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Structural analysis of p36, a Ca^{2+}/lipid-binding protein of the annexin family, by proteolysis and chemical fragmentation

Nils JOHNSSON and Klaus WEBER

Max Planck Institute for Biophysical Chemistry, Department of Biochemistry, Göttingen, Federal Republic of Germany

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Limited proteolysis of the core domain of the 36-kDa protein p36 by trypsin gives a first insight into the structural organization of the four annexin repeats. Trypsin opens only a single peptide bond, situated between residues 204 and 205. The two fragments (of 20 kDa and 15 kDa), each containing two annexin repeats, remain as a tight complex (nicked core), which binds phospholipids in a Ca^{2+}-dependent manner. After denaturation by 9 M urea, the nicked core is again formed upon renaturation provided both fragments are present. If the fragments are separated by chromatography in urea prior to renaturation, they show different behaviour. The 15-kDa C-terminal repeats aggregate, while the 20-kDa N-terminal repeats stay in solution. In comparison to p36, fragments with two (20-kDa fragment) or one (N-terminal CNBr fragment) annexin repeats show a conformational alteration in CD spectroscopy and hydrodynamics and display an increased susceptibility to proteases. In line with these differences, their Ca^{2+}-dependent affinity to phospholipids is more than 10–20-fold decreased. Thus the four annexin repeats form together an integrated domain with multiple contacts between the repeats. Although stable derivatives with less than four repeats can be obtained, their Ca^{2+}/phospholipid binding affinities are noticeably reduced.

In the last few years sequence analysis has defined a new family of Ca^{2+}/lipid binding proteins, variously referred to as annexins, calpactins or lipocortins. All members of this still expanding protein family bind to membranes and phospholipids in a Ca^{2+}-dependent manner. Their usually moderate Ca^{2+} affinity is increased by one or two orders of magnitude in the presence of phospholipids (for a review see [1]). The common sequence principle of this protein family is an array of some 70 residues which depending on the molecular mass, is tandemly repeated four or eight times [2–8]. Sequence similarities of 50–70% are characteristic for corresponding annexin repeats from distinct proteins, while a similarity of only 30% seems a characteristic value for the repeats within a single protein. All repeats reveal a 17-residue consensus motif, possibly involved in Ca^{2+}/lipid binding [9, 10]. Limited proteolysis delineates two distinct domains: the large protease-resistant core and a small N-terminal tail, which is very susceptible to proteolysis. The core domain covers the annexin repeats and displays the Ca^{2+}-binding and Ca^{2+}-dependent lipid-binding site(s) [11–13]. The tail is variable in length and sequence and harbors all phosphorylation sites so far identified in the annexins [6, 14–17]. It seems to exert a regulatory function on the core. Tail-less proteolytic derivatives of two annexins require less Ca^{2+} for phospholipid binding than their parent molecules [13, 18].

The 36-kDa protein p36, which is the major cytoplasmic target of *src*-related tyrosine-specific protein kinases, is a member of the annexin family [3, 9, 19, 20]. Its ability to form a tetrameric complex with a dimer of a unique 11-kDa subunit (p11) distinguishes p36 from other annexins [19]. The major p11-binding site of p36, originally located at the tail [12, 21], was subsequently shown to occur within the N-terminal 14 residues and to include the *N*-acetyl group of serine 1 [22]. Peptides covering these residues bind via an amphiphatic α-helix to p11 in a manner reminiscent of that in which certain peptides of calmodulin target molecules interact with calmodulin [22]. Since p11 and calmodulin display consecutive EF-hand sequences, they belong to the same superfamily of proteins [23, 24]. In contrast to most other members of this superfamily, the EF-hands of p11 are non-functional by sequence criteria and the protein does not bind Ca^{2+} [11, 23]. Thus the interaction of p11 with its p36 ligand reflects a permanent 'on state': p11 decreases the Ca^{2+} requirement of p36 for negatively charged lipids below the levels typically found in resting cells [25]. This property of the p36/p11 complex correlates well with its location at the cytoplasmic face of the plasma membrane revealed in immunofluorescence and immunoelectron microscopy [26–28].

In contrast to the detailed characterization of the tail, knowledge of the four annexin repeats of the core of p36 is still limited. To analyse the organization of the core in a systematic manner, we have used limited proteolysis and chemical fragmentation to prepare derivatives of p36 which cover less than four repeats. Here we show that, in p36, interactions between all four annexin repeats are required for a strong Ca^{2+}-dependent binding to phospholipids. Fragments covering only one or two annexin repeats display Ca^{2+}/lipid binding but have markedly reduced affinities. The postulated interaction between the repeats can be directly demonstrated by the properties of a nicked core, which is obtained by trypsin treatment. It integrates repeats 1 plus 2 (20-kDa fragment) with repeats 3 plus 4 (15-kDa fragment) into a tight 20-kDa/15-kDa complex. Although the nicked core lacks the peptide

Correspondence to N. Johnsson, Max Planck Institute for Biophysical Chemistry, Postfach 2841, D–3400 Göttingen, Federal Republic of Germany

Enzymes. Chymotrypsin (EC 3.4.21.1); endoproteinase Glu-C (EC 3.4.21.19), trypsin (EC 3.4.21.4).

bond between residues 204 and 205, it shows the same Ca^{2+}/lipid binding properties as the intact core.

