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Identification and characterization of a novel type of annexin-membrane interaction: Ca^{2+} is not required for the association of annexin II with early endosomes

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SUMMARY

Annexin II, a member of a family of Ca^{2+} and membrane binding proteins, has been implicated in regulating membrane organization and membrane transport during endocytosis and Ca^{2+} regulated secretion. To characterize the mechanistic aspects of the annexin II action we studied parameters which determine the endosomal association of annexin II. Immunoblot analysis of subcellular membrane fractions prepared from BHK cells in the presence of a Ca^{2+} chelating agent reveals that annexin II remains associated with endosomal membranes under such conditions. This annexin II behaviour is atypical for the Ca^{2+} regulated annexins and is corroborated by the finding that ectopically expressed annexin II mutants with inactivated Ca^{2+} binding sites continue to co-fractionate with endosomal membranes. The Ca^{2+}-independent membrane association of annexin II is also not affected by introducing mutations interfering with the complex formation of annexin II with its intracellular protein ligand p11. However, a deletion of the unique N-terminal domain of annexin II, in particular the sequence spanning residues 15 to 24, abolishes the Ca^{2+}-independent association of the protein with endosomes. These results describe a novel, Ca^{2+}-independent type of annexin-membrane interaction and provide a first explanation for the observed preference of different annexins for different cellular membranes. In the case of annexin II this specificity could be mediated through specific membrane receptors interacting with a unique sequence in the annexin II molecule.

Key words: Ca^{2+}/phospholipid binding protein, Endocytosis, Membrane-cytoskeleton interaction

INTRODUCTION

The Ca^{2+}-dependent regulation of membrane-cytoskeleton and membrane-membrane interactions plays an important role in a number of biological processes ranging from the control of cell and organelle shape to that of certain membrane traffic events. Among the components thought to be involved in such Ca^{2+}-mediated processes are the annexins, members of a multigene family of Ca^{2+}/phospholipid-binding proteins widely distributed among species. Typically, all annexins share as a characteristic biochemical property the ability to bind to negatively charged phospholipids and cellular membranes in a Ca^{2+}-dependent manner, and some members of the family also interact Ca^{2+}-dependently with certain cytoskeletal elements (for reviews see Creutz, 1992; Raynal and Pollard, 1994). These annexin properties are displayed by a well conserved protein core domain which is resistant to limited proteolysis and comprises a four- or eightfold repetition of a segment of 70-80 amino acid residues, the annexin repeat. Each annexin repeat harbours one or two novel types of Ca^{2+} binding sites. These differ in architecture from the EF hand motif (Moews and Kretsinger, 1975) and involve in addition to other elements a highly conserved acidic amino acid whose carboxyl oxygens are crucially important for Ca^{2+} coordination (Huber et al., 1990; Weng et al., 1993; Jost et al., 1992, 1994). Unique within the individual members of the annexin family is the N-terminal domain which precedes the protein core and varies in length and sequence. It is thought to be of regulatory importance as it harbours in several annexins phosphorylation sites for different signal transducing kinases (for review see Raynal and Pollard, 1994).

Annexin II is implicated in several membrane transport steps. These include the Ca^{2+}-regulated secretion since annexin II, which is a prominent component in chromaffin granule preparations, is able to aggregate these vesicles at Ca^{2+} levels which are close to those observed in stimulated chromaffin cells, and partially restores the secretory responsiveness in permeabilized chromaffin cells (Ali et al., 1989; Sarafian et al., 1991; for review see Creutz, 1992). Such Ca^{2+}-regulated function of annexin II in the exocytotic pathway seems to depend on a complex formation of the protein with its intracellular ligand, the S100 protein p11. Complex formation, which leads to an annexin II_2p11_2 heterotetramer, is mediated through the N-terminal domain of annexin II (for review see

Weber, 1992) and is a prerequisite for anchoring annexin II in the cortical region of cultured cells (Thiel et al., 1992; Jost et al., 1994). Based on these findings it had been suggested that the annexin II-p11 complex may participate in Ca^{2+}-regulated exocytosis by linking exocytotic vesicles to the cortical cytoskeleton and/or the plasma membrane and thereby positioning the vesicle at the correct place in the cell (for reviews see Creutz, 1992; Gerke, 1996).

Several lines of evidence indicate that annexin II is also involved in endocytotic processes. The protein is found on isolated early endosomal membranes and is one of the few proteins transferred from a donor to an acceptor endosomal membrane in an in vitro fusion assay (Emans et al., 1993). Moreover, ectopic expression of a *trans*-dominant mutant for the annexin II-p11 complex, which leads to the intracellular aggregation of annexin II and p11, specifically affects early endosomes which are translocated to the site of the aggregates (Harder and Gerke, 1993). Finally, in a cell-free system purified annexin II reconstitutes in conjunction with arachidonic acid a Ca^{2+}-dependent fusion among endosomes which previously had been washed with the Ca^{2+} chelating EDTA (Mayorga et al., 1994).

The mechanism by which annexin II affects fusion properties and/or the organization and intracellular location of endosomal membranes is not known. To shed light on this mechanism and to elucidate the structural requirements for an annexin II-endosome interaction we analyzed the subcellular fractionation of ectopically expressed mutant derivatives of annexin II. We show that the co-fractionation of annexin II with endosomal membranes is not regulated by Ca^{2+}- and p11-binding. This Ca^{2+}-independent membrane association does, however, depend on the presence of amino acids 15 to 24 of the unique N-terminal domain of annexin II.

