Annexin II contains two types of Ca^{2+} -binding sites

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The annexins are a multigene family of Ca^{2+} -dependent phospholipid-binding proteins which contain novel types of Ca^{2+} sites. Using site-directed mutagenesis, we generated mutant proteins that show defects in the Ca^{2+} -binding sites in a particular member of this family, the src tyrosine kinase substrate annexin II. Analysis of the relative Ca^{2+} -binding affinities of annexin II mutants in a combined Ca^{2+} /phospholipid-binding assay revealed two distinct types of Ca^{2+} -binding sites. Three so-called type II sites are found in annexin repeats 2, 3 and 4 respectively. Two so-called type III sites are located in the first repeat and involve the glutamic acid residues at positions 52 and 95. Both types of sites were recently identified by X-ray crystallography in annexins V and I [Huber, Schneider, Mayr, Römisch and Paques (1990) *FEBS Lett.* **275**, 15–21; Weng, Luecke, Song, Kang, Kim and Huber (1993) *Protein Sci.* **2**, 448–458], indicating that similar principles govern Ca^{2+} binding to annexins in crystals and in solution. The two types of Ca^{2+} -binding sites differ not only in

their architecture but also in their affinity for the bivalent cation. The Ca^{2+} concentration needed for half-maximal phosphatidylserine binding is 5–10 μM for an annexin II derivative with intact type II but defective type III sites (TM annexin II) whereas a mutant protein containing defective type II but unaltered type III sites (CM annexin II) requires 200–300 μM Ca^{2+} for the same activity. Annexin II mutants with defects in the type II and/or type III sites also show different subcellular distributions. When expressed transiently in HeLa cells, TM annexin II acquires the typical location in the cortical cytoskeleton observed for the wild-type molecule. In contrast, CM annexin II remains essentially cytosolic, as does a mutant protein containing defects in both type II and type III Ca^{2+} -binding sites (TCM annexin II). This indicates that the intracellular association of annexin II with the submembraneous cytoskeleton depends only on the occupation of type II Ca^{2+} -binding sites.

INTRODUCTION

The annexins are a family of proteins that bind to negatively charged phospholipids in a Ca^{2+} -dependent manner. They are widely distributed throughout the animal and plant kingdoms, with significant sequence identities existing between the different members. Although the direct physiological functions of annexins are not known, their biochemical properties suggest that the proteins are involved in some aspect of membrane biology. Indeed, members of this family have been implicated in membrane fusion processes during exo- and endocytosis, in the maintenance of certain membrane structures through the provision of membrane-cytoskeleton links, in the formation and regulation of Ca^{2+} -specific ion channels, and in some aspects of lipid metabolism (for reviews, see Crompton et al., 1988; Klee, 1988; Moss et al., 1991; Creutz, 1992; Gruenberg and Emans, 1993).

In addition to sharing biochemical properties, all annexins exhibit as a characteristic structural feature a sequence element of 70–80 amino acids, which is repeated four (in the 35–40 kDa annexins) or eight (in 68 kDa annexin) times. These annexin repeats show intra- and intermolecular sequence similarities and form a core domain which is resistant to limited proteolysis and harbours the binding sites for Ca^{2+} and phospholipids. Crystallographic analysis carried out on annexins I and V has shown that the protein core has a globular structure and consists almost exclusively of tightly packed α -helices (Huber et al., 1990a; Weng et al., 1993). Each of the four annexin repeats is characterized by five α -helices (A–E), which are connected by turns or short loops. The N-termini of the different annexins which precede the protein core are diverse both in sequence and in length. They are likely to be of regulatory importance because phosphorylation sites for signal-transducing protein kinases are frequently found in

the N-termini of various annexins (for a review, see Moss et al., 1991). In one member of the family, annexin II, the N-terminal sequence also harbours the binding site for an intracellular protein ligand, p11 (for a review, see Weber, 1992). p11 binding alters the Ca^{2+} - and/or phospholipid-binding properties of annexin II, with the (annexin II)₂(p11)₂ complex having a more than 100-fold increased affinity towards Ca^{2+} /phospholipid compared with monomeric annexin II (Powell and Glenney, 1987).

