Fiedler *et al.*, 1995). Other annexins thought to be involved in membrane trafficking events include annexin III, which translocates to the periphagosomal region upon phagocytosis in neutrophils (Ernst, 1991), and annexin VI, which is associated with phagosomes purified from J774 macrophages (Desjardins *et al.*, 1994). Annexin VI has also been reported to be enriched on early endosomal membranes (Jäckle *et al.*, 1994), and it has been implicated in the budding of clathrin-coated vesicles (Lin *et al.*, 1992), although the significance of the latter finding has been questioned (Smythe *et al.*, 1994). Annexin I, a major substrate for the epidermal growth factor (EGF) receptor kinase (Fava and Cohen, 1985), has also been implicated in the endocytotic pathway, more specifically in the sorting of EGF receptors to the lysosome, which occurs in the multivesicular body (MVB) (Futter *et al.*, 1993).

Here, we used gradient fractionation of endosomal membranes prepared from baby hamster kidney (BHK) cells and immunoelectron microscopy to determine the subcellular distribution of annexin I. We show that annexin I is enriched in membrane fractions containing early endosomes and, to a lesser extent, in those containing late and multivesicular endosomes. This endosome association depends on the presence of micromolar concentrations of Ca^{2+} and is specifically affected when certain truncations are introduced into the N-terminal domain of the molecule.

MATERIALS AND METHODS

Purification of Annexin I

Annexin I was purified from pig, cow, pigeon, and rat lung essentially as described by Glenney *et al.* (1987). Briefly, an ethylenebis(oxyethylenenitrilo) tetra-acetic acid (EGTA) extract of the tissue was applied to a DE-52 (Whatman) column equilibrated in buffer A (10 mM imidazole-HCl, pH 7.2, 1 mM dithiothreitol [DTT], 1 mM NaN$_3$). The flowthrough fraction that contained the annexin I was applied to a hydroxylapatite (Bio-Rad, Richmond, CA) column, and bound proteins were eluted with a linear gradient of 0–0.2 M sodium phosphate in buffer A. Pure annexin I was released at 0.1–0.13 M phosphate. For long-term storage, annexin I was dialyzed against buffer A containing 50% glycerol and stored at −20°C.

Human annexin I was enriched in an EGTA extract from A431 cells. Cells from six confluent 100-mm dishes were washed three

times with phosphate buffered saline (PBS) and scraped into 2 ml of buffer B (30 mM imidazole-HCl, pH 7.5, 150 mM NaCl, 2 mM MgCl$_2$, 1 mM DTT, 0.5 mM benzamidine, 0.5 mM phenylmethyl-sulfonyl fluoride [PMSF], 10 mg/l trypsin inhibitor, 2 mg/l E-64 [an inhibitor for Ca^{2+}-activated proteases, Peptide Institute]). After homogenization with a glass Teflon homogenizer, CaCl$_2$ was added to a final concentration of 5 mM. The suspension was stirred for 30 min at 4°C, again homogenized, and then centrifuged for 40 min at 100,000 × g. The resulting pellet was washed with buffer B containing 2 mM CaCl$_2$ and finally resuspended in buffer B containing 10 mM EGTA. The suspension was stirred for 30 min at 4°C and again centrifuged (30 min, 100,000 × g). The supernatant (EGTA extract) containing annexin I was concentrated by chloroform/methanol precipitation (Wessel and Flügge, 1984) and used for Western blot analysis.

Limited Proteolysis

Trypsin treatment of annexin I purified from pig lung was performed at an enzyme-to-substrate ratio of 1:150 in a buffer containing 100 mM Tris-HCl, pH 8.5, and 5% acetonitrile. After incubation at 37°C for the times indicated, the reaction was stopped by boiling the sample in SDS sample buffer (Laemmli, 1970). The tryptic fragments were separated by SDS-PAGE using the tricine system (Schägger and von Jagow, 1987) and transferred by electrophoretic blotting to polyvinyldifluoride membranes (ProBlott Membranes; Applied Biosystems, Foster City, CA) for N-terminal sequence analysis (Bauw et al., 1989) or to nitrocellulose (Towbin et al., 1979) for immunoblotting, respectively. Protein sequencing was performed using an automated gas-phase sequenator (Knauer model 810).

For cyanogen bromide (CNBr) cleavage, porcine annexin I was concentrated by chlorofom/methanol precipitation (Wessel and Flügge, 1984), dissolved in 70% formic acid, and incubated with CNBr for 16 h at room temperature. Subsequently, the mixture was dried under vacuum, and the resulting fragments were separated by reverse-phase high-pressure liquid chromatography on a Vydac 218 TP52 column. After washing with 3 ml of solvent A (0.1% trifluoroacetic acid in water), peptides were eluted with a 30-ml linear gradient of 10–60% solvent B (90% acetonitrile in 0.1% trifluoroacetic acid) at a flow rate of 1 ml/min. The peak fractions were analyzed by N-terminal sequencing and immunoblotting with the monoclonal annexin I antibodies.

Cloning of the Pig Annexin I cDNA

The annexin I cDNA was isolated by immunoscreening using a porcine lung cDNA library in λgt11 (Clontech, Palo Alto, CA). After plating of the library and induction of the β-galactosidase fusion proteins with 10 mM isopropyl-β-D-thiogalactopyranoside [IPTG], plaque DNA was transferred to nitrocellulose membranes and the membranes were incubated for 1 h at room temperature with a mixture of hybridoma supernatants containing the annexin I antibodies (diluted 1:2 in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Tween 20, 0.4% pig hemoglobin). Alkaline phosphatase-coupled goat anti-mouse antibodies (Dianova, Hamburg, Germany; diluted 1:2000 in the same buffer; incubation for 1 h at room temperature) were used as the second antibodies, and antibody-reactive clones were detected using 0.33 mg/ml nitroblue tetrazolium and 0.2 mg/ml bromochloroinoyl phosphate in 0.1 M Na$_2$CO$_3$, pH 10.2. Positive plaques were picked, replated, and rescreened until all plaques were immunoreactive with the antibody mixture. The cDNA was isolated as described (Yamamoto et al., 1970), cloned into pBluescript KS(+), and sequenced by the dideoxy method (Sanger et al., 1970) using a T7 sequencing kit (Pharmacia, Piscataway, NJ).

Construction of Full-Length and Partial Annexin I cDNAs

The immunoscreening yielded only partial cDNA clones lacking the first 21 nucleotides. To obtain a full-length annexin I cDNA, the 5'

part of the protein-coding region was reinstalled by polymerase chain reaction (PCR). The forward primer was based on the porcine annexin I amino acid sequence obtained by N-terminal sequence analysis of one CNBr fragment and on the nucleotide sequence of the bovine cDNA (Ernst, 1996; EMBL accession number X56649). This primer introduced an EcoRI cleavage site upstream of the ATG start codon (5'-GCTAGAATTCAAAAATGGCAATGGTATCTGA-GTTCCTTAAGCAGGCCTGGTTTATTGAC). The reverse primer was based on the isolated cDNA sequence and introduced an additional EcoRI cleavage site downstream of the stop codon (5'-GCTAGAATTCAGAAAGGAGTTAGCTAGAC).

For epitope mapping of the monoclonal annexin I antibodies, four constructs were generated by PCR, which were truncated in the 5' part of the protein-coding region. The following forward primers, which introduced an EcoRI cleavage site 5' of the novel start codon, were used: construct A (5'-GCTAGAATTCGTGAGCCCCTATC-CTACCTTCAAT); B (5'-GAAGCCTCCCATGAATTCATAACAGT-GAAAGGTAAAGGTG); C (5'-GAAAGGTGTGGATGAATTCAC-CATCATTGAAAT); D (5'-CCAGGAGAAAGGAGAATTCCTG-GATGAAGCTCTTG). In each case, the reverse primer introduced an EcoRI cleavage site downstream from the stop codon (5'-GAGT-TAGCTAGACTAGAATTCTGCTGAAGAAAT). The PCR reactions were performed with Taq polymerase (MBI Fermentas, St. Leon-Rot, Germany) using pBluescript KS(+) containing the isolated annexin I cDNA as a template. The PCR products were gel-purified and digested with EcoRI. Subsequently, the full-length cDNA was cloned into appropriately linearized pBluescript for further mutagenesis (pBluescript AnxI, see below), into the pkk223-3 vector (Pharmacia) for bacterial expression (pKK AnxI), and into the pCMV5 vector (Andersson et al., 1989) for mammalian expression (pCMV AnxI). The N-terminally truncated constructs were cloned in frame with the fusion-protein part of the bacterial expression vector pET23a (Novagen, Madison, WI).

