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Two novel brain proteins, CaBP33 and CaBP37, are calcium-dependent phospholipid- and membrane-binding proteins

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Two acidic Ca^{2+}-binding proteins (CaBP33 and CaBP37) purified from bovine brain have been characterized in terms of immunological properties, heat-sensitivity, electrophoretic mobility, and Ca^{2+}-dependent binding to negatively charged phospholipids and to brain membranes. They were induced to bind to membranes by homogenization of brain tissue in the presence of $CaCl_2$. The membrane-bound CaBP33/CaBP37 mixture resisted extraction with detergents and was solubilized with high concentrations of EGTA/KCl. However, apparent Ca^{2+}-independent binding of the two proteins to membranes seemed to occur as well. This latter fraction of membrane-bound CaBP33 and CaBP37 could be solubilized with Triton X-100, indicating that brain membranes normally contain the two proteins as intrinsic components.

CaBP33/CaBP37; Calcium; Phospholipid; Membrane; Binding; Annexin; (Brain)

1. INTRODUCTION

The second messenger calcium ion is involved in the regulation of a number of activities within animal cells, including cell division, shape changes and motility, the conductance and transmission of nerve impulses, secretion, and several enzymatic activities [1]. The Ca^{2+} concentration in the cytoplasm of resting cells is kept ≤ 0.1 μM by the Ca^{2+} pump, mitochondria, and a number of cytoplasmic proteins which, thanks to their high affinity for Ca^{2+} binding, act as Ca^{2+} buffers [1]. In addition, cells possess a set of proteins characterized by affinities for Ca^{2+} in the range 1–100 μM, which act as amplifiers of the Ca^{2+} signal. The ubiquitous intracellular Ca^{2+} receptor, calmodulin, is one example of Ca^{2+}-binding proteins capable of amplifying the Ca^{2+} signal [2]. Calmodulin belongs to the superfamily of Ca^{2+}-binding proteins thought to have evolved from a common ancestor and defined as Ca^{2+}-regulatory proteins of the EF-hand type [2]. Other members of this superfamily are parvalbumin, troponin C, the intestinal Ca^{2+}-binding protein, S-100 proteins, and many others [2–4]. It is now becoming clear that individual cell types express their own set of EF-hand proteins, that this event is developmentally regulated, and that the uneven distribution of these proteins (with the exception of calmodulin) contributes to the specificity of individual cell types. The biological, physicochemical, and functional aspects of EF-hand proteins have been extensively reviewed [2–6].

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In recent years, a novel superfamily of Ca^{2+}-binding proteins has been identified. The members of this superfamily share the ability to bind Ca^{2+} and to bind to membranes (phospholipids) (see [7–9] for reviews). On structural grounds, they are characterized by the so-called endonexin fold, i.e. a highly conserved cluster of 17 amino acids that is repeated several times in individual primary sequences. The Ca^{2+}-binding affinities of Ca^{2+}- and phospholipid-binding proteins in solution are not high, but increase upon binding to targets. This novel superfamily of Ca^{2+}-binding proteins is now known as the annexin superfamily.

Given the central role played by Ca^{2+} in cellular activation, it would be advisable to identify, isolate, and characterize as many Ca^{2+}-binding proteins as possible.

Recently, we have identified two novel Ca^{2+}-binding proteins in bovine brain [10]. These proteins were called CaBP33 and CaBP37 on the basis of their molecular weights as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration. We show here that CaBP33 and CaBP37 are Ca^{2+}-dependent phospholipid- and membrane-binding proteins.

2. MATERIALS AND METHODS

The CaBP33/CaBP37 mixture was purified from bovine brain as in [10]. To separate the two proteins from one another, the mixture was loaded onto a column of DEAE Sephadex A-50 (1 × 10 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol (buffer I), which was washed first with buffer I and then with buffer I containing 0.15 M NaCl. Finally, the column was developed with a linear NaCl gradient (0.15–0.30 M in buffer I, 100 ml total vol.).