MATERIALS AND METHODS

Preparation of fragments and sequence analysis

Porcine intestinal protein 1 was purified according to Gerke and Weber [19] with minor modifications [22]. The p36 and p11 subunits were separated and renatured as described [29]. For the preparation of the 20-kDa and 15-kDa tryptic fragments, p36 was treated with trypsin in 20 mM Tris/HCl pH 7.5, 2 mM Ca^{2+} for 90 min at room temperature with an enzyme/substrate ratio of 1:200 (by mass). Proteolysis was stopped by adjusting the solution to 1 mM phenylmethylsulfonylfluoride (Fluka) and 0.2 mM diisopropylfluorophosphate (Sigma). Lyophilized protein was dissolved in 9 M urea, 20 mM Tris/HCl pH 7.4 and applied on a DEAE-cellulose column (Whatman, Maidstone, UK) equilibriated in the same solvent. Flow-through fractions were dialyzed against p36 renaturation buffer (20 mM Tris/HCl, 100 mM NaCl, 0.5 mM EGTA, 1 mM dithiothreitol pH 7.5) for 16 h at 4 °C. The precipitated protein was washed twice with water. Subsequent gel electrophoresis showed that the precipitate was nearly (95%) pure 15-kDa fragment. The 20-kDa fragment was eluted from the DEAE-cellulose column with 0.3 M NaCl in 9 M urea, 20 mM Tris/HCl pH 7.4. It was renatured by dialysis against renaturation buffer. Gel electrophoresis showed that it was pure renatured 20-kDa fragment.

The 20-kDa and 15-kDa fragments were characterized by CNBr cleavage. After lyophilization the resulting CNBr peptides were dissolved in 6 M guanidine·HCl and applied to a C_{18} reversed-phase HPLC column (Vydac), which was developed with a linear acetonitrile gradient in 0.1% trifluoroacetic acid. Peptides were sequenced using an Applied Biosystem gas-phase sequenator (model 470A) with an on-line phenylthiohydantoin analyzer. The sequences obtained could be aligned along the sequence of bovine p36 predicted by cDNA cloning [30]. Thus the 20-kDa fragment covers residues 28–204 of p36, while the 15-kDa fragment covers residues 205–338.

The N-terminal CNBr fragment (residues 1–117) of p36 was isolated by gel filtration in 9 M urea on Sephadex G-200 (see Results). Protease-susceptibility studies of the 20-kDa fragment and the N-terminal CNBr fragment were done on an analytical scale in 20 mM Tris/HCl pH 7.5, 100 M NaCl, 0.5 mM dithiothreitol at room temperature using an enzyme/substrate ratio of 1:200 (by mass). Aliquots were withdrawn after different times and proteolysis stopped by boiling in the sample buffer used for gel electrophoresis. The 20-kDa fragment was treated with endoproteinase Glu-C (Boehringer, Mannheim, FRG) and the CNBr fragment was incubated with chymotrypsin; p36 served as control for both digestion conditions.

Circular dichroic spectroscopy

Measurements were performed at 20 °C on a Jobin mark V, interfaced to a microcomputer. Cuvettes of 1-mm pathlength were used. Spectra were taken in steps of 0.2 nm and each signal was automatically sampled and signal-averaged. Proteins were in 20 mM phosphate pH 7.5. Concentrations of 15.5 μM, for p36, 29 μM for the 20-kDa fragment and 29 μM for the CNBr fragment were used. The concentrations of p36 and the CNBr fragment were measured by quantitative amino acid analysis, that of the 20-kDa fragment was determined by the Bradford assay using p36 as a standard.

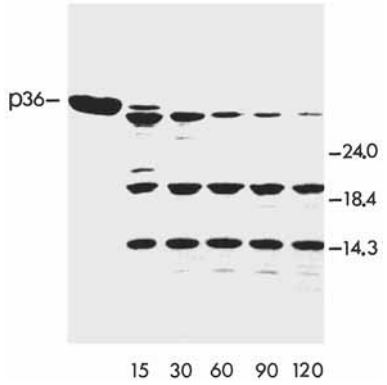

Fig. 1. *Gel electrophoresis of proteolytic fragments of p36.* Limited proteolysis of p36 in 20 mM Tris/HCl, 100 mM NaCl, 2 mM Ca^{2+}, 0.5 mM dithioerythrol, pH 7.5, at 20 °C for times between 15–120 min by trypsin (1:200, by mass) was monitored by gel electrophoresis. Note the rapid loss of the N-terminal tail (compare the first two lanes), which converts p36 to the 34-kDa core and the relative stability of the 20-kDa and 15-kDa fragments. A small amount of the core is still left after the longest trypsin treatment. Numbers at the bottom indicate time in minutes, those at the right molecular mass in kDa