Two novel types of Ca^{2+} -binding sites, which differ from the well-characterized EF-hand, have been identified in the annexin I and V crystals (Huber et al., 1990b; Weng et al., 1993). In the so-called type II sites, Ca^{2+} co-ordination in an individual annexin repeat is provided by peptide oxygens from the loop between helices A and B and by carboxyl oxygens from an acidic amino acid located 40 residues downstream of a conserved glycine present in the interhelical loop. Type III sites, on the other hand, possess a different architecture which, in most cases, involves the loop between helices D and E and one nearby acidic residue. In both sites, Ca^{2+} co-ordination is completed by water molecules in the crystals. Although these principles of Ca^{2+} binding are conserved in the two annexins crystallized, the location of the Ca^{2+} sites with respect to the individual repeats differs between annexin I and annexin V. Thus, whereas type III sites are occupied by Ca^{2+} ions in repeats 1 and 4 of annexin I, only the type III site in repeat 1 of annexin V is occupied by lanthanum. Functional, i.e. Ca^{2+} -labelled, type II sites have been identified in repeats 1, 2, 4 and recently also in repeat 3 of annexin V (Huber et al., 1990b; Berendes et al., 1993; Concha et al., 1993) but only in repeats 2, 3 and 4 of annexin I (Weng et al., 1993). Despite a detailed understanding of the architecture of these novel Ca^{2+} sites in annexin crystals, their affinities towards the bivalent cation are not known and cannot be deduced from the crystal structure.

To determine these affinities and to analyse whether the novel principles of Ca^{2+} binding observed in crystals of annexin I and V are conserved in solution and in other annexins, we characterized Ca^{2+} -binding sites in the tyrosine kinase substrate annexin II following a site-directed mutagenesis approach. This has already led to the identification of three acidic amino acids involved in the formation of the type II Ca^{2+} -binding sites present in repeats 2, 3 and 4 (Jost et al., 1992). In this paper, we show that sites resembling type III are also present in annexin II and involve the glutamic acid residues in positions 52 and 95 of the first repeat. When compared with the type II sites, and type III sites have a more than 30-fold reduced affinity towards Ca^{2+} in a Ca^{2+} -dependent phospholipid-binding assay. Thus the two types of Ca^{2+} -binding sites described in the annexin I and annexin V crystals are also present in an annexin in solution and show a significant difference in their affinity towards the bivalent cation.

MATERIALS AND METHODS

Cloning and expression of human annexin II

The cDNA encoding human annexin II was cloned into the bacterial expression vector pDS10 (Bujard et al., 1987) and expressed in *Escherichia coli* XL1 blue cells as described previously (Thiel et al., 1991a). The cloning strategy used resulted in the recombinant protein having six additional amino acids which are encoded by the expression vector and fused to the N-terminus (Thiel et al., 1991a). All recombinant annexin II molecules generated also contained Ala65Glu. This substitution led to the reconstitution of the epitope for the monoclonal antibody H28 (Osborn et al., 1988; Thiel et al., 1991b) and thus facilitated the immunological detection of recombinant annexin II.

Purification of wild-type or mutant annexin II molecules was carried out as described previously (Thiel et al., 1991a). As the recombinant protein was present in insoluble inclusion bodies in *E. coli*, the inclusion bodies were isolated from the lysed bacteria and solubilized by sonication in a buffer containing 10 M urea. Following treatment of this material with Q-Sepharose (Pharmacia) and CM 52 (Whatman) in the presence of 10 M urea, the recombinant annexin II was renatured by dialysis against 100 mM NaCl, 2 mM $\text{Na}_2\text{S}_2\text{O}_3$, 1 mM EGTA, 1 mM dithiothreitol and 20 mM imidazole-HCl, pH 7.5. Final purification was achieved by ion-exchange chromatography on CM 52 and a Mono-S f.p.l.c. column (Pharmacia) in a buffer containing 25 mM sodium acetate, 2 mM $\text{Na}_2\text{S}_2\text{O}_3$ and 1 mM dithiothreitol, pH 5.6. During the final Mono-S chromatography, wild-type annexin II and the various mutant proteins eluted at different salt concentrations because of the replacement of acidic amino acids by uncharged residues in the mutant molecules. The purification scheme led to the isolation of the different annexin II derivatives in high purity (> 95% pure) and with a comparable yield (approx. 5 mg of protein/l of cell culture). The protein concentration of annexin II derivatives was determined spectrophotometrically [$A_{1\text{cm}, 280}^{1\%} = 7.9$].