MATERIALS AND METHODS

Expression constructs

The cDNA encoding human annexin II (Huang et al., 1986) served as a template for oligonucleotide-directed mutagenesis (Kunkel, 1985) which was employed to generate mutant cDNAs encoding defective Ca^{2+} (CM) and/or p11 (PM) binding sites (Jost et al., 1994). The mutants containing a deletion of the region encoding the N-terminal 14 or 24 amino acid residues were constructed by PCR using the wild-type (WT) cDNA as a template and the oligonucleotides Δ1 (5′ CTGTGCAAGCTCGAATTCGAGATGTCTCACTCTACACC 3′) or Δ2 (5′ CTACACCCCCGAATTCATATATGTCTGTCAAAGCC 3′) as sense and the oligonucleotide Δ3 (5′ GACCTGTTATCTAGAAG-CATGGTG 3′) as antisense primers, respectively. All annexin II cDNAs also contained a single nucleotide exchange resulting in a glutamic acid for alanine replacement at amino acid position 65. This substitution installed the epitope for the monoclonal antibody H28 and thus enabled us to use this antibody to specifically detect the recombinantly expressed annexin II derivatives (Thiel et al., 1991). For expression in BHK cells the individual annexin II cDNAs were cloned into the pCMV5 vector to yield the constructs pCMV-WT, pCMV-PM, pCMV-CM, pCMV-PMCM, pCMV-Δ1-14, and pCMV-Δ1-24. The presence of the individual mutations was verified by dideoxy sequencing (Sanger et al., 1977).

Cell culture and transfection

Hamster BHK cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% fetal calf serum (Boehringer, Mannheim). Transient transfection employed a modified calcium phosphate precipitation method (Chen and Okayama, 1987) using 20 μg of the respective plasmid DNA per 100 mm dish of cells grown to 50% confluency. After addition of the DNA, cells were incubated for 12 to 16 hours at 35°C in 3% CO_2, then washed with PBS and cultivated in fresh medium using normal culture conditions. Between 30 and 40% of the total cell population was expressing the exogenous protein 40 hours following transfection, as revealed by routine immunofluorescence analysis with the monoclonal antibody H28.

Fractionation of endosomal membranes

Labeling and fractionation of endosomes was carried out essentially as described by Gorvel et al. (1991) and Aniento et al. (1996). Briefly, four 100 mm dishes of untreated BHK cells or six dishes of transfected cells were incubated at 37°C with 2 mg/ml horseradish peroxidase (HRP) in IM (internalization medium: Dulbecco's modified Eagel's medium supplement with 10 mM Hepes, pH 7.4). To label early endosomes the HRP incubation was carried out for five minutes. Late endosomal labeling was achieved by treating the cells for five minutes with HRP in IM followed by a 45 minute chase in IM supplement with 2 mg/ml bovine serum albumin. Subsequently, the cells were lysed in HB buffer (0.25 M sucrose, 3 mM imidazole-HCl, pH 7.4) followed by a low speed centrifugation to yield a post-nuclear supernatant (PNS). The PNS was brought to 40.6% sucrose, 3 mM imidazole-HCl, pH 7.4, and placed at the bottom of a SW 60 centrifugation tube (Beckman). This load was overlaid with 1.5 ml of 35% sucrose, 3 mM imidazole-HCl, pH 7.4, then with 1 ml 25% sucrose, 3 mM imidazole-HCl, pH 7.4, and finally with 0.5 ml HB buffer. In some experiments, the sucrose solutions were supplemented with 1 mM EDTA to reduce the free Ca^{2+} concentration. The step gradient was centrifuged at 35,000 rpm for 60 minutes at 4°C. Fractions containing late and mulitvesicular endosomes were collected at the 25% sucrose-HB interface and early endosomes were enriched at the 25%-35% sucrose interface. Fractions containing heavy membranes (HM) were collected at the 35%-40.6% sucrose interface. The activity of endocytosed HRP present in the different fractions was analyzed as described (Gorvel et al., 1991) and latency was measured according to Bomsel et al. (1990). Proteins present in the different fractions were concentrated by chloroform/methanol precipitation (Wessel and Flügge, 1984) and 10 μg of each fraction (as determined according to Bradford, 1976) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) and analyzed by immunoblotting (Towbin et al., 1987).

Immunofluorescence analysis of annexin II and transferrin receptor distribution

BHK cells grown on glass coverslips were co-transfected with an expression construct encoding the human transferrin receptor (hTfR) cDNA (pCMV-hTfR; Harder and Gerke, 1993) and with one of the expression constructs encoding wild-type (WT) or mutant annexin II derivatives (pCMV-WT, pCMV-PMCM, pCMV-Δ1-24). At 40 hours post transfection the cells were incubated for 1 hour in serum-free medium and then for 30 minutes in serum-free medium containing 20 μg/ml human transferrin (Boehringer) to obtain an efficient hTfR internalization into early and recycling endosomes. Subsequently, the coverslips were washed briefly in cold PBS and placed, cells facing down, for 10 minutes at 4°C onto a 50 μl drop of a solution containing activated streptolysin O (SLO; obtained from Dr S. Bhakdi, University of Mainz, FRG; Bhakdi et al., 1993). SLO was employed at 5 μg/ml in intracellular transport buffer (ICT; 78 mM KCl, 4 mM $MgCl_2$, 8.37 mM $CaCl_2$, 10 mM EGTA and 1 mM DTT; Burke and Gerace, 1986). EGTA in the ICT buffer was omitted in experiments analyzing the Ca^{2+}-dependent association of Δ1-24 annexin II with endosomes. Following incubation at 4°C the excess SLO that did not bind to the plasma membrane was removed by two washes with cold ICT. For permeabilization the coverslips were placed on pre-warmed drops of ICT for 10 minutes at 37°C. The effectiveness of permeabilization could be