Overexpression and Purification of Recombinant Annexin I

Expression of recombinant annexin I was achieved by tranforming Escherichia coli BL21 (DE3)pLysS cells with the following plasmids: pKK Anx I, pET Anx I A, pET Anx I B, pET Anx I C, pET Anx I D. Cells containing the expression plasmids were grown at 37°C in 50 ml of Luria broth (LB) containing 150 μg/ml ampicillin and 34 μg/ml chloramphenicol. Overexpression was induced by addition of 0.4 mM IPTG at late log-phase. After 4 h, total protein extracts were analyzed by SDS-PAGE and immunoblotting with annexin I antibodies.

Recombinant wild-type annexin I was purified from a 200-ml culture. Cells were harvested by centrifugation and resuspended in 10 ml of 50 mM imidazole-HCl, pH 7.4, 300 mM NaCl, 2 mM MgCl$_2$, 2 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.5 mM benzamidine, 10 μM E-64, 10 mg/l trypsin-inhibitor. The cells were lysed by repeated (3 times) freezing and thawing followed by sonification. The lysate was clarified by centrifugation (100,000 × g, 30 min, 4°C), dialyzed against 10 mM imidazole-HCl, pH 7.4, 10 mM NaCl, 1 mM EGTA, 1 mM NaN$_3$, and applied to a DE-52 (Whatman) column. The flowthrough was collected and subjected to Ca^{2+}-dependent liposome binding as described previously (Ivanenkov et al., 1994). Annexin I bound Ca^{2+} dependently to the liposomes and was released by EGTA. For further purification, the protein was concentrated by chlorofom/methanol precipitation and subjected to preparative SDS-PAGE. Unproteolysed annexin I was exised, electroeluted, and used for rabbit immunization.

Site-specific Mutagenesis

Oligonucleotide-directed mutagenesis (Kunkel, 1985) was used to construct AnxI Δ1-13 and AnxI Δ1-26. A uracil-containing, single-stranded annexin I cDNA template was prepared by infecting E. coli CJ236 cells carrying pBluescript AnxI with the helper virus M13KO7

and isolating the pBluescript AnxI DNA from purified phages (Sambrook *et al.*, 1989). The mutagenesis reactions were performed with a Muta-Gene M13 in vitro mutagenesis kit (Bio-Rad) according to the manufacturer's protocol using Δ1-13 (5'-CTTAAGCAGGC-CTGGTTTGAATTCAAAAATGGTCATTGACAATGAA-GAGCAAG) and Δ1-26 (5'-CATTAAAACTGTGAAAGAAT-TCAAAAATGGTCGGATCCAAAGGTGGTCCT) oligonucleotides as primers. After transformation into *E. coli* XL1 blue, the pBluesript constructs containing the mutant annexin I cDNAs were isolated, and the mutations were verified by dideoxy sequencing (Sanger *et al.*, 1977) using the T7 sequencing kit (Pharmacia). The mutant cDNAs were digested with *EcoRI* and cloned into the eucaryotic expression vector pCMV5 (pCMV Δ1-13, pCMV Δ1-26).

Cell Culture and Transfection

Human A431 and BHK cells were grown in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (Boehringer Mannheim, Mannheim, Germany) at 37°C and 5% CO_2 in humidified atmosphere. The BHK cells were transiently transfected using the modified calcium phosphate precipitation method described by Chen and Okayama (1987). The cells were transfected at 50% confluence in 100-mm dishes with 20 µg pCMV AnxI, pCMV Δ1-13 or pCMV Δ1-26, respectively. All plasmids used for transfection were purified on Qiagen columns following the manufacturer's protocol. After incubation for 12–16 h at 35°C in 3% CO_2, the cells were washed with PBS and then cultivated in fresh medium using normal culture conditions. Forty hours after starting the transfection, 30–40% of the total cell population was expressing the exogenous protein, as monitored by immunofluorescence.

Fractionation of Endosomes

The fractionation of endosomes was performed essentially as described by Gorvel *et al.* (1991) and Aniento *et al.* (1996). Briefly, four 10-cm dishes of BHK cells or six dishes of transfected BHK cells were treated with 2 mg/ml horseradish peroxidase (HRP) in IM (internalization medium: DMEM supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid [HEPES], pH 7.4) at 37°C. HRP was endocytosed for 5 min to label early endosomes. To label late endosomes, the cells were treated for 5 min with HRP and then the HRP was chased along the endocytotic pathway for 45 min by incubating the cells in IM supplemented with 2 mg/ml bovine serum albumin (Gorvel *et al.*, 1991). Subsequently, the cells were homogenized in homogenization buffer (HB) (0.25 M sucrose, 3 mM imidazole-HCl, pH 7.4) containing protease inhibitors (10 µg/ml aprotinin, 1 µg/ml pepstatin A, 1 µg/ml antipain, 1 µg/ml leupeptin, 10 µg/ml trypsin inhibitor, 0.5 mM benzamidine, 10 µM E-64), and the mixture was centrifuged to yield a postnuclear supernatant (PNS). The PNS was brought to 40.6% sucrose, 3 mM imidazole-HCl, pH 7.4, and loaded at the bottom of a SW60 centrifugation tube (Beckman, Fullerton, CA). The load was overlaid with 1.5 ml of 35% sucrose, 3 mM imidazole-HCl, pH 7.4, then with 1 ml of 25% sucrose, 3 mM imidazole-HCl, pH 7.4, and finally with 0.5 ml of HB buffer. All sucrose solutions contained the same cocktail of protease inhibitors as the HB buffer. In some experiments, all solutions were supplemented with 0.5 mM EDTA. The step gradient was centrifuged at 35,000 rpm for 60 min at 4°C in a SW60 rotor. Late endosomes/endosomal carrier vesicles were collected at the 25% sucrose–HB buffer interface, and early endosomes were present at the 25–35% sucrose interface. A fraction containing heavy membranes was collected at the 35%–40.6% sucrose interface. The activity of the endocytosed HRP present in the different fractions was analyzed as described (Gruenberg and Howell, 1986). Latency was measured as described by Bomsel *et al.* (1990). For analysis of the annexin distribution among the different fractions, proteins present in the individual fractions were concentrated by chlorofom/methanol precipitation (Wessel and Flügge, 1984), and 10 µg of each

fraction (as determined according to Bradford, 1976) were separated in 12.5% SDS gels (Laemmli, 1970) and analyzed by immunoblotting. The distribution of the early endosome-associated protein EEA1 was documented by immunoblotting of the gradient fractions separated in 7.5% SDS gels.

Immunolocalization

MDCK II cells grown on a filter support were incubated with 5 nm BSA gold in the apical and basal medium for 10 min at 37°C to label early endosomes as described previously (Parton *et al.*, 1989). Cells were fixed with 8% paraformaldehyde in phosphate buffer, pH 7.35, and then processed for frozen sectioning. Ultrathin sections were labeled with affinity-purified polyclonal antibodies to annexin I followed by 10 nm protein A-gold.

For pre-embedding labeling of perforated cells, MDCK II cells grown on filters were incubated with 10 mg/ml HRP (Sigma, St. Louis, MO). They were then washed and perforated mechanically using nitrocellulose as described by Simons and Virta (1987). Cells were then further incubated in KOAC buffer (25 mM HEPES, pH 7.4, 115 mM potassium acetate, 2.5 mM $MgCl_2$) for 5 min at 4°C to allow cytosol to leak out of the cells. During this time, the filters were cut into two pieces; one piece was fixed in 8% paraformaldehyde in 250 mM HEPES, pH 7.4, and the other was incubated for 30 min at 4°C in KOAC buffer containing 10 mM EGTA before fixation. The filter pieces with attached perforated cells were then labeled with the annexin I antibody followed by 5 nm protein A-gold, as described by Ikonen *et al.*, 1996. Fixation with glutaraldehyde, visualization of HRP reaction product, and embedding in Epon have been described (Parton *et al.*, 1989).

Antibodies

Monoclonal antibodies (mAbs) were obtained by standard procedures (Osborn *et al.*, 1988). Briefly, BALB/c mice were immunized with annexin I purified from bovine lung by M. von Ehrenstein. After fusion, the selection of suitable hybridomas was performed by an ELISA assay. Hybridomas were subcloned by limiting dilution and then grown further to yield culture supernatant. A total of 10 independent isolates were used for further studies.

The polyclonal annexin I antibody was raised in rabbits against gel-purified recombinant annexin I. Annexin II was detected using the mAb HH7 (Thiel *et al.*, 1991). The polyclonal annexin VI antibodies have been described (Shadle *et al.*, 1985). Antibodies against the early endosome-associated protein EEA1 (Mu *et al.*, 1995) were kindly provided by Dr. Ban-Hock Toh (Monash Medical School, Melbourne, Australia). Immunoblotting was performed as described by Towbin *et al.* (1979) using 50 mM Tris, 50 mM boric acid as transfer buffer. Peroxidase-coupled secondary antibodies (Dako, Glostrup, Denmark) were used, and immunoreactive bands were detected using enhanced chemiluminescence (ECL) (Amersham-Buchler, Braunschweig, Germany) or a dye method using chloronaphthol.