Phospholipid binding

The preparation of the phospholipid affinity column followed the procedure of Uchida and Filburn [31] using cholesterol and dioleoylglycerophosphoserine in the given ratio. Approximately 40 μg protein was loaded on 1.5 ml of the affinity matrix in 20 mM imidazole/HCl pH 7.5, 100 mM NaCl, 2 mM NaN_3, 2 mM Ca^{2+}. The column was washed with 10 vol. loading buffer and the bound protein was eluted with buffer containing 8 mM EGTA. Fractions of 400 μl were collected and concentrated for SDS/PAGE. Liposomes were prepared by sonication of 10 mg/ml dipalmitoylglycerophosphoserine (kindly provided by Dr H. J. Eibl, Göttingen) in water. Binding experiments were performed in 0.2 ml 20 mM Tris/HCl pH 7.5, 100 mM NaCl, 2 mM NaN_3 at the indicated lipid and Ca^{2+} concentrations. After incubation for 15 min at room temperature samples were centrifuged at 200000 *g* for 15 min. Bound protein was extracted with 0.2 ml buffer containing 8 mM EGTA. Determination of the fraction of bound and unbound protein was performed by SDS/PAGE and gel scanning (see [25]) or by the Bradford assay [13].

Miscellaneous techniques

Analytical ultracentrifugation was performed in a Beckman model E centrifuge equipped with Flossdorf optics. Proteins were in 20 mM phosphate pH 7.5 containing 100 mM NaCl. Protein concentrations were 18 μM for p36 and 29 μM for the 20-kDa fragment. Difference spectra were recorded on a Cary 118 spectrophotometer between 320–250 nm. Proteins were in 20 mM Tris/HCl pH 7.8, 100 mM NaCl, 50 μM EGTA. After recording the baseline, Ca^{2+} was added to the sample cuvette and the spectrum was recorded again. Analytical gel filtration was on a Superose S12 column connected to an FPLC system (Pharmacia). Gel filtration was in 20 mM imidazole/HCl pH 7.5, 100 mM NaCl, 2 mM

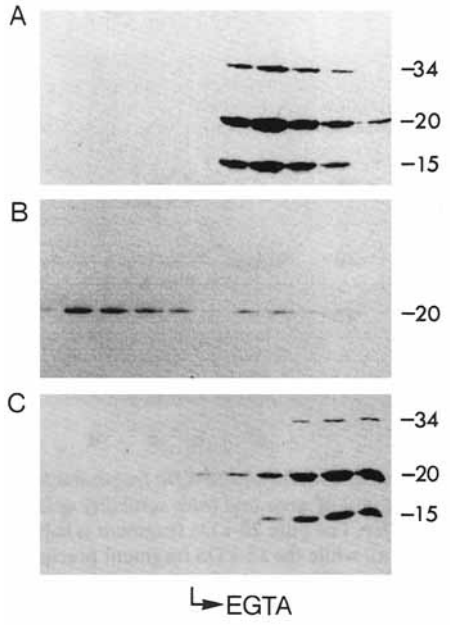

Fig. 2. *Phospholipid-affinity chromatography of the nicked core (A), its 20-kDa fragment (B) and the renatured 20-kDa/15-kDa complex (nicked core) (C).* Fractions of the flow-through (presence of Ca^{2+}) and the EGTA eluate (indicated by the arrow) are shown. The 20-kDa/15-kDa complex binds as strongly to the affinity column as the 34-kDa core (A, C), but only 10–15% of the isolated 20-kDa fragment was found in the EGTA eluate (B)

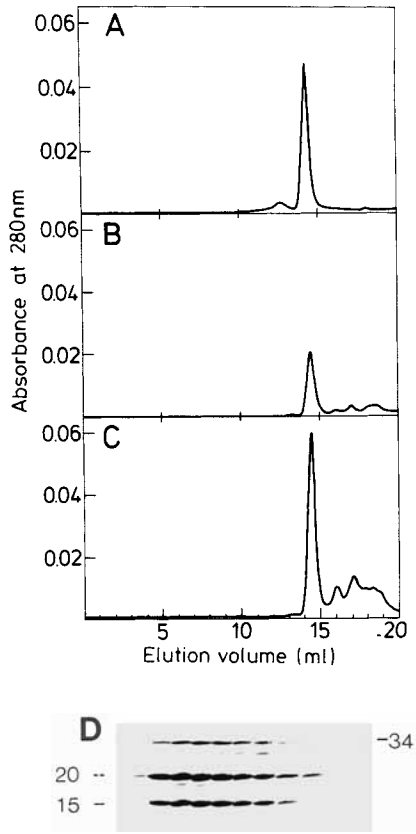

Fig. 3. *Gel filtration on Superose S12 of p36 (A), p36 treated with trypsin (20-kDa/15-kDa complex) (B), and the complex after urea denaturation and subsequent renaturation (C).* The peak fractions from experiment B were analyzed by gel electrophoresis (D). Note the co-elution of 20-kDa, 15-kDa fragments and the remaining 34-kDa core at 14.5 ml, while intact p36 elutes at 14.3 ml