measured by LDH release. The cells were then washed for an additional 10 minutes with cold ICT to obtain an efficient depletion of the cytosol. For double immunofluorescence staining of the transfected hTfR and annexin II, the cells were fixed for 2 minutes in −20°C cold methanol, washed with PBS and incubated with the first antibodies in a humid chamber for 45 minutes at room temperature. Mouse anti-human CD71 (IgG2A; Pharmingen) was employed to label the hTfR and the mouse monoclonal H28 (IgG1; Osborn et al., 1988) was used to specifically detect the ectopically expressed annexin II derivatives. The coverslips were then washed 3× 10 minutes with PBS and incubated for 45 minutes with the corresponding fluorescently labeled secondary antibodies (isotype-specific antibodies directed against mouse IgG1 and IgG2A, respectively). After three final PBS washes the coverslips were mounted in Moviol 4-88 (Hoechst). Cells were examined with a Zeiss axiophot photomicroscope and photography employed Kodak P3200 film.

Antibodies

The polyclonal rabbit antibodies directed against annexins II and IV as well as the mouse monoclonal anti-annexin II antibody H28 have been described (Gerke and Weber, 1984; Osborn et al., 1988). 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). Peroxidase coupled antibodies (Dako) were used as secondary antibodies and immunoreactive bands were visualised using the ECL chemoluminescence system (Amersham-Buchler). For double immunofluorescence analysis of transfected cells ectopically expressing hTfR and annexin II, FITC-coupled goat anti-mouse IgG2A and Texas red-coupled goat anti-mouse IgG1 (Southern Biotechnology Associated Inc.) were employed as secondary antibodies.

RESULTS

The association of annexin II with endosomal membranes in the presence and absence of Ca^{2+}

To analyze whether and how Ca^{2+} ions affect the association of annexin II with endosomal membranes we probed subcellular fractions of BHK cells prepared in the absence or presence of the Ca^{2+} chelating EDTA with an annexin II antibody. In these experiments an enrichment of annexin II in a given subcellular fraction was assessed by comparing the respective immunoblot signal to that obtained when an equal amount of total protein from the starting material for gradient fractionation, the post nuclear supernatant (PNS), was analyzed. Fig. 1 reveals that annexin II is enriched in fractions containing early endosomal membranes and to a lesser extent in those containing late endosomes when cell lysis and subsequent gradient fractionation are carried out in the absence of EDTA (as already shown by Emans et al., 1993). Unexpectedly the cofractionation with early endosomes is not affected by including EDTA in all buffers used in the subcellular fractionation indicating that the annexin II binding to early endosomes still occurs at submicromolar Ca^{2+} concentrations (Fig. 1). A Ca^{2+}-independent association of annexin II with endosomes is also corroborated by ultrastructural analyses of mechanically perforated MDCK cells. When these cells are incubated in a physiological buffer in the absence of Ca^{2+} prior to fixation they retain the majority of their endosome associated annexin II although the soluble protein and a substantial fraction of the plasma membrane associated annexin II is lost (Harder et al., 1997).

Further immunoblot analysis reveals that annexin IV is another annexin present in BHK cell fractions containing endosomal membranes (Fig. 1). However, in contrast to annexin II, annexin IV is enriched to an equal extent in fractions containing early and in those containing late endosomes and its co-fractionation with the respective membrane fractions is sensitive to Ca^{2+} chelation (Fig. 1). Thus, annexin IV exhibits a behaviour typical for an annexin, i.e. a Ca^{2+}-regulated interaction with membranes, whereas annexin II appears to associate with the endosomal membranes in a manner atypical for an annexin. To verify the enrichment of early endosomes in the different experiments, the gradient fractions obtained were also subjected to immunoblot analysis with an antibody against EEA1, a protein of 180 kDa specifically associated with early endosomes (Mu et al., 1995; a representative example revealing the specific enrichment of EEA1 in fractions containing early endosomes is shown in Fig. 1). Moreover, in all endosome fractionation experiments the enrichment and integrity of the different endosomal membranes was routinely monitored by following the fate of HRP internalized from the fluid phase (see Materials and Methods for details).

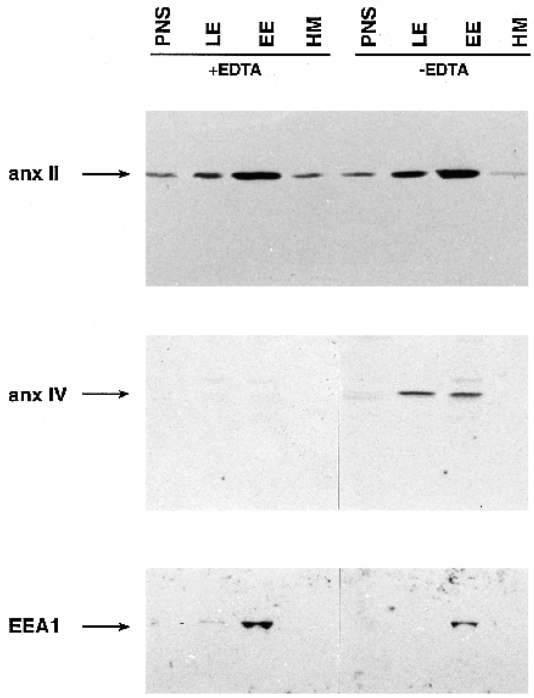

Fig. 1. Association of annexin II and annexin IV with different membrane fractions from BHK cells. A post-nuclear supernatant (PNS) was prepared from BHK cells and subjected to flotation gradient fractionation in the presence (+) or absence (–) of 1 mM EDTA. Fractions enriched in late (LE) and early endosomes (EE) as well as those containing heavy membranes (HM) were collected. Equal amounts of protein from these fractions (as determined according to Bradford, 1976) were subjected to SDS-PAGE and subsequent immunoblotting using polyclonal antibodies directed against annexins II (upper panel) and IV (middle panel). A control immunoblot using antibodies against the early endosome-associated protein EEA1 is shown in the bottom panel. Note that annexin II is enriched in fractions containing endosomal, in particular early endosomal, membranes and that this enrichment is not affected by the inclusion of the Ca^{2+} chelating EDTA. In contrast, annexin IV is equally enriched in fractions containing early and late endosomal membranes and this association is sensitive to Ca^{2+} chelation.