RESULTS

Epitope Mapping Reveals the Existence of a Highly Immunogenic Region between the N-Terminal Domain and the First Annexin Repeat
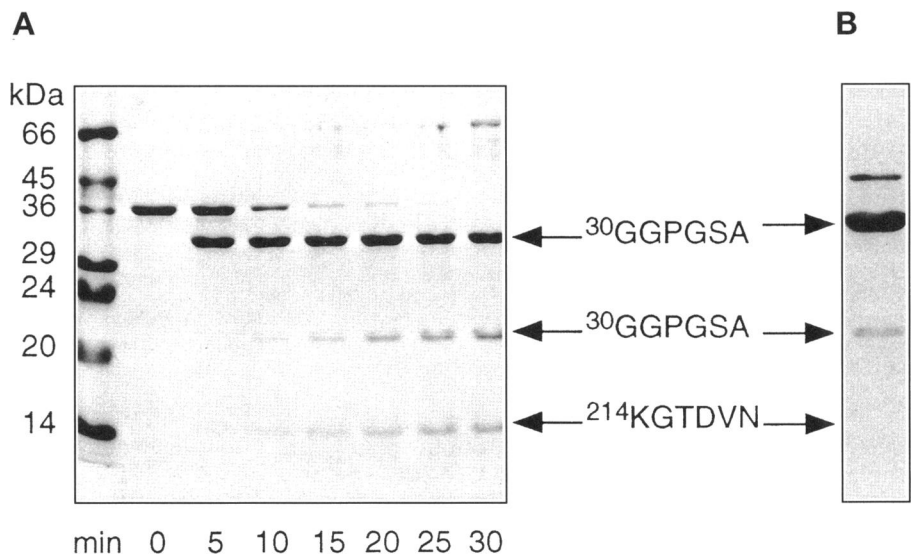
To obtain tools for analyzing the intracellular distribution and structural aspects of the annexin I molecule, we generated a series of mAbs against native porcine annexin I. Because structural information as to the folding of the polypeptide chain in solution can be obtained by describing antigenic regions likely to be exposed on the protein surface, we mapped the

epitopes for these mAbs. In a first set of experiments, we compared the reactivity of 10 different hybridoma supernatants with annexins I from human, porcine, bovine, rat, and pigeon tissue, respectively. Although all antibodies reacted in immunoblot analyses with bovine and porcine annexin I, none recognized the human, rat, or pigeon protein (our unpublished results). Similarly, all antibodies showed the same reactivity against different peptide fragments obtained after enzymatic or chemical cleavage of porcine annexin I. Limited trypsin treatment, for example, leads to the generation of a tryptic protein core starting at residue 30 and, after longer incubation times, to a second cleavage that occurs in the third annexin repeat between residues 213 and 214 (Figure 1A). Only the core and the N-terminal fragment starting at residue 30 were decorated by the antibodies, and the C-terminal core fragment starting at residue 214 was not labeled (a typical example for one mAb is shown in Figure 1B). Cleavage with CNBr yielded six fragments, of which only the most N-terminal one reacted with the panel of mAbs (our unpublished observation). All trypsin and CNBr cleavage sites were mapped by N-terminal sequence analysis of the respective fragments and alignment of these sequences with the deduced amino acid sequence of porcine annexin I. The latter was obtained by sequence analysis of the pig annexin I cDNA isolated by immunoscreening of a porcine lung cDNA library (see

MATERIALS AND METHODS). The porcine annexin I sequence on which the tryptic and CNBr fragments are indicated is given in Figure 2.

Inspection of the sequence between residues 30 (start of the reactive trypsin fragment) and 127 (end of the reactive CNBr fragment), which had to contain at least part of the epitope, revealed only four positions (residues 30, 49, 56, and 67, respectively) differing between the immunoreactive (cow, pig) and the non-reactive annexin I species (human, rat, pigeon). To map more precisely the residues conferring this species specificity, we generated N-terminally truncated porcine annexin I mutants (ΔA, ΔB, ΔC, ΔD), which were selected to contain 4, 3, 2, or only 1 of the residues differing between the species (Figure 2). cDNAs encoding the different mutants as well as full length annexin I were cloned into a procaryotic expression vector and expressed in *E. coli*. Probing of bacterial lysates containing the recombinant proteins with the panel of mAbs reveals that only full-length annexin I and the largest mutant protein are immunoreactive. (Figure 3B shows the representative result for one of the mAbs.) All other N-terminally truncated derivatives—although expressed at similar levels in the transformed bacteria (Figure 3A) and recognized by a polyclonal annexin I antibody (Figure 3C)—did not react with the mAbs. Again, all 10 mAbs show an identical reaction pattern with the N-terminal deletion mutants, indicating that all antibodies recognize the same epitope. In conjunction with the limited cross-

Figure 1. Limited tryptic digestion of porcine annexin I and analysis of tryptic fragments by immunoblotting with an mAb. (A) Annexin I purified from porcine lung was treated with trypsin at an enzyme-to-substrate ratio of 1:150. At the times indicated (0, 5, 10, 15, 20, 25, and 30 min), the reaction was stopped and the products were analyzed by SDS-PAGE. The tryptic fragments generated after 30 min were also transferred to polyvinylidifluoride membrane and characterized by N-terminal sequence analysis. The sequences are given in the one-letter code. The number indicates the position of the N-terminal residue of the respective fragment within the annexin I chain. Molecular weight markers of the molecular masses indicated are given in the first lane. Note that limited trypsin proteolysis leads to the generation of a relatively stable protein core, which starts at residue 30 with the sequence GGPGSA. The tryptic core is partially cleaved after longer incubation times, giving rise to an N-terminal fragment of ~20 kDa, starting with the same sequence as the core (^{30}GGPGSA), and a C-terminal fragment of ~15 kDa, starting at position 214 with the sequence KGTDVN. (B) The reaction products obtained after 30 min of trypsin treatment were transferred to nitrocellulose membrane and subjected to immunoblotting with the mAbs raised against porcine annexin I. The representative result for one antibody is shown. Note that the intact protein and the tryptic fragments starting at glycine 30 are recognized. In contrast, the C-terminal tryptic fragment starting at lysine 214 remains unlabeled.
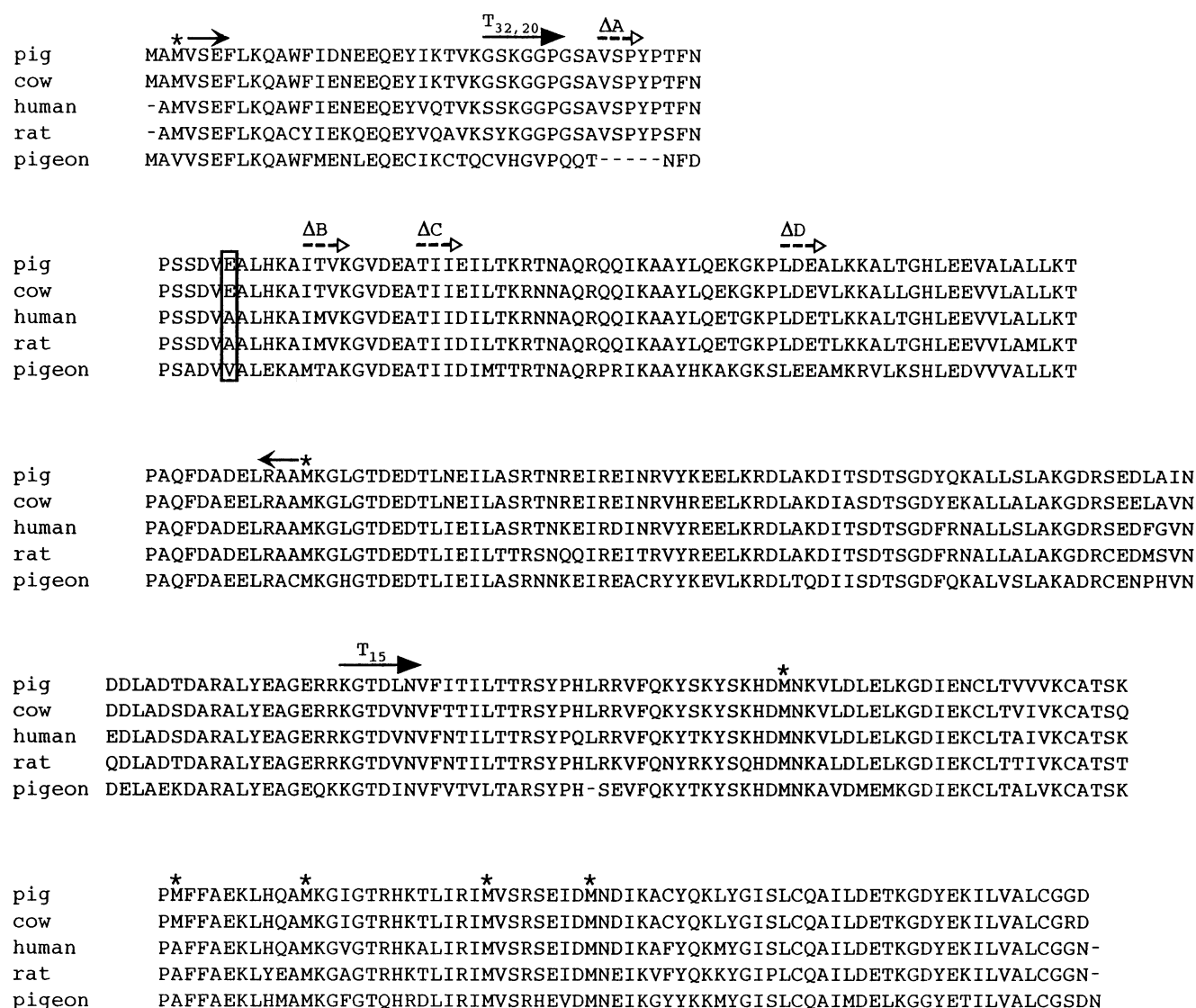
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                              T32,20        ΔA
                    *                        
pig       MAMVSEFLKQAWFIDNEEQEYIKTVKGSKGGPGSAVSPYPTFN
cow       MAMVSEFLKQAWFIENEEQEYIKTVKGSKGGPGSAVSPYPTFN
human     -AMVSEFLKQAWFIENEEQEYVQTVKSSKGGPGSAVSPYPTFN
rat       -AMVSEFLKQACYIEKQEQEYVQAVKSYKGGPGSAVSPYPSFN
pigeon    MAVVSEFLKQAWFMENLEQECIKCTQCVHGVPQQT-----NFD