NaN_3, 0.5 mM EGTA at a flow rate of 0.5 ml/min. Fractions of 0.15 ml were collected. Protein was precipitated with chloroform/methanol according to Wessel and Flügge [32]. Calibration of the column was performed under identical conditions using bovine serum albumin (Behringwerke, Marburg, FRG), chymotrypsinogen, myoglobin, ovalbumin (all from Sigma), RNase A, dextran Blue (Pharmacia) and vitamin B12 (Bio-Rad) as standards. Ca^{2+} concentrations were measured using an Orion Ca^{2+}-sensitive electrode (model 93–20) following the instructions of the manufacturer (Orion Co., MA, USA).

RESULTS

Limited proteolysis of p36 by trypsin

Proteolysis of p36 proceeds in two steps. Due to the preferential action on the N-terminal tail domain, trypsin initially converts p36 into its 34-kDa core, which is subsequently cleaved into two fragments of apparent molecular mass of 20 and 15 kDa (Fig. 1). To map the fragments along the p36 sequence, a 90-min digest separated on a gel was transferred to an Immobilon membrane and both fragments were sequenced for eight cycles. The 20-kDa fragment starts with alanine 28, while the 15-kDa fragment begins at lysine 205. To determine the carboxy-termini, we cleaved the purified fragments (see below) with CNBr and separated the resulting peptides by HPLC (see Materials and Methods). Sequence analysis showed that the 20-kDa fragment ends at arginine 204, whereas the 15-kDa fragment retains the C-terminus of the intact p36. Thus only the peptide bond at the carboxy side of arginine 204 is opened when the 34-kDa core is converted by trypsin into the two fragments. Given the general structure of Ca^{2+}/lipid binding proteins [33], the 20-kDa fragment contains annexin repeats 1 and 2, while the 15-kDa fragment starts 16 residues within annexin repeat 3, covering the rest of this repeat as well as the entire annexin repeat 4. To test for the interaction with phospholipids, a trypsin digest of p36 was loaded on a phospholipid affinity column of immobilized phosphatidylserine in 2 mM Ca^{2+}. After washing with 10 vol. of loading buffer, a solution containing 8 mM EGTA was applied to strip the bound protein. Fractions were analyzed by gel electrophoresis. Both fragments bound quantitatively to the column (Fig. 2) as did both p36 (not shown) and the 34-kDa core (Fig. 2).

The 20-kDa/15-kDa complex: preparation of a 20-kDa derivative with two annexin repeats

Gel filtration of the tryptic digest on Superose S12 showed that the 20-kDa and 15-kDa fragments and the small amount of 34-kDa core that remained uncleaved, co-elute at 14.5 ml, i.e. 0.2 ml later than intact p36 (Fig. 3). Ion exchange chromatography confirmed that the 20-kDa and 15-kDa fragments form a tight complex (nicked core; Fig. 4). Since the amino acid sequences predict a negative net charge for the 20-kDa fragment and a positive net charge for the 15-kDa fragment, the digest was subjected to anion-exchange chromatography on DEAE-cellulose. Contrary to the charge prediction, the 20-kDa fragment did not bind to the resin and instead eluted with the 15-kDa fragment in the flow-through fractions, which also contained the trace of 34-kDa core.

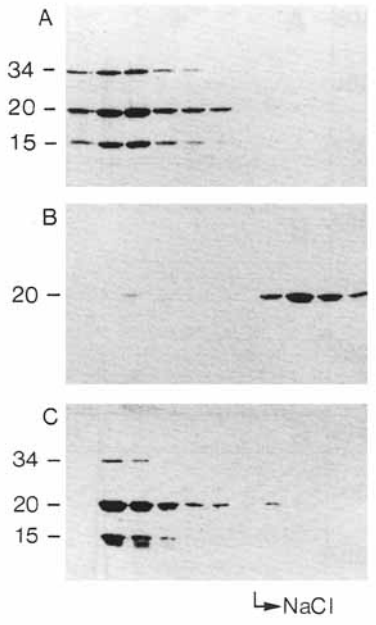

Fig. 4. *Anion-exchange chromatography of the 20-kDa/15-kDa complex (nicked core) on DEAE-cellulose as monitored by gel electrophoresis.* Trypsin nicked core containing equal amounts of 20-kDa and 15-kDa fragments was not retained by DEAE-cellulose and was found, together with the uncleaved 34-kDa core, in the flow-through (A). The 20-kDa fragment was separated from the 15-kDa fragment and the 34-kDa core by chromatography on DEAE-cellulose in the presence of 9 M urea (see Fig. 3) and renatured by dialysis. The native 20-kDa fragment was bound by DEAE-cellulose and released by 300 mM NaCl (B). Trypsin-nicked core was denatured by 9 M urea and subsequently renatured by dialysis. The reformed 20-kDa/15-kDa complex was again not retained by DEAE-cellulose (C; compare with A). Note that under native conditions the 20-kDa fragment can bind to DEAE-cellulose only in the absence of the 15-kDa fragment