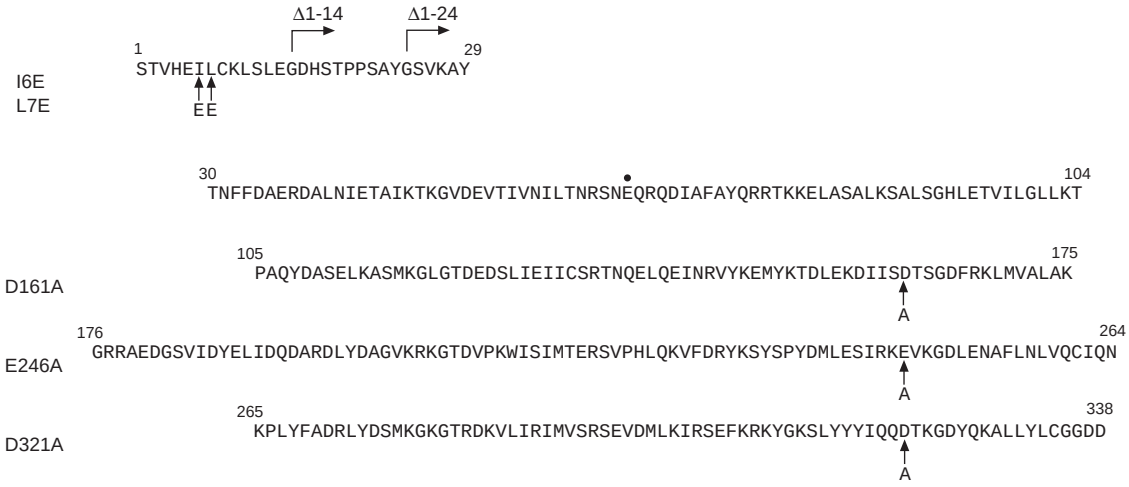

Fig. 2. Amino acid sequence of human annexin II and of annexin II mutants used in this study. The sequence (Huang et al., 1986) is given in the one letter code. The dot indicates a Glu for Ala replacement which is shared by all annexin II derivatives described here and which reconstitutes the epitope for the monoclonal antibody H28 (Osborn et al., 1988; Thiel et al., 1991). Vertical arrows mark the amino acid substitutions introduced to inactivate the type II Ca^{2+} binding sites (D161A, E246A, D321A, generating CM annexin II) and the p11 binding site (I6E, L7E, generating PM annexin II). The positions of the novel N-termini of the truncation mutants, Δ1-14 and Δ1-24 annexin II, are indicated by horizontal arrows.

Intact Ca^{2+} and p11 binding sites are not required for an endosomal association of annexin II

As the subcellular fractionation data suggested a novel type of annexin-membrane interaction for the annexin II-endosome association (one not sensitive to Ca^{2+}) we analyzed the structural requirements for this association in more detail. Therefore we employed a transfection approach to express ectopically in BHK cells certain annexin II mutant proteins. The subcellular distribution of these derivatives was determined by flotation gradient fractionation of a PNS prepared from the transfected cells and subsequent immunoblot analysis of the different fractions using a monoclonal antibody specifically recognizing the ectopically expressed but not the endogenous annexin II (Thiel et al., 1991).

In a first approach we analyzed the behaviour of an annexin II mutant with inactivated type II Ca^{2+} binding sites. These sites are located in repeats 2, 3 and 4 of annexin II and they were rendered inactive by introducing alanine in place of the crucial acidic amino acids serving as so-called cap residues in this type of Ca^{2+} binding site (D161A, E246A, D321A; CM annexin II; Fig. 2). The impairment of the Ca^{2+}-sites had been verified previously by our biochemical analyses (Thiel et al., 1992; Jost et al., 1992). An expression construct encoding wild-type (WT) annexin II was used in control experiments. Fractionation of BHK cells transiently expressing the WT annexin II reveals that this ectopically expressed protein shows the co-fractionation with endosomal membranes already observed for the endogenous annexin II (Fig. 3). Moreover, this co-fractionation is observed both in the absence and in the presence of a Ca^{2+} chelating agent (not shown).