                          ΔB        ΔC                            ΔD
                          -->       -->                           -->
pig       PSSDVEALHKAITVKGVDEATIIEILTKRTNAQRQQIKAAYLQEKGKPLDEALKKALTGHLEEVALALLKT
cow       PSSDVEALHKAITVKGVDEATIIEILTKRNNAQRQQIKAAYLQEKGKPLDEVLKKALLGHLEEVVLALLKT
human     PSSDVAALHKAIMVKGVDEATIIDILTKRNNAQRQQIKAAYLQETGKPLDETLKKALTGHLEEVVLALLKT
rat       PSSDVAALHKAIMVKGVDEATIIDILTKRTNAQRQQIKAAYLQETGKPLDETLKKALTGHLEEVVLAMLKT
pigeon    PSADVVALEKAMTAKGVDEATIIDIMTTRTNAQRPRIKAAYHKAKGKSLEEAMKRVLKSHLEDVVVALLKT


                         <----*
pig       PAQFDADELRAAMKGLGTDEDTLNEILASRTNREIREINRVYKEELKRDLAKDITSDTSGDYQKALLSLAKGDRSEDLAIN
cow       PAQFDAEELRAAMKGLGTDEDTLNEILASRTNREIREINRVHREELKRDLAKDIASDTSGDYEKALLALAKGDRSEELAVN
human     PAQFDADELRAAMKGLGTDEDTLIEILASRTNKEIRDINRVYREELKRDLAKDITSDTSGDFRNALLSLAKGDRSEDFGVN
rat       PAQFDADELRAAMKGLGTDEDTLIEILTTRSNQQIREITRVYREELKRDLAKDITSDTSGDFRNALLALAKGDRCEDMSVN
pigeon    PAQFDAEELRACMKGHGTDEDTLIEILASRNNKEIREACRYYKEVLKRDLTQDIISDTSGDFQKALVSLAKADRCENPHVN


                     T15
pig       DDLADTDARALYEAGERRKGTDLNVFITILTTRSYPHLRRVFQKYSKYSKHDMNKVLDLELKGDIENCLTVVVKCATSK
cow       DDLADSDARALYEAGERRKGTDVNVFTTILTTRSYPHLRRVFQKYSKYSKHDMNKVLDLELKGDIEKCLTVIVKCATSQ
human     EDLADSDARALYEAGERRKGTDVNVFNTILTTRSYPQLRRVFQKYTKYSKHDMNKVLDLELKGDIEKCLTAIVKCATSK
rat       QDLADTDARALYEAGERRKGTDVNVFNTILTTRSYPHLRKVFQNYRKYSQHDMNKALDLELKGDIEKCLTTIVKCATST
pigeon    DELAEKDARALYEAGEQKKGTDINVFVTVLTARSYPH-SEVFQKYTKYSKHDMNKAVDMEMKGDIEKCLTALVKCATSK


              *         *              *         *
pig       PMFFAEKLHQAMKGIGTRHKTLIRIMVSRSEIDMNDIKACYQKLYGISLCQAILDETKGDYEKILVALCGGD
cow       PMFFAEKLHQAMKGIGTRHKTLIRIMVSRSEIDMNDIKACYQKLYGISLCQAILDETKGDYEKILVALCGRD
human     PAFFAEKLHQAMKGVGTRHKALIRIMVSRSEIDMNDIKAFYQKMYGISLCQAILDETKGDYEKILVALCGGN-
rat       PAFFAEKLYEAMKGAGTRHKTLIRIMVSRSEIDMNEIKVFYQKKYGIPLCQAILDETKGDYEKILVALCGGN-
pigeon    PAFFAEKLHMAMKGFGTQHRDLIRIMVSRHEVDMNEIKGYYKKMYGISLCQAIMDELKGGYETILVALCGSDN
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Figure 2. Comparison of annexin I protein sequences from different species. A cDNA encoding porcine annexin I was isolated from a porcine lung cDNA library, and the deduced amino acid sequence was compared with that of bovine (Ernst, EMBL data library accession number X56649), human (Wallner *et al.*, 1986), rat (Tamaki *et al.*, 1987), and pigeon annexin I (Horseman, 1989). The N termini of fragments generated by limited trypsin treatment ($T_{32,20}$ and T_{15}) are indicated by arrows above the sequences. Smaller arrows highlight the N and C terminus of the large, most N-terminal CNBr fragment. Methionine residues, i.e., CNBr cleavage sites, are denoted by stars above the sequences. The N termini of the truncated annexin I mutants (ΔA, ΔB, ΔC, ΔD) are indicated by dashed arrows. The box marks the residue identified as a crucial part of the species-specific epitope (glutamic acid in immunoreactive annexin I molecules and alanine or valine in the nonreactive species).

species reactivity of the mAbs, the mutant results unambiguously identify glutamic acid residue 49 as a crucial part of the epitope. Although the immunoreactive annexin I molecules from pig and cow contain this glutamic acid, its replacement by alanine or valine in human, rat, or pigeon annexin I (Figure 2) renders the protein nonreactive toward the mAbs. In the crystal structure of an N-terminally truncated derivative of human annexin I, which starts at residue 33, the side chain at position 49 is located at the lateral pe-

riphery of the compact disk formed by the protein core (Weng *et al.*, 1993). A peripheral localization is consistent with our epitope mapping, indicating that the three-dimensional folding of the molecule in solution is comparable to that of the crystallized protein in the region of the epitope.