Site-specific mutagenesis

Point mutations were generated by oligonucleotide-directed mutagenesis (Kunkel, 1985). Synthetic oligonucleotides carrying the desired mutations were prepared on a 8750 Milligen Biosearch DNA synthesizer and purified on denaturing polyacrylamide gels. Mutagenesis reactions were carried out with a Muta-Gene M13 *in vitro* mutagenesis kit (Bio-Rad) according to the manufacturer's protocol using uracil-containing annexin II cDNA cloned into M13 mp18 vector as single-stranded DNA template.

All mutations were confirmed by dideoxy sequencing of the entire cDNA (Sanger et al., 1977). The mutant annexin II cDNA was excised with *Bam*HI and *Hind*III and cloned into the appropriately linearized pDS10 vector. Multiple mutations were introduced by sequential mutagenesis reactions or by annealing several mutant oligomers simultaneously.

Purification of porcine annexin II and the (annexin II)₂(p11)₂ complex

The heterotetrameric complex comprising two annexin II and two p11 chains was purified from porcine intestinal epithelium according to Gerke and Weber (1984). The complex was dissociated in the presence of 9 M urea and the subunits were separated on a Sephacryl S 200 column as described previously (Gerke and Weber, 1985).

Phospholipid binding

Ca^{2+} affinities of the different annexin II mutants were compared in a Ca^{2+} -dependent liposome pelleting assay (Thiel et al., 1991a). Phospholipids [phosphatidylserine (PtdSer) or phosphatidylinositol (PtdIns)] were dissolved in chloroform, dried in a N_2 stream and sonicated in water containing 40 μM EGTA, pH 8.0, to generate liposomes. The liposomes were subsequently pelleted by centrifugation (200 000 g, 15 min) and resuspended in water. Binding experiments were performed with either PtdSer or PtdIns liposomes (1 mg of phospholipid/ml) in 50 mM imidazole-HCl, pH 7.5, 150 mM NaCl, 2 mM MgCl_2 , 20 μM EGTA and 0.1 μM –2 mM CaCl_2 . Free Ca^{2+} concentrations in individual buffers were determined using Fura-2 (Grynkiewicz et al., 1985). The binding reaction was carried out for 20 min at room temperature. Subsequently, the liposomes were pelleted (200 000 g, 15 min) and Ca^{2+} -dependently bound annexin II was released by the addition of 10 mM EGTA. The released protein was recovered by centrifugation and subjected to SDS/PAGE. Densitometric analysis of the Coomassie Blue-stained band yielded a relative value for the amount of released annexin II.

Limited proteolysis

Limited α -chymotrypsin treatment was performed essentially as described previously (Thiel et al., 1991a). Wild-type annexin II and the different mutants were incubated with the enzyme at an enzyme:substrate ratio of 1:70 in a buffer containing 50 mM imidazole-HCl, pH 7.5, 150 mM NaCl and 20 μM EGTA. After 10 min at room temperature, the reaction was stopped by adjusting the sample to 1 mM phenylmethanesulphonyl fluoride (PMSF).

Transfections

The cDNAs encoding wild-type annexin II or different mutant proteins were cloned into the eukaryotic expression vector pCMV5 which utilizes the strong cytomegalovirus promoter (Andersson et al., 1989). Transient transfections were carried out with HeLa cells grown in Dulbecco's DME (Gibco-BRL) medium supplemented with 10% (v/v) fetal calf serum. The CaPO_4 method modified according to Chen and Okayama (1987) was employed as described previously (Thiel et al., 1992).

Serial extraction of transfected HeLa cells

HeLa cells cultivated for 63 h at 37 °C and 5% CO_2 following transfection were harvested using a rubber policeman and

homogenized in a Triton/Ca²⁺ buffer {10 mM imidazole-HCl, pH 7.5, 75 mM KCl, 2 mM MgCl₂, 0.5 mM CaCl₂, 1 mM NaN₃, 0.5% (v/v) Triton X-100, 1 mM PMSF and 1 μ M E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane]} using a Dounce homogenizer. An aliquot of this homogenate was mixed with an equal volume of SDS sample buffer (Laemmli, 1970), boiled and centrifuged to yield a total protein fraction. The remaining homogenate was centrifuged for 15 min at 22000 *g* to separate the Triton-soluble fraction from the insoluble material. The pellet was re-extracted with the Triton/Ca²⁺ buffer, and the Triton-soluble pools of both extractions were combined, mixed with an equal volume of SDS sample buffer and boiled. Proteins present in the Triton-insoluble material were extracted by treatment with 0.5% (v/v) SDS and 2 mM EGTA. Aliquots of the individual fractions (total protein, Triton-soluble and Triton-insoluble) were adjusted to identical amounts of exogenous annexin II derivative in the total protein fraction and then subjected to SDS/PAGE (Laemmli, 1970) and immunoblotting (Towbin et al., 1979) using mouse monoclonal antibodies directed against annexin II or p11 (H28 and H21, respectively; Osborn et al., 1988).