A very similar result is obtained when the subcellular distribution of the mutant protein with inactivated Ca^{2+}-sites is revealed by gradient fractionation, i.e. CM annexin II also co-fractionates with early endosomal membranes (not shown). To exclude the possibility that CM annexin II associates with endosomes through a p11-mediated binding to endogenous (intact) annexin II, we decided to analyze the subcellular distribution of a CM derivative with an inactivated p11 binding site. p11 binding is mediated through the N-terminal 14 amino acids of annexin II with the hydrophobic side chains at positions 6 and 7 (Ile and Leu, respectively) representing major contact sites (Johnsson et al., 1988; Becker et al., 1990). Therefore, we introduced glutamic acid residues in place of Ile-6 and Leu-7 (I6E, L7E; Fig. 2) to inactivate the p11 binding site. This PM (p11-minus) mutation was combined with the CM replacements generating PMCM annexin II. As revealed by ligand blotting and Ca^{2+}-dependent liposome pelleting this mutant protein was impaired in both p11 and Ca^{2+} binding (not shown). Gradient analysis of BHK cells expressing PMCM annexin II shows that this mutant continues to co-fractionate with early endosomal membranes (Fig. 3). Since PMCM annexin II remains monomeric these results show unambiguously that Ca^{2+} binding to annexin II is not required for its cofractionation with early endosomal membranes. Interestingly, the somewhat lesser but reproducibly observed enrichment of endogenous (Fig. 1) and transfected WT annexin II (Fig. 3) in fractions containing late endosomes is not seen in the case of the PMCM derivative (Fig. 3). Likewise, the CM annexin II mutant shows an enrichment only in early endosomal but not in late endosomal fractions (not shown). This indicates that the cofractionation of annexin II with late endosomal membranes requires intact Ca^{2+} binding sites in the protein and thus differs mechanistically from the Ca^{2+}-independent association of annexin II with early endosomes.

The signal for localizing annexin II Ca^{2+}-independently to endosomal membranes resides in the N-terminal domain

To identify the region in the annexin II molecule mediating the Ca^{2+}-independent association with endosomes we generated two N-terminally truncated derivatives which were again expressed in BHK cells and subjected to the fractionation protocol described above. We chose the N-terminal domain for a more detailed analysis since this region is highly variable within the individual annexins and thus likely to be involved in mediating specific properties. In a first truncation mutant we deleted the entire p11 binding site, i.e. amino acids 1-14 (Johnsson et al.,

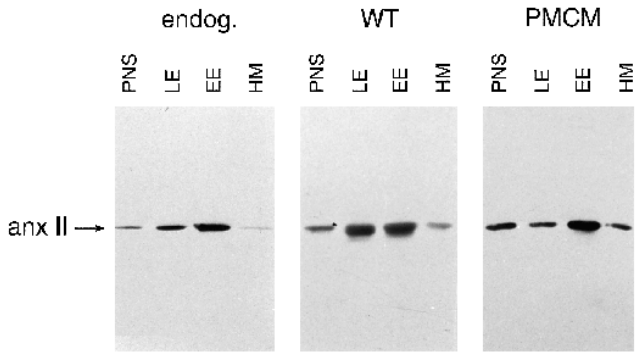

Fig. 3. Flotation gradient analysis of membranes from BHK cells ectopically expressing annexin II derivatives. BHK cells were transfected with expression constructs encoding wild-type (WT) annexin II or a mutant derivative containing inactivated Ca^{2+} binding sites as well as an inactivated p11 binding site (PMCM). Membranes present in a PNS from such cells were subjected to sucrose gradient fractionation and fractions enriched in late endosomes (LE), early endosomes (EE), and heavy membranes (HM) were analyzed by immunoblotting using a monoclonal antibody (H28) specifically recognizing the ectopically expressed annexin II. The distribution of endogenous annexin II (endog.) among the membrane fractions from non-transfected BHK cells was determined by immunoblotting with a polyclonal annexin II antibody and is given for comparison. Preparation of the PNS and gradient fractionation were carried out in the absence of EDTA to specifically analyze the consequences of the Ca^{2+}-site mutations. Note that PMCM annexin II which is incapable of forming the heterotetrameric annexin II-p11 complex and which does not bind Ca^{2+} continues to co-fractionate with endosomal membranes.

1988), and introduced a novel start by replacing the amino acids at positions 14 and 15 (Gly and Asp) by methionine and serine (Fig. 2). As expected from the properties displayed by PMCM annexin II, i.e. a protein mutant incapable of binding p11, the Δ1-14 annexin II derivative continues to co-fractionate with endosomal membranes in the presence of 1 mM EDTA (Fig. 4A). This shows again that p11 binding is not required and further reveals that the entire p11 binding site is dispensable for a Ca^{2+}-independent association with endosomes.

An additional 10 amino acid residues were deleted in the second truncation mutant, Δ1-24 annexin II, in which Gly-24 was replaced by the novel start-methionine (Fig. 2). The rationale for choosing this mutation was twofold. First, we wanted to delete or render inactive two phosphorylation sites of potential regulatory importance (Tyr-23 phosphorylated by $pp60^{src}$ and Ser-25 phosphorylated by protein kinase C; for review see Gerke, 1992). Second, since Lys-27 is part of the discontinous epitope of the monoclonal annexin II antibody H28 we decided to leave this residue unaffected (Thiel et al., 1991). This enabled us to detect the Δ1-24 derivative by immunoblotting with the H28 antibody. Subcellular fractionation of BHK cells ectopically expressing Δ1-24 annexin II reveals that this derivative fails to co-fractionate with endosomal membranes in the presence of a Ca^{2+} chelating agent (Fig. 4B). An enrichment of Δ1-24 annexin II in membrane fractions containing endosomes is, however, observed when the preparation of the PNS and the subsequent gradient analysis are carried out in the absence of EDTA (Fig. 4B). Thus, the annexin II core starting at residue 25 displays the Ca^{2+}-sensitive interaction with BHK membranes typical for an annexin, showing a preference for early and to a lesser extent late endosomes. However, the Ca^{2+}-independent association with these structures requires the sequence spanning residues 15 to 24. Fig. 4 also shows an immunoblot analysis employing EEA1 antibodies on gradient fractions prepared from BHK cells expressing the Δ1-24 annexin II mutant. This control reveals that the fractionation properties of early endosomes are not affected by the ectopic expression of the annexin II mutant. Thus, the loss of Ca^{2+}-independent membrane binding in the case of the Δ1-24 mutant protein is solely due to the deletion of an important sequence in the protein and not caused by any putative secondary effects on the endosomal membranes in the transfected cells.