Annexin I Is Associated with Early Endosomes in a Ca²⁺-dependent Manner

As annexin I had been implicated in the endocytotic

Figure 3. Immunoblot analysis of N-terminally truncated annexin I derivatives. Full-length (wild-type) porcine annexin I or the N-terminally truncated ΔA (A), ΔB (B), ΔC (C), or ΔD (D) derivatives were expressed recombinantly in E. coli, and the total bacterial lysates were subjected to SDS-PAGE followed by staining of the gel with Coomassie blue (A) or by electrophoretic transfer of the proteins to nitrocellulose membranes (B and C). The transferred proteins were analyzed by immunoblotting with the mAbs generated against annexin I from procine lung (one representative example is shown in B) or with a polyclonal rabbit antibody raised against bacterially expressed porcine annexin I (C). Lysates obtained from bacteria transformed with the parental expression plasmid alone were included as a control (lane 0). Molecular weight markers of the molecular masses indicated are given in the leftmost lanes.
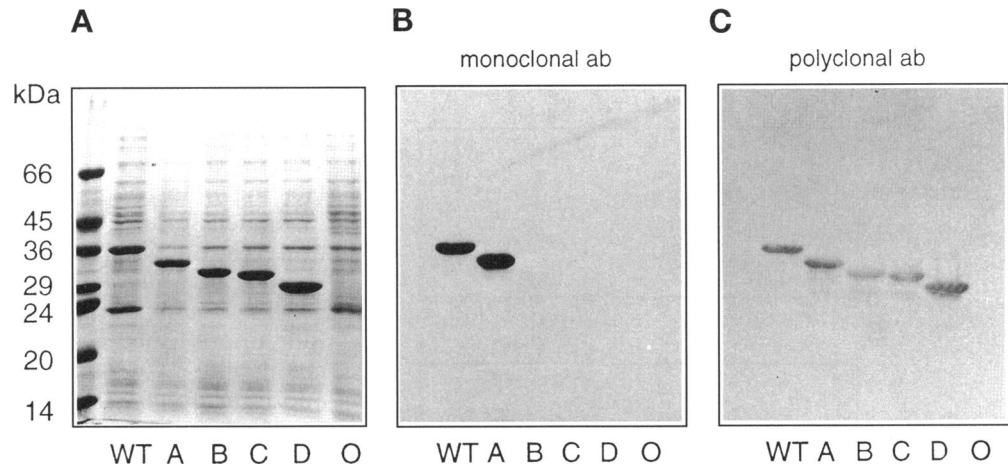
pathway (Futter et al., 1993), we analyzed whether it is enriched on certain endosomal membranes. Therefore, we prepared a PNS from BHK cells, separated fractions containing early and late endosomes on a flotation gradient (Gorvel et al., 1991; Aniento et al., 1996), and analyzed the different fractions by immunoblotting with a polyclonal annexin I antibody. In this and all subsequent endosome fractionation experiments, the enrichment and integrity of endosomes was routinely monitored by following the fate of HRP internalized from the fluid phase. Early endosomes were labeled by HRP uptake for 5 min, and a HRP treatment for 5 min followed by a 45 min chase in marker-free medium was used to label late endosomes. After gradient fractionation of the PNS, the endocytosed HRP was recovered at the 25%–35% sucrose interface after 5 min internalization (early endosomal membranes) and at the 25%–8% sucrose interface after the subsequent 45 min chase (late endosomal membranes). In all gradient analyses described here, the specific activity of HRP in the two endosomal fractions was enriched ~15- to 20-fold over that in the corresponding PNS, indicating the enrichment of early and late endosomes, respectively, in the different gradient fractions. Moreover, the fractionation of early endosomes was validated by including in the immunoblot analyses of the gradient fractions an antibody against EEA1, a protein of 180 kDa specifically associated with early endosomes (Mu et al., 1995) (see Figure 4D for a representative control).

Figure 4A shows that annexin I is indeed enriched in fractions containing endosomal membranes, in particular in those enriched in early endosomes. Interestingly, the presence of annexin I in these fractions seems to depend on the presence of Ca^{2+}, as it is lost when the preparation of the PNS and the subsequent gradient fractionation are performed in the presence of a Ca^{2+} chelator (Figure 4A). In contrast, the gradient fractionation of another annexin known to be present on early endosomes, i.e., annexin II (Emans et al., 1993), is not affected by the presence of EDTA, indicating that the association of annexin II with endosomal membranes is Ca^{2+}-independent (Figure 4B).

BHK cells contain several other members of the annexin family, including annexins IV and VI (our unpublished results). Because annexin VI had been identified on endosomal membranes prepared from rat liver (Jäckle et al., 1993), we also analyzed the BHK cell fractions for the presence of this annexin. Figure 4C shows that annexin VI is also found in fractions containing early and late endosomes. However, a particular enrichment when compared with the starting material, i.e., the PNS, is not observed. In contrast to annexin I, but similar to annexin II, the fractionation properties of annexin VI are not affected by Ca^{2+} chelation (Figure 4C), indicating that the intracellular membrane associations of different annexins are regulated differently by the divalent cation.

To corroborate that the cofractionation in the flotation gradient represents the association of annexin I with early endosomes, we examined the subcellular localization of the protein using ultrathin frozen sections of MDCK cells. Early endosomes in these cells were labeled before frozen sectioning by internalization of 5-nm BSA-gold from the apical and basal medium of the filter-grown MDCK cells. The ultrastructural analysis clearly reveals that annexin I is present on early endosomal membranes (Figure 5, C and D). Moreover, the plasma membrane (Figure 5, A and B) and tubular elements close to the Golgi that may correspond to the trans-Golgi network (our unpublished observation) are labeled with the annexin I antibody. MDCK cells were chosen for the immunolocalization because this cell type enabled us to examine the Ca^{2+} dependence of the intracellular membrane binding of annexin I by an independent prefixation labeling tech-
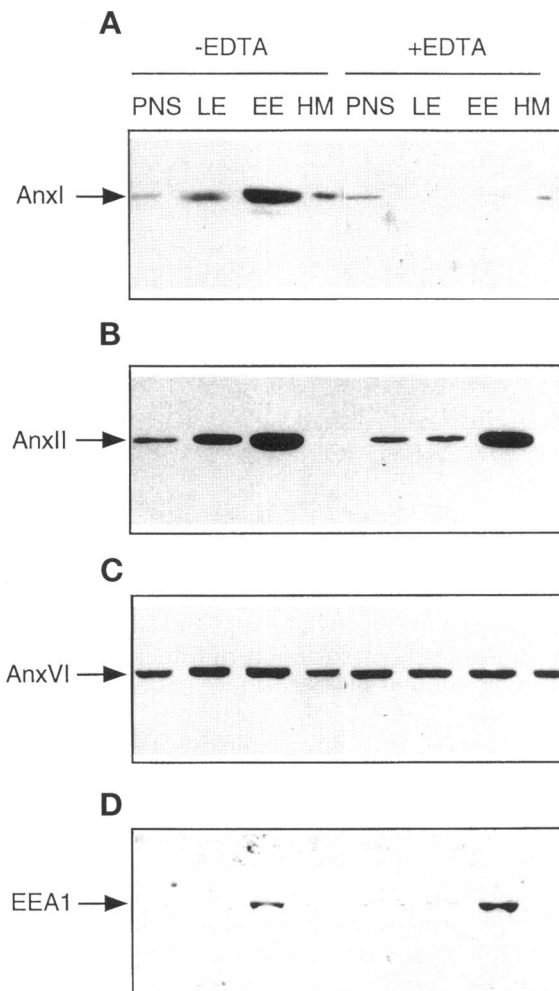
Figure 4. Distribution of different annexins among membrane fractions from BHK cells. A PNS prepared from BHK cells was subjected to fractionation of membranes on a flotation gradient (Gorvel *et al.*, 1991; Aniento *et al.*, 1996) in the absence (−) or presence (+) of EDTA. Fractions enriched in late and multivesicular endosomes (LE), early endosomes (EE), and heavy membranes (HM) were collected. Equivalent amounts of these fractions and of the PNS were subjected to SDS-PAGE and subsequent immunoblotting with polyclonal antibodies directed against annexin I (A), annexin II (B), annexin VI (C), and the early endosome-associated protein EEA1 (D) (Mu *et al.*, 1995), respectively. The latter was included as a compartment marker for early endosomes and clearly shows the enrichment of early endosomes in the respective gradient fractions. Note that annexin I is enriched in fractions containing early endosomes and that this cofractionation is abolished in the presence of a Ca^{2+} chelator. Addition of EDTA does not change the amount of annexin I present in the PNS. The PNS represents the starting material before gradient separation containing both membrane-bound and soluble protein and thus, also, the annexin I released from membranes by Ca^{2+} chelation. In contrast to annexin I, the presence of annexin II in the endosomal membrane fractions is not affected by the addition of EDTA.

nique. Therefore, the early endosomes of MDCK cells were labeled with HRP as a fluid phase marker, and

the cells were then mechanically perforated using nitrocellulose to partially rip off the apical surface (Simons and Virta, 1987). Cells fixed immediately after rip-off show specific annexin I labeling of the plasma membrane, HRP labeled endosomes, and unlabeled vesicles (Figure 6A). However, the specific antibody labeling is lost when the ripped-off MDCK cells are incubated in an EGTA-containing buffer before fixation (Figure 6B). In contrast, the early endosomal staining obtained in ripped-off MDCK cells with antibodies to annexin II is unaffected by Ca^{2+} chelation (our unpublished results). These ultrastructural data are in line with our gradient fractionation analyses and indicate that the annexin I identified in fractions enriched in early endosomes (Figure 4A) is indeed associated with these membranes and not with other contaminating but cofractionating structures.