When the 20-kDa/15-kDa complex was dissociated in buffer containing 9 M urea, an easy separation of the fragments on DEAE-cellulose was obtained. Now the 15-kDa fragment and the 34-kDa core appeared in the flow-through of the column, while the 20-kDa fragment was retained and could be eluted after addition of salt to 300 mM. Since p36 is easily renatured from urea [29], the separated fragments were extensively dialyzed against renaturation buffer. After high-speed centrifugation, the 15-kDa fragment was pelleted and the 34-kDa core was found in the supernatant. In contrast to the 15-kDa fragment, the 20-kDa material remained soluble (Fig. 5). As expected from its amino acid composition, the renatured pure 20-kDa fragment bound to DEAE-cellulose (Fig. 4).

When the 20-kDa/15-kDa complex was made 9 M in urea, subsequent dialysis against renaturation buffer showed no precipitation of the 15-kDa fragment. Both fragments eluted as a single peak at 14.5 ml from Superose S12 (Fig. 3) and were not bound by DEAE-cellulose (Fig. 4). The combined results show that trypsin treatment leads to a nicked core. The 20-kDa/15-kDa complex is reformed after urea denaturation, provided the two fragments are renatured together. While the separated 20-kDa fragment can renature on its own, the separated 15-kDa fragment aggregates. Thus the 20-kDa fragment can complement the 15-kDa fragment during the folding process when the mixture of the two fragments is renatured. This renatured 20-kDa/15-kDa complex again binds to the phospholipid affinity matrix in a Ca^{2+} dependent manner (Fig. 2).

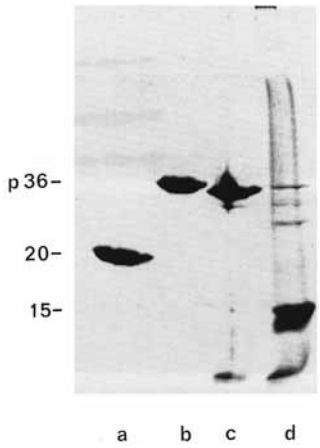

Fig. 5. *Separation of 20-kDa and 15-kDa fragments by anion-exchange chromatography in 9 M urea and their solubility after dialysis against renaturation buffer.* The pure 20-kDa fragment is soluble after dialysis from urea (lane a), while the 15-kDa fragment precipitates after dialysis (lane d). The small amount of p34 found in the 15-kDa fraction remains soluble after removal of urea (lane c). Lane b shows the control for p36

Preparation of a derivative with a single annexin repeat

Since limited proteolysis generated only fragments covering at least two annexin repeats, we used chemical cleavage of p36 to prepare a fragment consisting of only one annexin repeat. Porcine p36 has its first methionine at position 117. Therefore the N-terminal CNBr fragment covers the N-terminal tail domain, the complete annexin repeat 1 and some 14 residues of repeat 2. With a molecular mass of 13 kDa it is the only large CNBr fragment and therefore is easily purified when the fragment mixture is subjected to gel filtration on Sephadex G-200 in 9 M urea. The purified 13-kDa fragment has the predicted amino acid composition (Table 1) and reacts with a monoclonal antibody whose discontinuous epitope includes residues 23–27 in the tail as well as residues around position 65 in repeat 1 of porcine p36 [34]. The fragment is soluble after removal of urea. Unlike the 20-kDa fragment, it cannot rescue the 15-kDa fragment when added prior to the removal of urea. Dialysis of the mixture leads to precipitated 15-kDa fragment while the 13-kDa fragment stays in the supernatant (not shown). Cleavage of p36 at its cysteines with 2-nitro-5-thiocyanobenzoic acid leads to a fragment corresponding to annexin repeat 4 (residues 261–334). Unfortunately this fragment also aggregated upon renaturation and therefore could not be used for further studies.