To corroborate the results obtained by subcellular fractionation we also collected morphological data on the subcellular localization of different annexin II mutants. Therefore, BHK cells were co-transfected with expression plasmids encoding the annexin II derivatives and the human transferrin receptor (hTfR) which was included as a marker for early endosomes in the transfected cells. At 40 hours following transfection the cells were permeabilized with streptolysin O (SLO) and then incubated in an intracellular transport buffer (ICT) to release

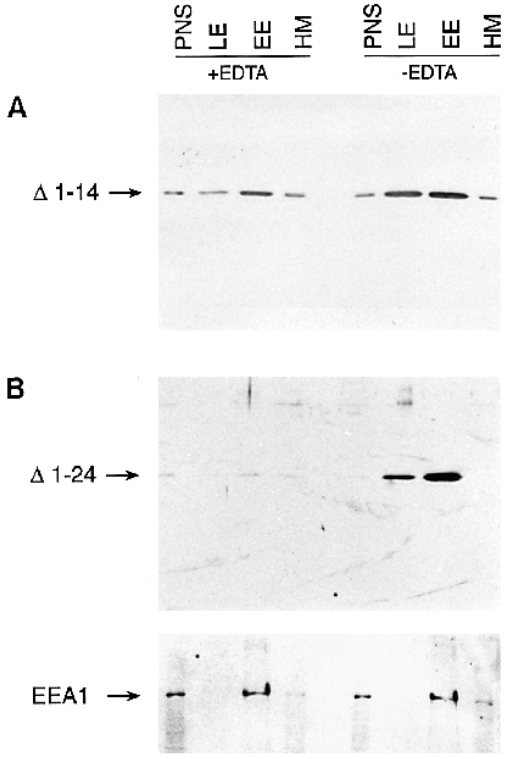

Fig. 4. Effect of N-terminal deletions on the Ca^{2+}-independent co-fractionation of annexin II with endosomal membranes. Cellular membranes (LE, EE, HM) obtained by gradient fractionation of a PNS prepared from BHK cells ectopically expressing Δ1-14 (A) or Δ1-24 (B) annexin II were subjected to immunoblotting using the H28 monoclonal antibody specifically recognizing the mutant proteins. Subcellular fractionation was carried out in the presence (+) or absence (–) of 1 mM EDTA. Note that the deletion of amino acids 1-24 but not that of residues 1-14 renders the molecule incapable of co-fractionating with endosomal membranes in the presence of EDTA. A control immunoblot with the EEA1 antibody of the fractions obtained from Δ1-24 annexin II-transfected cells is shown in the bottom part of B and reveals that the gradient fractionation of early endosomes is not affected in these cells.

the cytosolic fraction of annexin II (see Materials and Methods) before subjecting them to double immunofluorescence using antibodies specifically recognizing the ectopically expressed hTfR and annexin II derivatives, respectively. Fig. 5 reveals that WT annexin II and the PMCM mutant protein co-localize to a large extent with hTfR positive structures thus showing the early endosomal localization already established for endogenous annexin II at the light and electron microscope level (Emans et al., 1993; Harder and Gerke, 1993). In line with our biochemical analyses this annexin II distribution is observed after incubating the permeabilized cells prior to fixation in ICT buffer containing the Ca^{2+} chelating EGTA (Fig. 5). In contrast, the Δ1-24 mutant protein fails to show any endosomal or other intracellular membrane association under such conditions (Fig. 5). However, when Ca^{2+} is not chelated during the incubation of the permeabilized cells in ICT buffer the Δ1-24 derivative remains associated with intracellular structures which to a large extent are hTfR positive (Fig. 5). These data are in line with the subcellular fractionation (Fig. 4) and thus support the conclusion that the unique N-terminal domain of annexin II is required for its Ca^{2+}-independent association with endosomal membranes. When this domain is truncated the resulting Δ1-