An antiserum against the mixture was raised in rabbits by multisite injection of 0.3–0.5 mg of protein in complete Freund adjuvant,

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followed by injections of proteins in incomplete adjuvant 15 and 40 days later. Animals were bled two weeks after the last injection. The specificity of the antiserum was assessed by immunoblotting [11] using the CaBP33/CaBP37 mixture and a bovine brain cytosolic fraction.

Binding of the CaBP33/CaBP37 mixture to liposomes of different phospholipid composition was done exactly as in [12].

SDS-PAGE (10% acrylamide) and PAGE in the absence of SDS (20% acrylamide) were done as in [13,14], respectively. Gels were stained with Coomassie blue.

S-100b and S-100a_0 proteins were purified from rat brain and porcine heart, respectively [15].

Other experimental details are given in section 3 and in the legends to figures.

3. RESULTS AND DISCUSSION

CaBP33 and CaBP37 could be separated from one another by DEAE Sephadex A-50 chromatography, the left and right tails of the chromatogram containing virtually pure CaBP33 and CaBP37, respectively (fig.1a–c). In polyacrylamide gels run under native conditions, the unfractionated mixture migrated significantly slower than S-100b and S-100a_0 proteins, as two distinct but poorly separated bands (fig.1d–f), as expected on the basis of the results illustrated in fig.1a–c.

The antiserum raised against the unfractionated mixture proved specific to CaBP33 and CaBP37 by immunoblotting when tested on a bovine brain cytosolic fraction (fig.1g,h,g′,h′). No binding of this antiserum was detected when a rat lung cytosolic fraction was analyzed (fig.1j,j′). On the other hand, this antiserum recognized a 53 kDa doublet in a rat liver cytosolic fraction (fig.1i,i′) and a 53 kDa polypeptide in a rat kidney cytosolic fraction (fig.1k,k′).

Subjecting rat brain and porcin heart to the procedure used to purify bovine brain CaBP33 and CaBP37 [10] resulted in the purification of CaBP33 exclusively, in the case of rat brain (fig.1m), and of a mixture of CaBP33 and CaBP37 in which CaBP33 was predominant, in the case of porcine heart (fig.1n). Rat brain CaBP33 was much less immunoreactive than the homologous bovine brain protein, by immunoblotting (fig.1m′). In the case of porcine heart CaBP33 and CaBP37, the antiserum turned out to interact exclusively with CaBP33 (fig.1n′).

Bovine brain CaBP33 and CaBP37 proved substantially heat-stable (fig.1o–r,o′–r′). They resisted to 70°C for 5 min. When heated at 100°C for 5 min, the two proteins could be partially precipitated. The fraction of them that remained in solution did not appear to have undergone denaturation, since the anti-CaBP33/CaBP37 antiserum still bound to both proteins (fig.1r,r′).

The electrophoretic mobility of the CaBP33/CaBP37 mixture in SDS gels was the same irrespective of whether or not 2-mercaptoethanol was present during boiling before electrophoresis (fig.1s,t).