Immunofluorescence

Following transfection, HeLa cells were grown on glass coverslips for 63 h at 37 °C and 5% CO₂. Subsequently, the cells were fixed for 10 min in 3.7% (v/v) formaldehyde in PBS at room temperature followed by methanol for 6 min at -10 °C. Alternatively, cells were treated for 2 min with ice-cold 0.5% Triton X-100, 100 mM KCl, 3 mM MgCl₂, 1 mM CaCl₂, 200 mM sucrose, 10 mM Hepes, pH 6.8, 1 mM PMSF and then for 2 $\times$ 5 min in the same solution without Triton X-100 prior to fixation in formaldehyde (3.7% in PBS for 4 min at room temperature) and methanol (6 min at -10 °C). The latter treatment yielded Triton cytoskeletons with a preserved submembranous compartment (cortical cytoskeleton; Osborn et al., 1988; Thiel et al., 1992). Incubations with the primary antibodies were carried out at 37 °C for 45 min and employed an affinity-purified polyclonal rabbit antibody directed against annexin II (Gerke and Weber, 1984) at a concentration of 10 μ g/ml in PBS and 1% BSA and the mouse monoclonal H28, which was used as undiluted hybridoma culture supernatant. After treatment with fluorescein isothiocyanate-labelled goat anti-mouse IgGs and rhodamine-coupled goat anti-rabbit IgGs (Dianova, Hamburg, Germany) as secondary antibodies (37 °C, 45 min), the cells were mounted in Moviol and analysed with a Zeiss Axiophot microscope. Photography used Kodak Tri-X film.

RESULTS

We had shown previously that recombinant annexin II expressed in *E. coli* has, except for p11 binding, the same biochemical and physicochemical properties as the authentic protein isolated from mammalian cells (Thiel et al., 1991a). Notably, the recombinant molecule has the same affinity for Ca²⁺/phospholipid in Ca²⁺-dependent PtdSer (Thiel et al., 1991a) and PtdIns (results not shown) binding assays. This indicates that recombinant and authentic annexin II are structurally and biochemically equivalent and that it is valid to study the structural principles underlying Ca²⁺/phospholipid binding by performing a mutational analysis of annexin II synthesized in bacteria.

Two different types of Ca²⁺-binding sites are present in annexin II

In a recent mutagenesis study, we could identify three acidic amino acid residues in human annexin II (Asp-161, Glu-246 and

Asp-321) that are involved in the formation of three separate Ca²⁺-binding sites located in repeats 2, 3 and 4 respectively (Jost et al., 1992). Because these residues correspond in a linear sequence alignment to acidic amino acids that provide crucial Ca²⁺-co-ordination sites in the crystals of annexin V or annexin I, respectively (Huber et al., 1990b; Weng et al., 1993), they most likely participate in the formation of type II Ca²⁺-binding sites in annexin II. Inactivation of the three type II sites in annexin II through the replacement of the three crucial acidic residues by alanine led to a mutant protein (-2/-3/-4 annexin II, here referred to as CM annexin II) which showed a markedly reduced affinity for Ca²⁺ in a Ca²⁺-dependent PtdSer-binding assay (Jost et al., 1992). The mutant protein was, however, still able to interact with phospholipid liposomes, with significant binding occurring at Ca²⁺ concentrations exceeding 100 μ M. This indicated the presence of (an) additional Ca²⁺-binding site(s). To map and characterize these Ca²⁺ site(s), we employed site-directed mutagenesis on the CM annexin II cDNA and introduced alanine in place of two glutamic acid residues (Glu-52 and Glu-95, Figure 1) which correspond in a linear sequence alignment to acidic amino acids involved in the complexation of lanthanum ions in annexin V crystals (Huber et al., 1990b). These lanthanum ions label two minor Ca²⁺ sites which recently have also been identified as type III sites in annexin I crystals (Weng et al., 1993).