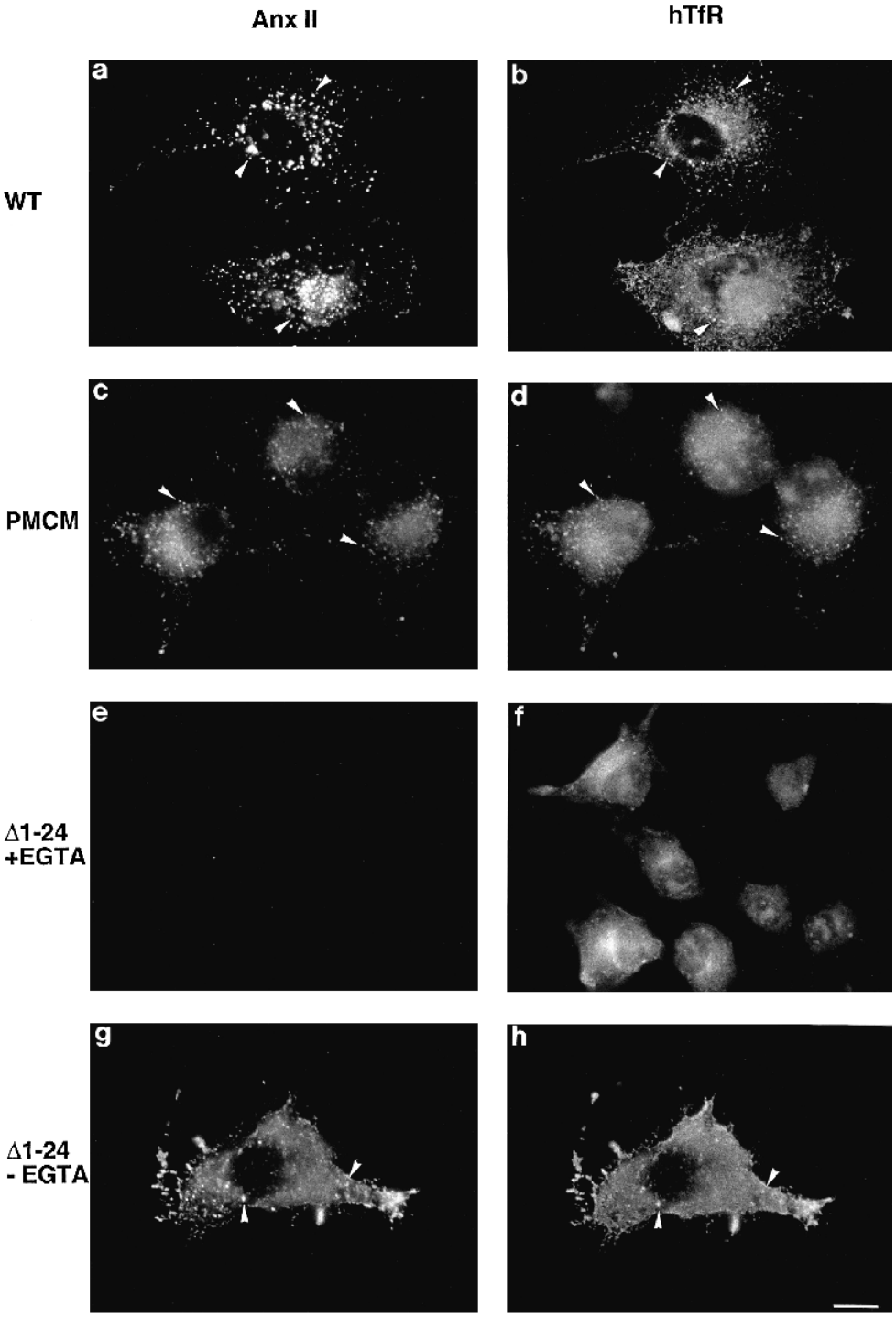

Fig. 5. Localization of ectopically expressed annexin II derivatives and human transferrin receptor in SLO-permeabilized BHK cells. BHK cells were co-transfected with expression constructs encoding the human transferrin receptor (hTfR) and one of the annexin II derivatives (WT, PMCM, or Δ1-24 annexin II, respectively). At 40 hours following transfection efficient hTfR uptake was induced by exogenously supplemented transferrin. Subsequently the cells were permeabilized with SLO and depleted of cytosol by incubation in an intracellular transport buffer (ICT) containing EGTA (see Materials and Methods). This treatment releases the cytosolic annexin II pool which to some extent masks the membrane-bound fraction in immunofluorescence analyses of directly fixed cells (Osborn et al., 1988; Harder and Gerke, 1993). In the case of cells expressing the Δ1-24 annexin II the cytosol was released either in the presence (e,f) or absence (g,h) of 10 mM EGTA to analyze the effect of Ca^{2+} on the intracellular distribution of this mutant. The cells were then fixed and processed for double immunofluorescence using antibodies directed against annexin II (a,c,e,g) and hTfR (b,d,f,h), which only recognized the ectopically expressed and not the endogenous proteins. Note that WT and PMCM annexin II co-localize to a large extent with hTfR positive endosomal structures in a manner not affected by the EGTA which is included in the ICT buffer (arrowheads in a,b and c,d, respectively). In contrast, Δ1-24 annexin II fails to show any membrane association under such conditions (e and f). A co-localization of Δ1-24 annexin II with hTfR positive endosomes is, however, observed when EGTA is omitted during cytosol depletion, i.e. in the presence of Ca^{2+} (g and h).