Calcium/lipid binding properties of derivatives with one or two annexin repeats

The Ca^{2+}-dependent phospholipid binding of p36 and its fragments was first tested with the phospholipid affinity column of immobilized phosphatidylserine. In contrast to the nicked p36 core, the N-terminal 13-kDa CNBr fragment showed no binding (data not shown) and the 20-kDa fragment revealed strongly reduced binding. Only 10–15% of the loaded 20-kDa fragment was retained by the affinity column (Fig. 2). To vary the concentration of lipid more precisely, we made use of the liposome pelleting assay introduced for p36 by Glenney [35]. Fig. 6 shows that both 20-kDa and 13-kDa fragments bind to the negatively charged liposomes, although the lipid concentration needed for half-maximal binding is

Table 1. *Amino acid composition of the 13-kDa fragment by hydrolysis (A) and by sequence (B)*
Cys and Trp were not determined (n.d.)

Amino acid	Amount in 13-kDa fragment	
	A	B
	mol/mol protein	
Asp	9.8	6
Asn		5
Thr	9.5	10
Ser	10.8	11
Glu	12.2	9
Gln		4
Pro	2.7	2
Gly	5.1	5
Ala	15.6	14
Val	6	6
Met	–	1
Ile	6.5	7
Tyr	4	4
Phe	2.2	2
Lys	8.7	9
His	3.1	3
Arg	5.5	5
Trp	n.d.	–
Cys	n.d.	–

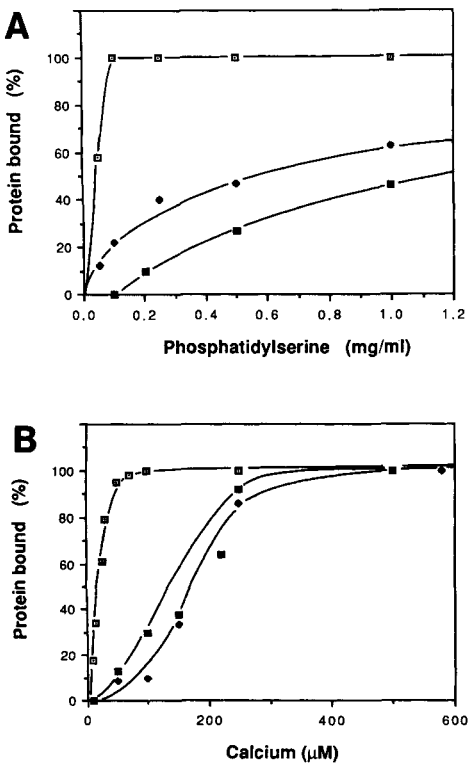

Fig. 6. *Binding of p36 and its fragments to phospholipids monitored by the liposome precipitation assay using dipalmitoylglycerophosphoserine.* (A) Binding at increasing phospholipid concentrations; (B) binding at increasing Ca^{2+} concentrations. The binding in B refers to the maximal binding at saturating Ca^{2+} concentrations set to 100%. The Ca^{2+} concentrations indicated reflect the free Ca^{2+} concentrations (see text). (□) p36; (■) 20-kDa fragment; (●) N-terminal CNBr fragment

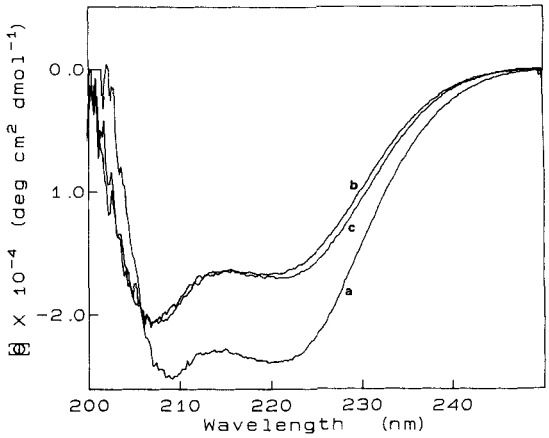

Fig. 7. *CD spectra of (a) p36, (b) its tryptic 20-kDa fragment and the (c) N-terminal CNBr fragment in 20 mM phosphate pH 7.5*

increased by factors of more than 10 and 20, respectively, when compared with p36. To relate these results to the observations made with the lipid affinity column (see above), we stress that most likely a high concentration of exposed negatively charged lipids seems critical for binding of fragments, which carry only one or two instead of four annexin repeats.

Fig. 6 also shows binding results obtained at constant dipalmitoylglycerophosphoserine concentration (1 mg/ml) with varying Ca^{2+} concentrations. When compared to p36, the Ca^{2+} requirement for half-maximal binding of the two fragments is increased: p36 requires 250 μM Ca^{2+} for half-maximal binding under these conditions whereas both fragments need Ca^{2+} levels of about 700 μM in the buffer. Since the assays had to be performed at high phospholipid concentrations, we tested the influence of the phosphatidylserine liposomes on the free Ca^{2+} concentrations using *a* Ca^{2+}-sensitive electrode. Phospholipid at 1 mg/ml decreases the free Ca^{2+} concentration significantly. When extrapolated to lipid-free buffer, the Ca^{2+} concentration for half-maximal binding decreased to approximately 20 μM for p36 and to 200 μM for the two fragments. Although clearly an oversimplification, this subtraction procedure for the lipid-bound Ca^{2+} leads to a value for p36 which is in good agreement with results measured at a fivefold lower phospholipid concentration [25].