The resulting annexin II mutant carrying the E52A and E95A mutations in addition to the CM substitutions (D161A, E246A and D321A) was expressed in *E. coli* and purified following a protocol developed for the isolation of recombinant wild-type annexin I (Thiel et al., 1991a). The purified mutant protein, which we will refer to as TCM annexin II (for *total Ca²⁺ minus*), shows the same characteristic stability towards limited proteolysis as wild-type and CM annexin II, indicating a general equivalence of the overall three-dimensional folding (not shown). Yet significant differences are observed when the Ca²⁺/phospholipid-binding properties of different annexin II mutants are compared in Ca²⁺-dependent liposome pelleting assays. In these experiments, we employed 33 kDa protein cores generated by limited chymotrypsin treatment (amino acid residues 30–338) to exclude a potential effect of the altered N-terminal tails of the recombinant annexin II derivatives (see the Materials and methods section). Whereas the 33 kDa chymotryptic protein core of wild-type annexin II shows half-maximal PtdSer-liposome binding at 5–10 μ M Ca²⁺ (Jost et al., 1992), the 33 kDa core of CM annexin II requires at least 30-fold higher Ca²⁺ concentrations for the same effect (Figure 2; Jost et al., 1992) and the TCM mutant protein core has lost totally the ability to interact with PtdSer-liposomes in the presence of Ca²⁺ (Figure 2). Even when PtdIns-liposomes, which reduce the Ca²⁺ concentration for half-maximal binding of wild-type annexin II and the CM mutant protein by a factor of 2–3 (M. Jost, K. Weber and V. Gerke, unpublished work), are employed in the assay, significant binding of TCM annexin II is not observed (results not shown). Thus residues Glu-52 and Glu-95 are most likely involved in the formation of Ca²⁺-binding sites that are responsible for the residual Ca²⁺-dependent phospholipid binding of CM annexin II.

The E52A and E95A mutations were also introduced into a wild-type background, i.e. by replacing Glu-52 and Glu-95 with Ala in wild-type and not CM annexin II. The resulting molecule, which we will refer to as TM annexin II, enabled us to study directly the impact of these mutations on the Ca²⁺/phospholipid-binding properties. When analysed in the Ca²⁺-dependent liposome pelleting assay, TM annexin II shows the same behaviour as wild-type annexin II, i.e. half-maximal PtdSer-liposome bind-

Figure 1 Positions of amino acid substitutions in the sequence of human annexin II

The N-terminal domain (residues 1–29) and the four annexin repeats (residues 30–104, 105–175, 176–264, 265–338) are given in separate lines. The endonexin fold is underlined. Mutations introduced to inactivate different Ca^{2+} sites are indicated by arrows pointing upwards. CM annexin II harbours three point mutations, D161A, E246A and D321A, and thus carries defects in the three type II Ca^{2+} -binding sites. TCM annexin II is a CM molecule containing two additional amino acid substitutions, E52A and E95A. TM annexin II only harbours the E52A and E95A mutations. The glutamic acid residues at positions 52 and 95 correspond in a linear sequence alignment to the acidic amino acids that were shown to be involved in lanthanum binding in annexin V crystals (Huber et al., 1990b). All mutant molecules contain the A65E substitution (arrow pointing downward), which reconstitutes the epitope for the monoclonal antibody H28.

Figure 2 Ca^{2+} -dependent binding of CM and TCM annexin II to PtdSer liposomes

The 33 kDa protein cores of CM and TCM annexin II, which were generated by limited chymotrypsin treatment of the purified proteins, were incubated with PtdSer liposomes in the presence of increasing Ca^{2+} concentrations. After pelleting of the liposomes, Ca^{2+} -dependently bound protein was released by the addition of EGTA to 10 mM and analysed in SDS/PAGE. Whereas CM annexin II shows half-maximal binding to the liposomes at $\sim 250 \mu\text{M}$ Ca^{2+} (see also Jost et al., 1992), the TCM mutant protein has lost the ability to interact with PtdSer liposomes even at $500 \mu\text{M}$ Ca^{2+} .