Taken together, these data indicated that CaBP33 and CaBP37 are two distinct proteins and that CaBP33 is not the result of limited proteolysis of CaBP37. Antisera were also raised against individual proteins. Strong immunological cross-reactivity was detected (not shown), indicating homology between the two proteins. This observation, the Ca^{2+}-binding properties of the two proteins, and the notion that several annexins are characterized by M_r values around 35 kDa [7–9] led us to investigate whether our proteins are brain annexins. To this end, two sets of experiments were done. In one set, the procedure used to isolate two novel 32 and 34 kDa annexins from bovine lung [12] was applied to bovine brain. Briefly, bovine brain tissue was homogenized in buffer I containing 0.1 M NaCl, 5 mM EGTA, 0.25 mM phenylmethylsulfonyl fluoride (PMSF), and centrifuged at 40 000 × g for 1 h at 4°C in a Kontron (Centrikon H-401) centrifuge. The supernatant was adjusted to 6 mM $CaCl_2$ at a constant pH of 7.5, incubated at 4°C for 15 min under agitation, and centrifuged. The pellet was washed first with the above medium minus EGTA plus 1 mM $CaCl_2$, and then with the above medium minus NaCl and EGTA plus 1 mM $CaCl_2$. The final pellet was resuspended in buffer I containing 5 mM EGTA, and centrifuged at 150 000 × g for 30 min at 4°C in a Kontron (Centrikon T 1055) centrifuge. The supernatant was analyzed for the presence of the CaBP33/CaBP37 mixture by SDS-PAGE and immunoblotting. Fig.1u,v,u′,v′ shows that the two proteins could be isolated by the above procedure.

In the other set of experiments, the interaction of the CaBP33/CaBP37 mixture with phospholipids was studied using liposomes of different phospholipid composition. No binding of the two proteins to liposomes of phosphatidylcholine (PC) was detected, irrespective of the presence or absence of Ca^{2+} (fig.2A). No binding was observed in the case of liposomes of phosphatidylserine (PS), phosphatidylinositol (PI), or cardiolipin (CL) in the absence of Ca^{2+} (fig.2A), whereas strong binding was registered in the presence of 0.1 mM free Ca^{2+} (fig.2A). The strongest binding was observed with CL liposomes (fig.2A). Thus we used CL liposomes to study the binding of the two proteins as a function of free Ca^{2+} concentration. No Ca^{2+}-dependent precipitation of the two proteins was registered in the absence of liposomes (fig.2B, lanes 1s,1p). Maximal binding was detected around 100 μM free Ca^2, with half-maximal binding at ~10 μM free Ca^{2+} (fig.2B, lanes 2s,2p–9s,9p). The Ca^{2+}-dependent binding of CaBP33 and CaBP37 to CL vesicles was completely reversed by EGTA (fig.2B, lanes 10s,10p). Also, Ca^{2+}-dependent binding of the two proteins to CL vesicles occurred even in the presence of 5 mM 2-mercaptoethanol (fig.2A,●,■). Collectively, these data indicate that CaBP33 and CaBP37 are Ca^{2+}-dependent phospholipid binding proteins.