Since binding of Ca^{2+} by p36 and its 34-kDa core can be monitored in the absence of lipids by difference spectroscopy [12, 29], we also tested the 20-kDa and 15-kDa fragments for this property. No effect was found for either even when 5 mM Ca^{2+} was used (not shown). Thus the C-terminal residues 206–338 covered by the 15-kDa fragment are either directly or indirectly responsible for the pronounced Ca^{2+}-dependent difference spectrum of p36.

Derivatives with one or two annexin repeats assume a conformation related to p36

If one demands functional Ca^{2+}/lipid-binding sites for the N-terminal annexin repeats in the intact protein, either incomplete folding or a reduced stability of the repeats in the two fragments could explain their low affinity for phospholipids. We therefore analyzed the conformation of the derivatives in more detail. Fig. 7 shows that the CD spectrum of p36 is strongly dominated by α-helix. Using the formula of Clark et al. [36], the ellipicity of -23800 deg cm^2 dmol^{-1} at 220 nm predicts an α-helix content of about 66%. The tryptic

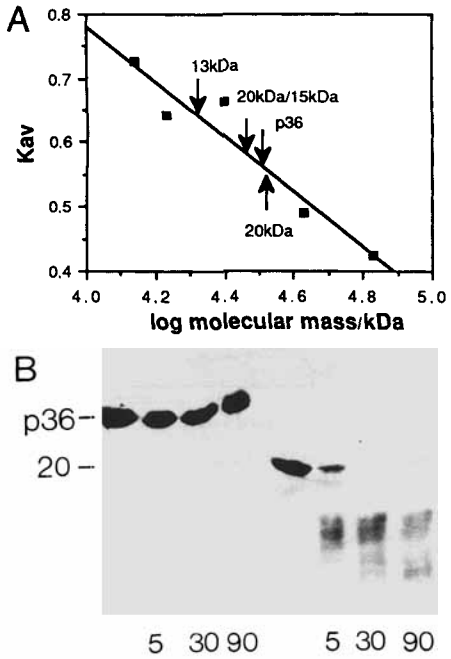

Fig. 8. *(A) Gel filtration of p36 and its derivatives on a calibrated Superose S12 column; (B) limited proteolysis of p36 and 20-kDa fragment by endoproteinase Glu-C.* (A) (■) K_{av} values for bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin and RNase. Note that the 20-kDa fragment elutes just prior to p36. (B) Limited proteolysis of p36 (first four lanes) and the 20-kDa fragment by endoproteinase Glu-C under identical conditions (see Methods). Samples were removed at the indicated times (in minutes). While p36 remained stable, the 20-kDa fragment is readily cleaved into smaller products.

20-kDa fragment and the N-terminal 13-kDa CNBr fragment have nearly identical CD spectra. They display an ellipicity of -17000 deg cm^2 dmol^{-1} and thus an α-helix content of only 46%. Fig. 7 also shows that the spectra of the two fragments are still closely related to the spectrum of intact p36. We conclude that proteins containing one and two annexin repeats can assume a conformation similar but not identical to that of p36, which has four such repeats.

In gel filtration on a calibrated Superose S12 column, both fragments elute earlier than expected from their calculated molecular masses (Fig. 8). The 13-kDa fragment elutes at a position typical for a globular protein with a molecular mass of 20-kDa while the 20-kDa fragment elutes 0.1 ml prior to the position of p36, i.e. corresponding to a globular protein of around 35-kDa. Several lines of evidence suggest that the fragments do not form dimers but, instead, have a more elongated and flexible shape compared to p36 which behaves more like a globular molecule. Chemical cross-linking with two different reagents [dimethylsuberimidate, ethylene glycol-bis(succinimidylsuccinate)] yielded no dimer species when monitored by gel electrophoresis. Analytical ultracentrifugation gave a sedimentation coefficient of 3.1 ± 0.2 S for p36, and 2.0 ± 0.2 S for the 20-kDa fragment. Since a hypothetical dimer of 20-kDa should have a sedimation coefficient of around 3.1 S or slightly higher, we conclude that the hydrodynamic data point to a more elongated and flexible shape of the fragment. This prediction is confirmed by a markedly increased proteolytic sensitivity of the fragments in comparison to p36. The core of p36 is known to be very resistant to chymotrypsin and endoproteinase Glu-C [12, 22]. In contrast, the two fragments are rapidly degraded (for treatment of the 20-kDa fragment with endoproteinase Glu-C see Fig. 8 B).