The Association of Annexin I with Early Endosomes Is Affected by Mutations within the N-Terminal Domain of the Molecule

Previous studies identified annexin I as a major substrate for the internalized EGF receptor kinase in multivesicular bodies (MVBs) of NIH 3T3 cells stably expressing the human EGF receptor (Futter *et al.*, 1993). Moreover, it was reported that annexin I associated with MVBs in a Ca^{2+}-independent manner and that this association was rendered Ca^{2+}-dependent upon phosphorylation by the EGF receptor kinase (Futter *et al.*, 1993). Our subcellular fractionation and immunocytochemical studies, however, have provided no evidence for a Ca^{2+}-independent interaction of annexin I with endosomal membranes and have localized the majority of annexin I to fractions enriched in early endosomes (see above). The specificity of annexin–membrane interactions and probably also its Ca^{2+} dependence is likely to be mediated through the regulatory N-terminal domain, which harbors the major phosphorylation sites (for review, Raynal and Pollard, 1994). Therefore, we analyzed whether this N-terminal domain of annexin I or certain modifications within it could be responsible for the different Ca^{2+} require-

Figure 5 (on facing page). Immunolocalization of annexin I in MDCK cells. MDCK II cells grown on filters were incubated with 5 nm of BSA-gold present in the apical and basal medium to label early endosomes (see MATERIALS AND METHODS) and then fixed and processed for frozen sectioning. Sections were labeled with the rabbit polyclonal antibody to annexin I followed by 10 nm of protein A-gold. Specific labeling is associated with the apical (ap in A), lateral (la in B and C), and basal plasma membrane domains. Note the nonuniform distribution of gold particles (arrowheads) on the cell surface. (C and D) Annexin I labeling associated with early endosomes labeled with 5 nm of BSA-gold (arrows). Note the typical morphology of the early endosomes in these cells; a ring-shaped cisterna surrounding an electron-lucent area is labeled on both faces with antibodies to annexin I. Bar, 100 nm.
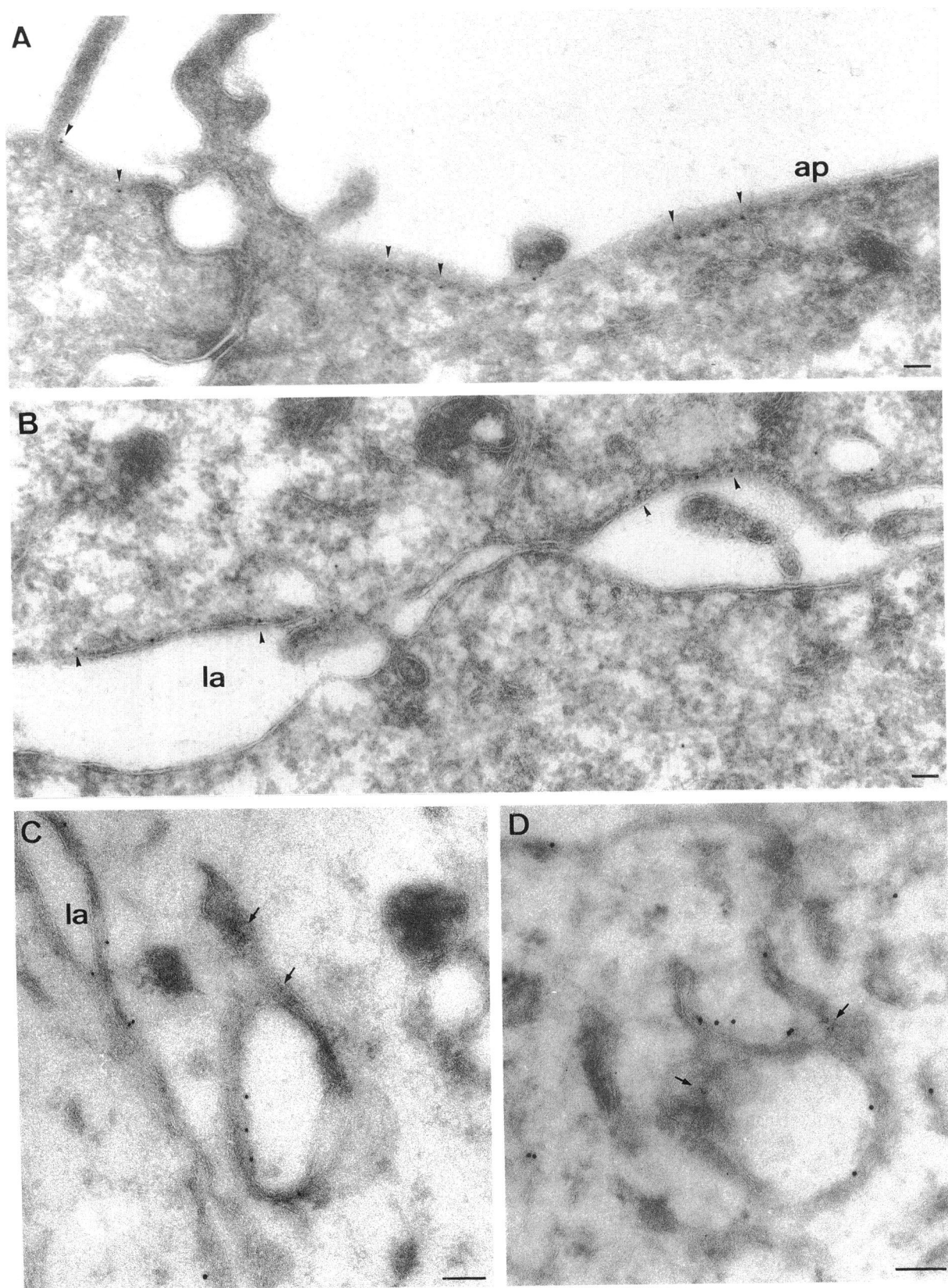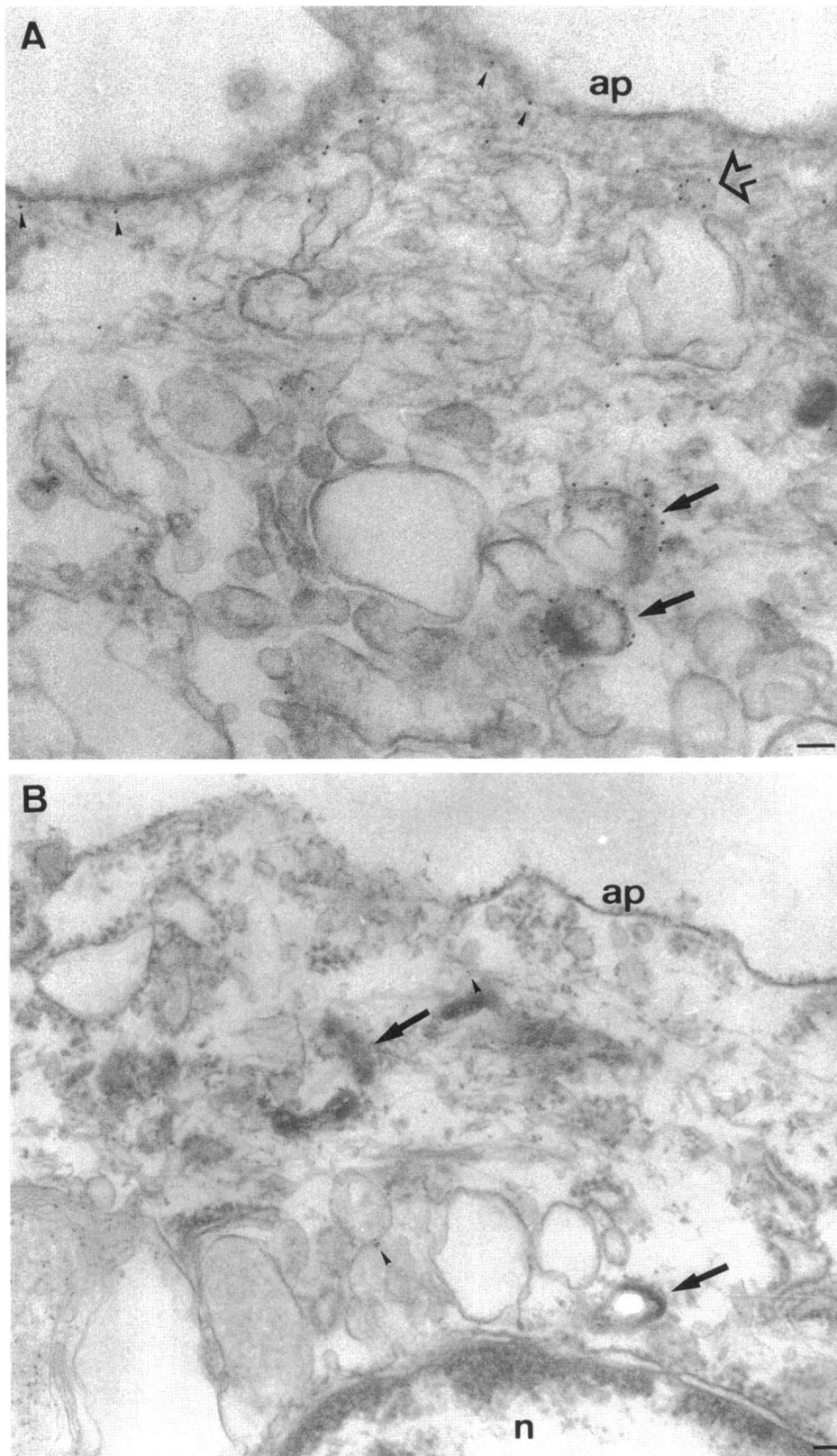
Figure 5 (cont).