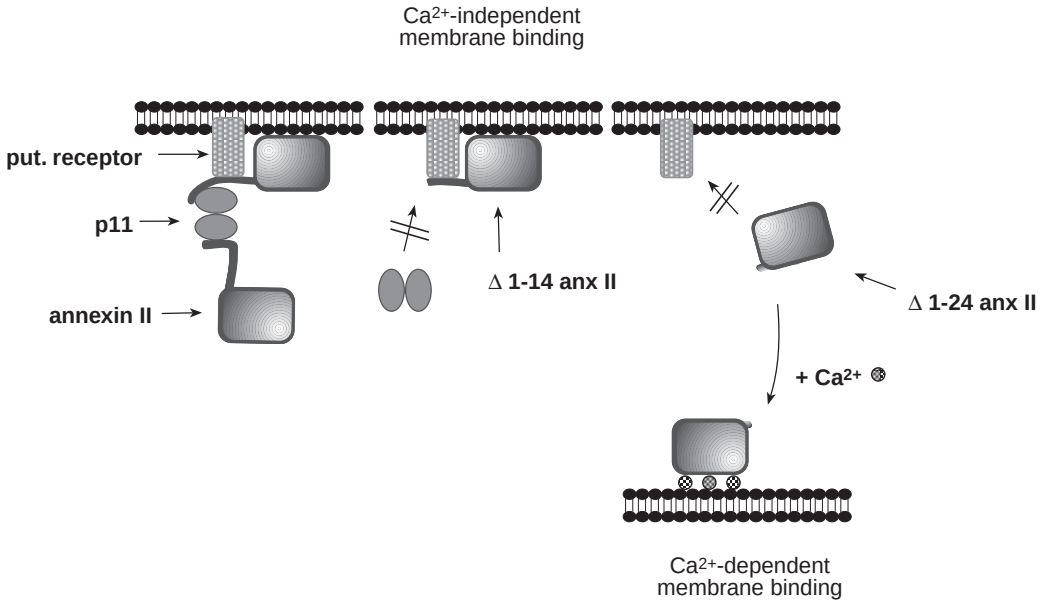

Fig. 6. Model depicting different modes of an annexin II-membrane interaction. The Ca^{2+}-independent association with a target membrane described here is mediated through the sequence spanning residues 15 to 24 which could bind to a receptor specific for this membrane. This interaction would leave the very N-terminal 14 residues of annexin II accessible for p11 binding. Removal of the entire N-terminal region encompassing residues 1 to 24 generates a mutant derivative (Δ1-24 annexin II) whose membrane association strictly depends on the presence of Ca^{2+}.

24 mutant protein requires Ca^{2+} for an interaction with endosomal membranes. Interestingly and as also seen in the gradient analysis the Ca^{2+}-dependent membrane association of the Δ1-24 mutant protein remains to be specific for early and to a lesser extent late endosomal membranes, most likely because the phospholipid composition of these membranes is best suited for interacting with the annexin II core.

DISCUSSION

Members of the annexin family of Ca^{2+}-regulated membrane binding proteins are present on most and possibly all intracellular membranes including the plasma membrane (Gruenberg and Emans, 1993). Specificity with respect to the target membrane of the individual annexins is thought to be conferred through the N-terminal domains which vary between the different members of the family. The Ca^{2+} sensitivity for a reversible and peripheral membrane binding, on the other hand, is most likely carried through the protein core comprising four or eight annexin repeats. These repeats harbour the type II and type III Ca^{2+} binding sites (for reviews see Huber et al., 1992; Swairjo and Seaton, 1994) and isolated core domains bind Ca^{2+}-dependently to phospholipid vesicles and chromaffin granules (Glenney, 1986; Johnsson et al., 1986; Drust and Creutz, 1988). Most likely, the EDTA-sensitive, i.e. Ca^{2+} regulated, co-fraction of Δ1-24 annexin II with endosomes (Figs 4, 5) is the consequence of such a Ca^{2+}-dependent interaction of the annexin II core domain with endosomal membranes. In this case specificity is probably carried through a certain phospholipid composition of the endosomal membranes which is better suited for binding the annexin II core than that of other cellular membranes.

A different mechanism must be responsible for mediating the Ca^{2+}-independent association of annexin II with early endosomes described here. The association depends on the presence of the unique N-terminal domain of the annexin II molecule with the sequence encompassing amino acids 15-24 being of critical importance. In contrast, p11 binding and the resulting annexin II-p11 complex formation are not required. This type of Ca^{2+}-independent binding to certain cellular membranes has not been observed for an annexin before. It could be mediated through a specific receptor for annexin II on early endosomal membranes. This putative receptor would bind to the N-terminal annexin II domain, most likely to the sequence spanning residues 15 to 24, in a manner not regulated by Ca^{2+} (Fig. 6). Such an interaction could be accompanied in the presence of Ca^{2+} by a binding of the annexin II core to endosomal membrane phospholipids. Moreover, it would leave the very N-terminal region (residues 1 to 14) accessible for p11 binding thus enabling the annexin II-p11 complex formation to occur on endosomal membranes. Alternatively, the second part of the N-terminal domain, i.e. amino acids 15-24, could be involved in establishing or stabilizing a conformation of the core domain which allows for a Ca^{2+}-independent interaction of this core domain with endosomal membranes. Future experiments, e.g. the identification of the putative annexin II receptor (protein or a certain lipid structure) on endosomes, have to resolve this question.

Within cells annexin II is not restricted to endosomes but is also found on the plasma membrane and/or in the cortical cytoskeleton colocalizing with molecules of the spectrin family (for review see Gerke, 1992). Interestingly, Ca^{2+} as well as p11 binding are required for establishing the tight association with the cortical cytoskeleton (Thiel et al., 1992; Jost et al., 1994) indicating that different structural requirements underlie the association of annexin II with endosomes and the cortical cytoskeleton, respectively. This could reflect the existence of functionally distinct annexin II pools and/or different modes of regulation. Cortical annexin II could be involved in stabilizing a peripheral localization of endosomes, possibly by providing a physical link between the endosomal membrane and the cortical cytoskeleton (Harder and Gerke, 1993). It may also serve an alternative or additional role in structuring or organizing endosomal membranes (or domains of the plasma membrane).

At least in certain cell types, e.g. adrenal chromaffin cells, annexin II has also been implicated in exocytotic processes, in particular in Ca^{2+} regulated secretion (Ali et al., 1989; Sarafian

et al., 1991). While the exact mechanism by which annexin II acts in exocytotic membrane transport is not known it has been suggested that a heterotetrameric annexin II-p11 complex could provide physical linkage between different chromaffin granule membranes and/or between the granule and the plasma membrane (Nakata et al., 1990; for review see Creutz, 1992). Conceptually, annexin II could therefore be involved in different membrane transport steps by serving a structural role in organizing membranes and/or membrane-cytoskeleton interactions. Specificity could then be guaranteed by certain phospholipid compositions allowing for a Ca^{2+}-dependent binding through the annexin II core domain or specific receptors (e.g. on endosomes) interacting Ca^{2+}-independently with a region in the N-terminal domain.

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