On the basis of these observations, we sought to

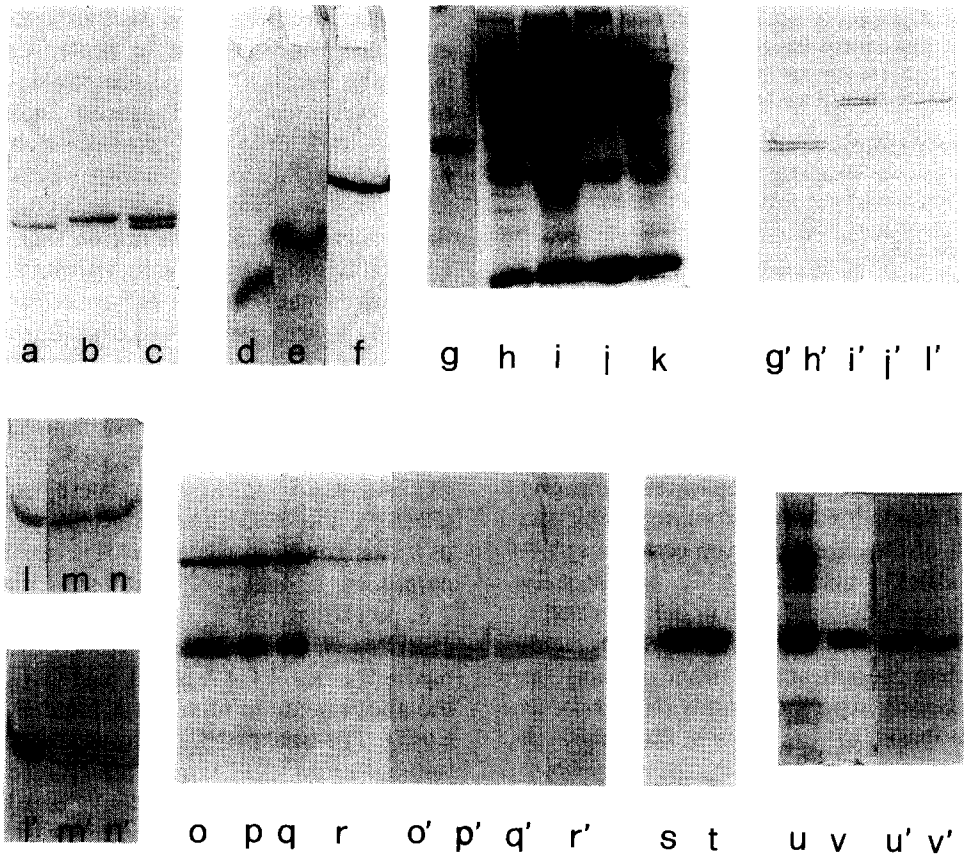

Fig.1. Characterization of CaBP33 and CaBP37. (a–c) SDS-PAGE of purified CaBP33 (a), purified CaBP37 (b), and CaBP33/CaBP37 mixture (c). (d–f) PAGE in the absence of SDS of S-100b protein (d), S-100a_0 protein (e), and CaBP33/CaBP37 mixture (f). (g–k') SDS-PAGE (g–k) and immunoblots (g'–k') of CaBP33/CaBP37 mixture (g,g'), bovine brain cytosol (h,h'), rat liver cytosol (i,i'), rat lung cytosol (j,j'), and rat kidney cytosol (k,k'). (l–n') SDS-PAGE (l–n) and immunoblots (l'–n') of bovine brain CaBP33/CaBP37 mixture (1,1'), rat brain CaBP33 (m,m'), and porcine heart CaBP33/CaBP37 mixture (n,n'). (o–r') SDS-PAGE (o–r) and immunoblots (o'–r') of a partially purified preparation of bovine brain CaBP33/CaBP37 mixture incubated for 5 min at 20°C (o,o'), 45°C (p,p'), 70°C (q,q'), or 100°C (r,r'). Proteins were cooled on ice and centrifuged. Samples of supernatants were analyzed by SDS-PAGE and immunoblotting. (s,t) Samples of CaBP33/CaBP37 mixture boiled in 2% (w/v) SDS minus (s) or plus (t) 2% (w/v) 2-mercaptoethanol prior to SDS-PAGE. (u–v') Isolation of the CaBP33/CaBP37 mixture from bovine brain by the procedure in [12]. SDS-PAGE (u–v) and immunoblots (u',v') of partially purified CaBP33 and CaBP37 (u,u') and purified CaBP33 and CaBP37 (v,v').

determine whether CaBP33 and CaBP37 also exist in a membrane-bound form and/or are capable of binding to brain membranes. Thus bovine brain tissue was homogenized in buffer I containing 1 mM $CaCl_2$, 0.25 mM PMSF (buffer II) and centrifuged. The pellet was washed twice with buffer II, and resuspended in buffer II containing 0.1 M KCl plus 1% (v/v) Triton X-100 (buffer III). After 1 h at 4°C under agitation, the suspension was centrifuged. The supernatant (Triton X-100-extract) was saved, and the pellet was washed twice with buffer III minus Triton X-100 before extraction with buffer I containing 5 mM EGTA (buffer IV). The suspension was centrifuged to obtain a supernatant (EGTA-extract) which was saved, and a pellet which was resuspended in buffer IV containing 0.9 M KCl. After 1 h at 4°C under agitation, the suspension was centrifuged to obtain a KCl-extract. All extracts were brought to 60% saturation with solid $(NH_4)_2SO_4$ and centrifuged. The supernatants were brought to 80% saturation with $(NH_4)_2SO_4$ and centrifuged. The pellets were resuspended in minimal vols of buffer I and dialyzed against buffer I. The dialysates were centrifuged to remove insoluble material. These experiments were repeated in the absence of added $CaCl_2$ and presence of 5 mM EGTA throughout.