Figure 6. Immunolocalization of annexin I in mechanically perforated MDCK cells. MDCK II cells grown on filters were incubated with HRP in the apical and basolateral medium for 10 min at 37°C. They were then washed and permeabilized by removal of part of the apical surface (ap) with nitrocellulose filters. The cells were washed again to remove cytosol and either fixed immediately with paraformaldehyde (A) or further incubated in buffer containing EGTA for 30 min (B). Subsequently, the cells were incubated with the polyclonal antibody to annexin I followed by 5 nm of protein A-gold before visualization of HRP reaction product and embedding in Epon. Prefixed cells (A) show annexin I labeling associated with HRP-labeled early endosomes (solid arrows), as well as with the plasma membrane (arrowheads) and unlabeled vesicles (open arrows). In contrast, in the cells incubated with EGTA, there is negligible labeling for annexin I (gold particles indicated by arrowheads). Bar, 100 nm.

Figure 7. Cofractionation of mutant annexin I derivatives with different endosomal membranes. BHK cells were transfected with expression plasmids encoding wild-type (WT) porcine annexin I or two N-terminally truncated derivatives (Δ1-13, Δ1-26). A PNS from the respective cells was subjected to fractionation on the flotation gradient (Gorvel *et al.*, 1991; Aniento *et al.*, 1996) performed in the absence (A–C) or presence (D) of EDTA. Equal amounts of the membrane fractions enriched in late and multivesicular endosomes (LE), early endosomes (EE), or heavy membranes (HM) were then analyzed by immunoblotting with one of the mAbs against annexin I (top panels) or with a polyclonal antibody against annexin II (bottom panels). Because of the limited cross-species reactivity, the mAb recognizes only the ectopically expressed porcine annexin I derivatives and not the endogenous hamster protein. Note that wild-type and Δ1-13 annexin I show the same subcellular fractionation as the endogenous protein, i.e., an enrichment in fractions containing early endosomal membranes, whereas the Δ1-26 mutant fails to cofractionate with early endosomes. The latter derivative is enriched in fractions containing late and multivesicular endosomes, and this association remains EDTA-sensitive. Note also that the distribution of annexin II, a protein known to be enriched on early endosomes (Emans *et al.*, 1993; Mayorga *et al.*, 1994; Aniento *et al.*, 1996), is not affected in the transfected cells.
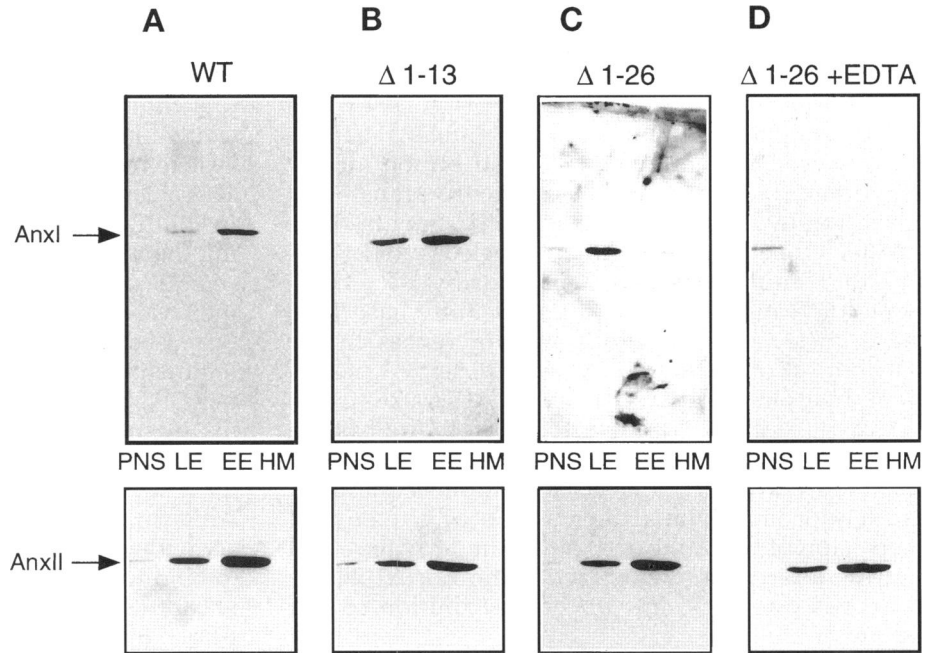
ments and specificities of the annexin I-endosome interactions observed in BHK cells and in NIH 3T3 cells overexpressing the EGF receptor. We used transient transfections to express annexin I derivatives harboring mutations in the N-terminal domain in BHK cells and analyzed the endosomal association of the respective mutant proteins using the flotation gradient described above. Because all expression constructs were based on the porcine annexin I cDNA, we could use the mAbs characterized in the first part of this study as specific probes. These antibodies recognize the ectopically expressed porcine annexin I derivatives, but do not react with the endogenous hamster protein.

Ectopically expressed wild-type porcine annexin I served as a control. As demonstrated in Figure 7A, this protein exhibits essentially the same enrichment in fractions containing endosomal membranes as the endogenous BHK annexin I. Similarly, an N-terminally truncated mutant protein missing residues 1-13 (Δ1-13), a region of the molecule which in annexin II harbors the binding site for the intracellular protein ligand p11 (Johnsson *et al.*, 1988), shows the distribution among endosomal fractions already observed for the endogenous molecule, i.e., a particular enrichment on early endosomal membranes (Figure 7B). However, when an additional 13 residues of the N-terminal domain are deleted (Δ1-26 mutant), the relative amount of mutant protein cofractionating with the different endosomal membranes is changed significantly. Δ1-26 annexin I is highly enriched in fractions containing

late and multivesicular endosomes, but absent from or present in only minor amounts in fractions containing early endosomes (Figure 7C). The distribution of ectopically expressed annexin I derivatives was analyzed in a number of independent experiments, and representative examples are shown in Figure 7. Slight variations are observed between experiments; for example, slightly more annexin I is present in the late endosome fraction from pCMV Δ1-13 transfected cells compared with the pCMV AnxI transfected cells in the experiments depicted in Figure 7. These are most likely caused by small differences in the individual membrane preparations (the enrichment of the endosomal membranes varied between 15- and 20-fold in the different experiments; see above). However, wild type and Δ1-13 annexin I were always found enriched in fractions containing early endosomes; at least 65% of the membrane-associated wild type and Δ1-13 annexin I cofractionated with early endosomes, whereas between 10 and 25% were found in fractions containing late and multivesicular endosomes. Δ1-26 annexin I, on the other hand, was always enriched in the late/multivesicular endosome fractions with 60–80% of the membrane-associated Δ1-26 annexin I being present in this fraction.

To exclude the possibility that the shift in the distribution of Δ1-26 annexin I is due to an alteration in the density of endosomal compartments possibly induced by the ectopic expression of the truncated annexin I, we probed all gradient frac-

tions from the transfected cells with antibodies to annexin II, a protein known to be enriched on early endosomes (Emans et al., 1993; Mayorga et al., 1994; Aniento et al., 1996) (see also Figure 4). As shown in the bottom panels of Figure 7, no significant changes are observed in the annexin II distribution among membrane fractions from the differently transfected cells. Moreover, the endosomal compartments of the transfected cells were routinely labeled by HRP uptake for 5 min (early endosomes) or by HRP treatment for 5 min followed by a 45 min chase (late endosomes), and no significant differences in the distribution of HRP activity were observed in gradient fractions prepared from pCMVΔ1-26–transfected, pCMVΔ1-13–transfected, pCMVAnxI-transfected, or nontransfected cells. These controls show that the flotation gradient distribution of the principle endosomal compartments is not affected by the expression of the truncated annexin I derivative and that the altered gradient fractionation of Δ1-26 annexin I is due to a shift in the distribution of this mutant protein.

As already observed for endogenous annexin I (Figure 4A), the association of Δ1-26 annexin I with endosomal membranes (in this case, late and multivesicular endosomes) is sensitive to EDTA (compare Figure 7, C and D). This indicates that the subcellular fractionation of annexin I, but not its Ca^{2+} dependence, is regulated through the N-terminal domain of the molecule. Interestingly, the portion of the N-terminal domain deleted in the Δ1-26 annexin I mutant contains the residue phosphorylated by the EGF receptor kinase (Tyr-21), as well as at least one putative protein kinase C phosphorylation site (Thr-24) (Wang and Creutz, 1994). However, the switch in cofractionation from early endosomes to late and multivesicular endosomes is not simply due to a removal of the phosphorylatable Tyr-21 in the Δ1-26 mutant, because a site-specifically mutated annexin I derivative containing a cysteine for tyrosine replacement at position 21 shows the same gradient fractionation characteristics as endogenous or ectopically expressed wild-type annexin I (our unpublished results). Thus, more complex phenomena or different post-translational modifications occurring in the region encompassing residues 13–26 must account for the shift from early to late endosomal distribution observed for the Δ1-26 mutant protein.