DISCUSSION

The annexins are based on a 70-residue motif, the annexin repeat, which is tandemly repeated either four or eight times. Although quantitative results on the number of Ca^{2+} sites differ distinctly, there is agreement that at least some annexins, including p36, bind more than one Ca^{2+} ion/molecule [11, 37, 38]. Thus it was suggested that each annexin repeat may provide a Ca^{2+}/lipid-binding site and that the repeats could act as autonomous domains [9, 10]. Therefore we initiated a study aiming at p36 derivatives containing less than four annexin repeats. During this analysis, we found an unexpectedly tight interaction between the repeats. It shows that the repeats are integrated into a functional domain. Nevertheless a single repeat can still show Ca^{2+}/lipid-binding, albeit at clearly reduced affinity.

Trypsin cleaves the 34-kDa core of p36 between the Arg-Lys peptide bond at position 204, one residue prior to the canonical glycine of the annexin repeat 3. In spite of this cleavage, the two fragments remain as a tight 20-kDa/15-kDa complex (nicked core), which retains the ability to bind to phospholipids at high affinity. Separation of the fragments requires denaturating agents such as urea. Once separated, the two halves of the molecule show different behaviour, although each fragment consists of approximately two annexin repeats. The 20-kDa fragment containing repeats 1 and 2 stayed soluble after renaturation whereas the 15-kDa fragment containing repeats 3 and 4 aggregated in physiological buffer. Reassociation of the 20-kDa and 15-kDa fragments to form the nicked core occurred only when both fragments were renatured together. These results argue for extensive contact zones of the two N-terminal repeats with the two C-terminal repeats. These contacts also seem important for the functional integrity of the annexin repeats in p36. Consequently, p36-derived fragments consisting of only one (13-kDa CNBr fragment) or two (tryptic 20-kDa fragment) annexin repeats revealed only very reduced but still Ca^{2+}-dependent binding to phospholipids. The tendency of the C-terminal part of p36 to aggregate restricted our analysis to the N-terminal repeats. CD spectroscopy suggests that the secondary structure of both N-terminal fragments (20-kDa, 13-kDa) is still quite similiar to that found in the intact p36 molecule, but the hydrodynamic measurements and the increased susceptibility to proteolytic attack argue for a more flexible and elongated structure of the fragments. The decrease in binding activity observed for the fragments seems to be caused by a certain loss of ordered structure when compared with the intact core or p36. This change in structure also explains the reduced affinity of a monoclonal antibody when tested on p36 and the N-terminal CNBr fragment. Although the epitope combines sequences on the tail and the first annexin repeat, the CNBr fragment covering both regions has an approximately 13-fold reduced affinity to the antibody [34].

The tryptic fragments of p36 described here are comparable to fragments derived from an elastase digest of lipocortin I (p35) [39], a different member of the annexins. The lipocortin fragments separated by SDS/PAGE were eluted from the gel, freed of SDS and tested for their phospholipase A2 inhibition activity at high Ca^{2+} concentrations. Huang et al. concluded that the region of the p35 molecule (lipocortin I) responsible

for phospholipase inhibition resides in an 18-kDa fragment, which by its sequence is equivalent to the tryptic 20-kDa fragment in our study. Since for annexins, phospholipase A2 inhibition and phospholipid binding are now thought to be highly correlated if not identical properties [40], this result is in agreement with the localization of a phospholipid binding site on the 20-kDa fragment of p36 (see above). No phospholipase inhibitory activity was detected for the carboxy-terminal 15-kDa fragment of p35, which corresponds to the tryptic 15-kDa fragment of p36. Huang et al. [39] therefore stressed a functional inequality of the annexin repeats. Since no independent test for proper renaturation of the 15-kDa fragment of lipocortin 1 was performed in this study a different interpretation is also possible. In analogy to the p36 fragments, the p35-derived 18-kDa and 15-kDa fragments may also form a tight complex. Consequently the C-terminal 15-kDa fragment of p35 might not fold correctly in the absence of the 18-kDa fragment. Since elastase and trypsin cut the two different annexins at exactly the same site, a very similar folding of both proteins can be anticipated.

Our analysis of the annexin repeats shows a noticeable overall analogy to the structure of EF-hand-type Ca^{2+}-binding proteins such as troponin, calmodulin or parvalbumin. These proteins are also built from tandemly repeated sequence motifs, here the EF-hand, which is approximately 30 residues in length [41]. Proteolytic fragments of troponin C covering a pair of EF-hands showed strong Ca^{2+} binding. This was markedly reduced once the EF-hand pair was further processed to single EF-hands [42]. This observation, which was confirmed by studies on parvalbumin [43], can be explained by the X-ray structure of the proteins [44, 45]. Two EF-hands are assembled into one domain. Once the hydrophobic contacts between the two hands are disrupted by further proteolysis, the single EF-hand is destabilized and its Ca^{2+} affinity consequently reduced. This behaviour corresponds to the minimal repeat number of two EF-hands/polypeptide. The characterized annexins possess either four or eight repeats. Our data support the view that in the annexins the four repeats are assembled into one domain, which has multiple Ca^{2+}/phospholipid-binding sites.

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