When bovine brain tissue was homogenized in the presence of added $CaCl_2$, BaCP33 and CaBP37 were recovered in all 3 membrane extracts (fig.3a–c,a'–c'). When bovine brain tissue was homogenized in the presence of EGTA, the two proteins were recovered exclusively in the Triton X-100-extract (fig.3d–f,d'–f').

Thus the two proteins can be induced to bind to brain membranes in the presence of Ca^{2+}. Once the binding had occurred, a significant fraction of CaBP33 and CaBP37 resists extraction with Triton X-100, but it is not simply extractable with EGTA, suggesting that CaBP33 and CaBP37 interact strongly with Triton X-100-resistant (cytoskeletal?) membrane proteins through Ca^{2+}, and that Ca^{2+} is no longer required for at least a fraction of these proteins to remain bound to

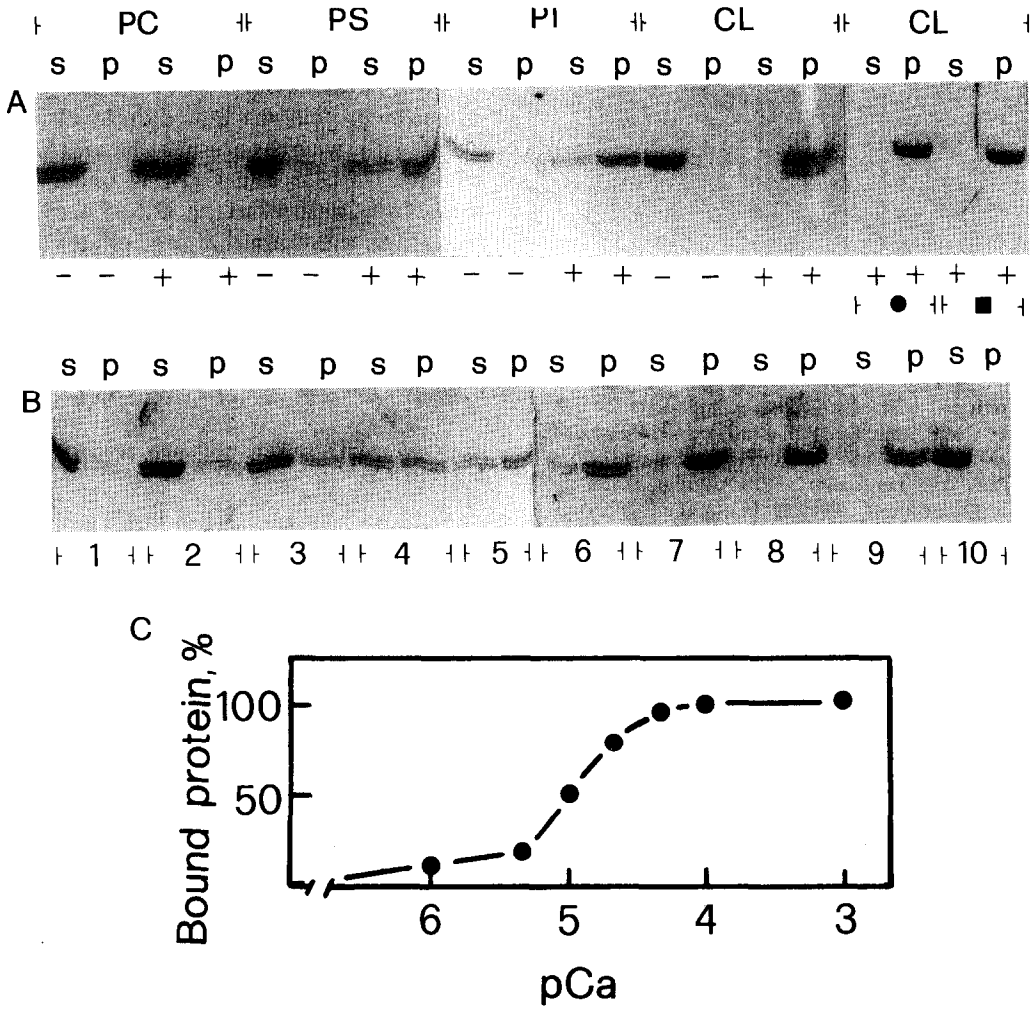

Fig.2. Binding of CaBP33 and CaBP37 to phospholipids. (A) Binding of the CaBP33/CaBP37 mixture to liposomes of different phospholipid composition. Liposomes made of phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), or cardiolipin (CL) were incubated at a concentration of 0.3 mg/ml with 10 μg of the CaBP33/CaBP37 mixture in 0.5 ml of 20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 1 mM $MgCl_2$, 1 mM EGTA in the absence (−) or presence (+) of 1.1 mM $CaCl_2$ for 15 min at 20°C, and centrifuged at 12000 × g for 10 min. Supernatants were saved, and pellets washed twice with the respective incubation buffer by resuspension and centrifugation. Individual final pellets were resuspended in 0.5 ml of 20 mM Tris-HCl, pH 7.5. Identical vols of supernatants (s) and pellets (p) were analyzed by SDS-PAGE. Symbols (●,■) refer to results of experiments in which the binding of the CaBP33/CaBP37 mixture to CL vesicles was studied in the presence of 100 μM