ing is observed at $5\text{--}10 \mu\text{M}$ Ca^{2+} (Figure 3). This indicates that mutation of the low-affinity Ca^{2+} sites that involve Glu-52 and Glu-95 does not interfere with Ca^{2+} binding to the type II sites residing in repeats 2, 3 and 4. Moreover, the E52A and E95A mutations do not lead to significant changes in the tertiary structure because several other biochemical properties, including the characteristic resistance towards limited proteolysis, are unaffected in TM compared with wild-type annexin II (results not shown). The combined Ca^{2+} /phospholipid-binding studies show that an impact of the E52A and E95A mutations on Ca^{2+}

Figure 3 Ca^{2+} -dependent PtdSer binding of WT and TM annexin II

Chymotryptic cores of wild-type (WT) annexin II and of a mutant protein containing the E52A and E95A substitutions (TM) were incubated with PtdSer liposomes in the presence of increasing Ca^{2+} concentrations. After liposome pelleting, Ca^{2+} -dependently bound protein was released by the addition of 10 mM EGTA and analysed in SDS/PAGE. Note that the TM mutant displays the same affinity towards Ca^{2+} /phospholipid as does WT annexin II.

binding to annexin II *in vitro* is only observed when the type II sites are mutated simultaneously (in the TCM mutant).

Type II but not type III Ca^{2+} -binding sites are required for the intracellular association of annexin II with the submembranous cell cortex

To study the importance of the Ca^{2+} -binding sites that involve Glu-52 and Glu-95 for the intracellular distribution of annexin II, we employed a eukaryotic expression system in combination with subcellular fractionation. The cDNAs encoding different annexin II derivatives—the wild-type molecule (WT) and mutants containing (a) a defective p11-binding site (PM; this mutant

Figure 4 Western blot analysis of extracts from HeLa cells transfected with different annexin II mutants

Cells were transfected transiently with pCMV 5 constructs containing either wild-type or mutant annexin II cDNAs. The cells were harvested 63 h after transfection and extracted with a buffer containing 0.5% (v/v) Triton X-100 and 0.5 mM Ca²⁺. Subsequently, proteins present in the Triton-insoluble fraction were released by treatment with 0.5% (v/v) SDS and 2 mM EGTA. Identical aliquots of both fractions (Triton-soluble and -insoluble) were analysed by SDS/PAGE and subjected to immunoblotting using the monoclonal antibody H28, which only recognizes the exogenous annexin II. The two mutant proteins containing a defective p11 binding site (PM and PMCM) show a slightly reduced electrophoretic mobility. This is most probably a consequence of the introduction of two additional negative charges in the N-terminal p11-binding domain (the I6E and L7E mutations), which could interfere with proper SDS binding. Note that only a small fraction of WT and TM annexin II is released upon Triton/Ca²⁺ treatment while the majority is found in the Triton-insoluble fraction. The mutants carrying defects in the type II Ca²⁺-binding sites (CM, PMCM and TCM) or in the p11-binding site (PM, PMCM) are essentially Triton-soluble.

protein harbours the two amino acid substitutions I6E and I7E, which render the p11-binding site inactive; Thiel et al., 1992), (b) defective type II Ca²⁺ sites (CM), (c) defective p11- and type II Ca²⁺-binding sites (PM CM), (d) defective type III Ca²⁺ sites (TM), and (e) defective type II and type III Ca²⁺ sites (TCM)—were cloned into the eukaryotic expression vector pCMV5 under the control of the cytomegalovirus promoter and transfected into HeLa cells. As transient transfections using the CaPO₄ method routinely yielded 60–70% HeLa cells expressing the exogenous annexin II, these populations were accessible to biochemical analysis. The transfected cells were subjected to serial extractions employing different detergents in the presence or absence of Ca²⁺. This allowed a separation of the cytosolic annexin II pool, which was extracted with a buffer containing 0.5% Triton X-100 and 0.5 mM Ca²⁺, from the cytoskeleton-associated annexin II, which was only solubilized in the presence of 0.5% (v/v) SDS and 2 mM EGTA (Zokas and Glenney, 1987).

Figure 4 shows an immunoblot analysis of such HeLa cell fractions probed with the monoclonal annexin II antibody H28. This has a limited cross-species reactivity and recognizes a discontinuous epitope which involves Glu-65. This glutamic acid is not present in the endogenous HeLa cell (human) annexin II (Thiel et al., 1991b), but has been introduced in all exogenous annexin II derivatives to reconstruct the H28 epitope (Figure 1). Thus the H28 immunoblot only reveals the distribution of these exogenous molecules. Figure 4 shows that wild-type annexin II and also the TM annexin II mutant, i.e. the molecule with defective type III Ca²⁺-sites, are present predominantly in the Triton-insoluble fraction, i.e. tightly associated with the cytoskeleton. In contrast, all other mutant proteins are essentially soluble (Figure 4).