DISCUSSION

Despite the accumulating evidence for an involvement of annexins in membrane transport and/or membrane organization, their precise mechanism of action is not known. Several of the proteins, including annexins I, II, III, and VII, are able to aggregate certain membrane vesicles in vitro and, at least in some cases, seem to be able to stimulate spontaneous membrane fusions. However, they are probably not truly fusogenic proteins because annexins require cofactors such as cis-unsaturated fatty acids or diacylglycerol to significantly increase the rate of membrane fusion in in vitro systems (for review, Creutz, 1992; Raynal and Pollard, 1994). Thus, annexins may act in overall membrane fusion by establishing a membrane contact and holding the membranes in place for the actual fusion process. Based on the ability of at least some annexins to interact with cytoskeletal elements, it has also been proposed that annexins serve a structural role by linking membranous structures to the cytoskeleton (Glenney, 1987; Gerke, 1989). The high abundance of annexins in some cell types, e.g., endothelial and certain epithelial cells, also provides circumstantial evidence for a structural rather than a specific catalytic role. Annexin I belongs to the group of annexins that interact with cytoskeletal elements, and it has been shown to bind to and even bundle actin filaments. However, in contrast to annexin II, which in its heterotetrameric, p11-complexed form exhibits F-actin binding and bundling activity at submicromolar levels of Ca^{2+} (Ikebuchi and Waisman, 1990), unphysiologically high Ca^{2+} concentrations are required for this effect in the case of annexin I (Glenney et al., 1987; Haigler and Schlaepfer, 1987). Although this finding questions the relevance of the actin-binding properties for the function of annexin I in vivo, the protein seems to colocalize, at least to some extent, with the cortical microfilament network in fibroblasts (Glenney et al., 1987). Because this cortical region of the cell also contains early endosomes and because annexin I shows a punctate and not a uniform distribution at the light microscope level (Glenney et al., 1987), it may be involved in establishing connections between the cortical cytoskeleton and early endosomal membranes. A similar role has been proposed for annexin II, another microfilament-binding annexin (Harder and Gerke, 1993).

Annexin I is able to aggregate membrane vesicles in vitro and thus may also or alternatively be involved in connecting membrane surfaces within the cell. Vesicle aggregation is not a general property of the annexins. Mutational studies with chimeric derivatives containing parts of annexin I and parts of the aggregation-inactive annexin V indicate that sequences of the N-terminal domain and of the first annexin repeat of annexin I, respectively, are critical for aggregation activity (Ernst et al., 1991; Andree et al., 1993). The region shared by all aggregation-competent chimera constitutes amino acids 41–46, i.e., a sequence directly neighboring the mAb epitope mapped in this study (Figure 2). Thus, we can expect this region to be easily accessible on the lateral surface of the folded molecule, as also indicated by the crystal structure analysis of a Δ1-32 annexin I derivative (Weng et al., 1993) and by the finding that the epitopes for a series of mAbs that

block phospholipid binding map to amino acids 42–99 of annexin I (Ernst, 1993).

Several biochemical properties of annexin I, including vesicle aggregation, are regulated by phosphorylation in the N-terminal domain. Protein kinase C phosphorylation at residues Thr-24, Ser-27, and/or Ser-28, for example, significantly inhibits the ability of annexin I to aggregate phospholipid vesicles and chromaffin granules by increasing the Ca^{2+} requirement for this reaction (Wang and Creutz, 1992; Johnstone et al., 1993). The effect on vesicle aggregation of EGF receptor-catalyzed tyrosine phosphorylation at Tyr-21, on the other hand, has not been elucidated, although it seems clear that this phosphorylation decreases the Ca^{2+} concentration required for phospholipid binding (Schlaepfer and Haigler, 1987; Ando et al., 1989). Moreover, phosphorylation by the EGF receptor converts a certain form of annexin I, which remains associated with membranes prepared from human placenta in the absence of Ca^{2+}, into a Ca^{2+}-regulated form whose membrane association is sensitive to chelation of the divalent cation (Sheets et al., 1987). The MVBs of NIH 3T3 cells expressing the human EGF receptor also contain a Ca^{2+}-insensitive form of annexin I, which is rendered Ca^{2+}-sensitive upon phosphorylation by the receptor kinase. Because this phosphorylation event is specific for MVBs and not observed for plasma membrane-bound annexin I, it has been proposed that MVB-associated annexin I is involved in driving inward vesiculation and that the release of annexin I through tyrosine phosphorylation is then required for the inward release of the vesicles (Futter et al., 1993).

This hypothesis is not easily reconcilable with our findings because we could not identify a Ca^{2+}-insensitive form of annexin I on endosomal fractions prepared from BHK cells. This is unlikely to be due to experimental artefacts, because another endosome-associated annexin, annexins II, continues to copurify with the endosomal membrane fractions in the absence of Ca^{2+}, thus serving as an internal control (Figure 4). Moreover, the majority of annexin I identified in our membrane fractionation approach copurifies with early endosomes and not with the fraction containing late endosomes and endosomal carrier vesicles or MVBs. Likewise, our ultrastructural characterization of the annexin I distribution provides evidence for an association with early endosomal membranes. The reason for this discrepancy is not clear, but it might relate to the fundamental differences in the experimental approaches taken. While Futter et al. (1993) analyzed the presence of annexin I in MVB fractions purified in the absence of Ca^{2+} by in vitro phosphorylation through the endogenous EGF receptor kinase and subsequent immunoprecipitation with an annexin I antibody, we used Western blotting of subcellular fractions and immunoelectron microscopy

to obtain a general picture of the annexin I distribution. The phosphorylation reaction is probably more sensitive, and a minor amount of Ca^{2+}-insensitive annexin I in MVBs, which would be below the level of detection in our experimental approaches, could have escaped our attention. Such MVB-associated annexin I could possibly vary with the amount of internalized EGF receptor, and it is likely to be higher in NIH 3T3 cells overexpressing the human EGF receptor than in BHK cells containing a considerably smaller number of receptor molecules. Nonetheless, we can conclude from our data that, at least in BHK and MDCK cells, the majority of membrane-associated annexin I resides on early endosomes and that this association depends on the presence of micromolar concentrations of Ca^{2+}. This early endosomal annexin I could fulfill a structural role in organizing endosomal membranes and/or establishing membrane–membrane contacts through its aggregating activity.

The cofractionation of annexin I with early endosomes critically depends on the presence of an intact N-terminal domain in the molecule. Whereas a truncation of amino acids 1-13 has no effect in our assay, the removal of an additional 13 residues in the Δ1-26 mutant leads to a derivative that no longer cofractionates with early endosomes but instead appears in a fraction containing late and multivesicular endosomes. The region of the molecule affecting the subcellular distribution of annexin I, i.e., residues 13–26, contains the site phosphorylated by the EGF receptor kinase as well as at least one protein kinase C phosphorylation site. However, phosphorylation at these residues does not seem to alter the subcellular localization, because mutant annexin I derivatives harboring a Cys for Tyr-21 or an Ala for Thr-24 and a Val for Ser-28 substitution, respectively, show the same behavior in the flotation gradient as wild-type annexin I (our unpublished results). This leaves two possible explanations for our observation. First, a specific receptor recognizing a site in the sequence between residues 13 and 26 is present in or on endosomal membranes. When the receptor binding site is removed within annexin I, the truncated protein shows a different binding characteristic and interacts preferentially with membranes present in the late endosome fraction, possibly because the lipid composition is better suited for binding the Δ1-26 derivative. Second, the N-terminal domain is intramolecularly communicating with the Ca^{2+}/phospholipid-binding protein core, thereby regulating its membrane-binding properties. The N-terminal domain or parts of it could, for example, sterically interfere with phospholipid binding, or they could induce conformational changes in the rest of the molecule, which would alter membrane-binding specificities in favor of early endosomal membranes. Biochemical evidence argues for a direct regulatory role of the N-terminal domain because

different N-terminally truncated variants of annexin I exhibit markedly different Ca^{2+} sensitivities in a phospholipid column binding assay (Ando et al., 1989), as well as in a chromaffin granule aggregation assay (Wang and Creutz, 1994). The effect of a truncation of the N-terminal region comprising residues 1-26 on the aggregation properties and the subcellular localization could be of physiological importance because Tyr-21 phosphorylation by the EGF receptor kinase renders the annexin I molecule more sensitive to proteolytic attack, which removes part of the N-terminal domain (Haigler et al., 1987; Chuah and Pallen, 1989). Future experiments need to clarify whether challenge with EGF indeed causes the generation of N-terminally truncated derivatives of annexin I and whether such derivatives translocate from early to late or multivesicular endosomes.

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