free Ca^{2+} in the absence (●) and presence (■) of 5 mM 2-mercaptoethanol. (B) Binding of the CaBP33/CaBP37 mixture to CL vesicles as a function of free Ca^{2+} concentration. Conditions were as described in (A), except that binding was studied in the presence of increasing free Ca^{2+} concentrations: lanes 2(s,p), 0.5 μM; lanes 3(s,p), 1 μM; lanes 4(s,p), 5 μM; lanes 5(s,p), 10 μM; lanes 6(s,p), 50 μM; lanes 7(s,p), 100 μM; lanes 8(s,p), 500 μM; lanes 9(s,p), 1000 μM. Lanes 1(s,p) refer to experiments in which the CaBP33/CaBP37 mixture was incubated at 1 mM free Ca^{2+} in the absence of liposomes. Lanes 10(s,p) refer to experiments in which the CaBP33/CaBP37 mixture was incubated with CL vesicles in the presence of 100 μM free Ca^{2+} for 15 min, after which EGTA was added to 5 mM before centrifugation. Note that no Ca^{2+}-induced precipitation of the CaBP33/CaBP37 mixture occurs in the absence of liposomes, and that EGTA completely reverses the binding of these proteins to CL vesicles. (C) Plot of the CaBP33/CaBP37 bound to CL vesicles vs the free Ca^{2+} concentration. The amounts of CaBP33 and CaBP37 bound to CL vesicles (see B) were measured by densitometric scanning of SDS gels illustrated in (B). Results are expressed as the percentage of bound protein.

their targets. Irrespective of the presence or absence of Ca^{2+}, however, a substantial fraction of the two proteins is recovered in the Triton X-100-extract, pointing to a tight binding of the CaBP33/CaBP37 mixture to the lipid bilayer and/or to intrinsic membrane proteins. This interaction seems to be Ca^{2+}-independent or, alternatively, Ca^{2+} is only required for this interaction to occur. Future studies will dissect the molecular aspects of the complex binding of CaBP33 and CaBP37 to brain membranes.