To corroborate the data obtained in the biochemical experiments, which employed serial extractions, and to elucidate the intracellular distribution of different annexin II derivatives *in situ*,

Figure 5 Distribution of different annexin II mutants in transiently transfected HeLa cells

HeLa cells were transfected with expression constructs carrying wild-type (a, b), TM (c, d), CM (e, f) or TCM (g, h) annexin II cDNAs. At 63 h after transfection, the cells were treated with Triton X-100 in the presence of Ca²⁺ and then fixed in formaldehyde/PBS and methanol (see the Materials and methods section). Annexin II was visualized by immunofluorescence microscopy using the mouse monoclonal antibody H28 which only recognized the exogenous annexin II (a, c, e and g) and a rabbit polyclonal serum which stained both exogenous and endogenous annexin II (b, d, f and h). Fluorescein isothiocyanate-labelled goat anti-mouse IgGs and rhodamine-coupled goat anti-rabbit IgGs were employed as secondary antibodies in the individual double-labelling experiments. Note that WT and TM annexin II remain associated with the reticular network of the submembrane cytoskeleton whereas mutant proteins containing defects in the type II Ca²⁺ sites (CM, TCM) are not found in this structure. The bar in A represents 10 μ m.

we performed immunocytochemical studies. HeLa cells that had received the expression constructs carrying either wild-type annexin II or the various type II and/or type III Ca²⁺ site mutants were processed for immunofluorescence analysis with the H28 antibody either after direct fixation or after detergent treatment and subsequent fixation. The latter method is particularly useful for the analysis of components of the cortical cytoskeleton because it removes large portions of potentially obscuring soluble proteins but leaves the cytoskeleton behind. When the detergent treatment is carried out in the presence of Ca²⁺, endogenous annexin II remains in a characteristic reticular network (Zokas and Glenney, 1987; Osborn et al., 1988), which is also stained with antibodies against non-erythroid spectrins,

typical components of this submembranous cytoskeleton (Greenberg and Edelman, 1983; Lehto et al., 1983).

H28 immunofluorescence of the directly fixed HeLa cells revealed a uniform staining pattern for wild-type annexin II and all mutant proteins analysed (not shown). Although this only indicated a submembranous and/or cytosolic location of the respective protein, immunofluorescence microscopy on the detergent-resistant cytoskeleton was more instructive. Here, reticular H28 staining was only observed in cells expressing the exogenous wild-type or TM annexin II whereas cells transfected with CM or TCM expression constructs failed to display a significant cytoskeleton-associated H28 labelling (Figure 5). To exclude the possibility that the latter cells lack a detergent-resistant reticular network, the same preparations were also stained with a polyclonal annexin II antibody which recognized both exogenous and endogenous annexin II. Figure 5 shows that this antibody labels the typical reticular network in cells expressing CM and TCM annexin II. These results are in agreement with our previous immunofluorescence analysis of rat fibroblasts transfected with the CM annexin II carrying expression construct (Thiel et al., 1992) and indicate that intact type II Ca^{2+} -binding sites are required for an association of annexin II with the submembranous cytoskeleton. Type III Ca^{2+} -binding sites, however, seem not to be involved in establishing this association, as a molecule containing mutations in the two type III Ca^{2+} -sites only (TM annexin II) shows the same properties as wild-type and endogenous annexin II and binds to the cortical cytoskeleton.

DISCUSSION

Proteins of the annexin family share three distinct features: Ca^{2+} -dependent binding to acidic phospholipids; the annexin repeat, a well-conserved segment of 70–80 amino acids present in either four or eight copies in all members described so far; and novel types of Ca^{2+} -binding sites which differ from the well-characterized EF-hand (see the Introduction for references). The typical annexin-type Ca^{2+} -binding site, which was originally described in annexin V crystals (Huber et al., 1990b) and which was referred to as a type II Ca^{2+} site in a recent study (to distinguish it from the type I EF-hand Ca^{2+} site; Weng et al., 1993), is characterized by two distinct elements of an individual annexin repeat: an interhelical loop, which connects helices A and B of a given repeat and contains carbonyl oxygens involved in Ca^{2+} co-ordination; and an acidic amino acid located 40 residues C-terminal to the conserved glycine of the A/B loop. This acidic residue is also known as the cap residue and provides carboxyl oxygens for Ca^{2+} complexation (Huber et al., 1990b). Sites of identical architecture have recently been identified in crystals of annexin I (Weng et al., 1993) and are most probably also present in annexin II in solution, as mutagenesis analyses revealed that the acidic cap residues of repeats 2, 3 and 4 as well as certain residues in the A/B loop of repeat 3 are indispensable for high-affinity Ca^{2+} binding (Thiel et al., 1991a; Jost et al., 1992). Ca^{2+} sites of yet another type of site, the so-called type III sites, were also identified in crystals of annexins V and I (Huber et al., 1990b; Weng et al., 1993). In these sites, Ca^{2+} co-ordinates to backbone carbonyl oxygens often located in the loop between helices D and E and one nearby acidic amino acid. By introducing alanine in place of two acidic residues in the first repeat of annexin II, which in a linear sequence alignment correspond to type III Ca^{2+} ligands in the annexin V crystals, we show here that two such sites are also formed in annexin II. Our biochemical analysis reveals for the first time that these type III sites are occupied in an annexin in solution but only in the presence of elevated Ca^{2+} concentrations (significant liposome binding of the CM annexin

II mutant, which contains intact type III sites, is only observed at Ca^{2+} concentrations exceeding 100 μM).

The type III Ca^{2+} -binding sites in annexin II involve the glutamic acid residues at positions 52 and 95. It seems unlikely that additional type III sites exist in annexin II as a mutant molecule containing crucial amino acid replacements in these type III sites (E52A and E95A) and in the three type II sites of repeats 2, 3 and 4 (D161A, E246A and D321A) fails to display Ca^{2+} -dependent properties. In contrast, annexin I, which harbours equivalent type II sites in repeats 2, 3 and 4 and the two type III sites in repeat 1, shows Ca^{2+} -binding in a third type III site (Weng et al., 1993). This type III site resides in repeat 4 and involves the glutamic acid residue at position 335. The equivalent position in annexin II is occupied by glutamine (Gln-327; Figure 3), a replacement which most likely renders this type III site inactive. Thus, although the principles of Ca^{2+} binding, i.e. the presence of type II and type III Ca^{2+} -binding sites, are shared by annexins I, II and V, the locations and the total number of these sites differ between the three proteins. In annexin V, three type II sites are located in repeats 1, 2 and 4 (Huber et al., 1990b) and a fourth site has recently been identified in repeat 3 (Berendes et al., 1993; Concha et al., 1993) whereas annexins I and II contain only three such sites in repeats 2, 3 and 4 (Jost et al., 1992; Weng et al., 1993). Similarly, whereas all three annexins contain the two type III sites in the first repeat, only annexin I harbours the additional type III site in repeat 4 (Huber et al., 1990b; Weng et al., 1993; this study). As all Ca^{2+} -binding sites reside in close proximity on one side of the individual annexin molecule (Huber et al., 1990b; Weng et al., 1993), and as this side is most probably involved in phospholipid binding (Brisson et al., 1991), the observed differences in the distribution and number of the type II and type III Ca^{2+} sites could probably account for the differences in Ca^{2+} affinity and phospholipid specificity reported for the individual annexins (see Moss et al., 1991, for review).

Whatever the actual role of annexin II in a given cell type, it is likely to be regulated by Ca^{2+} binding. Our transfection studies show that integrity of the type II Ca^{2+} -binding sites is a prerequisite for an association of annexin II with the cortical cytoskeleton, which most likely determines the intracellular function of at least the (annexin II)₂(p11)₂ complex (Thiel et al., 1992; this study). The type III Ca^{2+} -binding sites, on the other hand, are not required for the cytoskeletal association of annexin II, as a mutant molecule containing disruptions only in the two type III sites shows the typical cortical localization. This finding and the low affinity of the type III Ca^{2+} -binding sites (even in the presence of phospholipid, the K_d for Ca^{2+} bound in these sites is approx. 100 μM) raise the issues of whether type III Ca^{2+} -binding sites are occupied within the cell and whether their integrity is indispensable for the intracellular annexin II function. The generation and characterization of other annexin mutants carrying disruptions in one or more type III sites should answer at least some of these questions.

We thank Mary Osborn for critical reading of the manuscript. This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft (Ge 514/2-1).

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