Since several requirements, namely Ca^{2+}-dependent binding to phenyl-Sepharose [10], to negatively charged phospholipids, and to natural membranes, are fulfilled, we suggest that CaBP33 and CaBP37 may be reasonably defined as brain annexins. Of course, final conclusions in this respect can be drawn once the primary sequences of the two proteins are available.

Two proteins of M_r values similar to those of CaBP33 and CaBP37 have been identified in bovine brain using an antiserum against the 34 kDa calelectrin purified

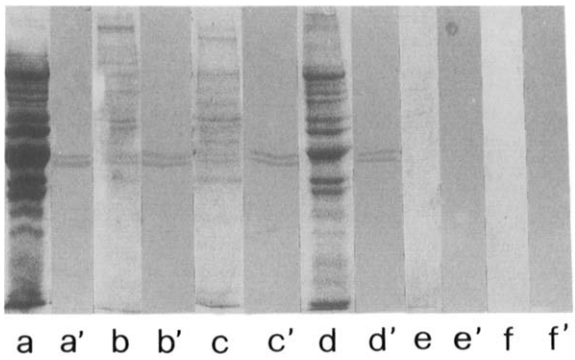

Fig.3. Electrophoretic and immunological characterization of membrane-associated CaBP33 and CaBP37. Fractions to be analyzed for the presence of the CaBP33/CaBP37 mixture were obtained from bovine brain tissue as described in section 2. SDS-PAGE (a–f) and immunoblots (a'–f') of the Triton X-100-xtract (a,a'), the KCl-extract (b,b'), and the EGTA-extract (c,c') from brain tissue obtained after homogenization in the presence of 1 mM $CaCl_2$, and of the Triton X-100-extract (d,d'), the KCl-extract (e,e'), and the EGTA-extract (f,f') from bovine brain tissue obtained after homogenization in the presence of 5 mM EGTA. Note that the Triton X-100-extract contain the CaBP33/CaBP37 mixture irrespective of the homogenizing conditions, whereas the presence of $CaCl_2$ during homogenization is a prerequisite for the two proteins to be recovered in the EGTA- and KCl-extracts. Similar experiments done in the presence of endogenous $CaCl_2$, i.e. no $CaCl_2$ or EGTA added, gave the same results as in a–c, a'–c' (data not shown).

from *Torpedo marmorata* [16]. Although in principle we cannot exclude the possibility that we have purified and characterized brain calelectrins, we note the following. (i) The anti-calelectrin antiserum cross-reacts with two 32 and 34 kDa annexins that are abundant in bovine lung (70 mg/kg of tissue) [12], whereas no binding of our anti-CaBP33/CaBP37 antiserum has been observed to polypeptides present in a rat lung cytosolic fraction. (ii) The anti-CaBP33/CaBP37 antiserum does not bind to the 67 kDa annexin found in the brain and liver [16] or to polypeptides of ~35 kDa in liver. It is known that the anti-34 kDa calelectrin antiserum and an antiserum raised against the 32.5 kDa calelectrin from liver and brain cross-react with the 67 kDa calelectrin of liver and brain [16]. (iii) Contrary to the 34 kDa calelectrin which does not bind to liposomes in the presence of 1 mM 2-mercaptoethanol [17], CaBP33 and CaBP37 bind to negatively charged phospholipids and to brain membranes in the presence of 5 mM 2-mercaptoethanol. Also, changes in the electrophoretic mobility of the 34 kDa calelectrin in SDS gels has been reported when the protein was boiled in the absence of reducing agents [18], whereas no such changes have been observed with CaBP33 and CaBP37 under the same conditions. (iv) Contrary to the 32.5 kDa calelectrin purified from liver and brain that precipitates at 70°C [16], our proteins are substantially heat-stable. Thus it can be reasonably concluded that we have identified and characterized novel brain Ca^{2+}-dependent phospholipid- and membrane-binding proteins (annexins).

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