Interaction of two brain annexins, CaBP33 and CaBP37, with membrane-skeleton proteins

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CaPB33 and CaPB37, two annexins purified from bovine brain, interact with a Triton X-100-resistant fraction (cytoskeleton) from bovine brain membranes in a Ca^{2+}-dependent way in vitro. The binding is saturable with respect to the CaBP33-CaBP37 concentration, half-maximal binding occurring at ~15 μg of the CaBP33-CaBP37 mixture/ml. The binding of these two annexins to the crude cytoskeleton preparation as a function of free Ca^{2+} concentration is biphasic, with half-maximal binding at ~50 μM and ~400 μM free Ca^{2+} for the first and the second component, respectively. By an overlay technique, CaBP33 and CaBP37 bind to a set of low M_r polypeptides (10–20 kDa) in the crude cytoskeleton preparation, with formation of an 85–90 kDa complex as investigated in cross-linking experiments. No binding of the CaBP33-CaBP37 mixture to either G- or F-actin has been observed Identification of the CaBP33-CaBP37-binding proteins in cytoskeletons would help elucidating the function(s) of these annexins in the brain

CaBP33-CaBP37, Annexin, Calcium, Cytoskeleton, Binding

1. INTRODUCTION

Two Ca^{2+}-binding proteins purified from bovine brain by Ca^{2+}-dependent affinity chromatography on phenyl-Sepharose [1] were shown to be Ca^{2+}-dependent phospholipid- and membrane-binding proteins [2]. Ca^{2+}-dependent phospholipid and membrane-binding proteins constitute a novel superfamily of Ca^{2+}-binding proteins known as annexins, that have been involved in the regulation of the structural organization of biological membranes, cell growth, exocytosis and endocytosis, and the inflammatory response (see (3–5) for reviews). The two proteins were called CaBP33 and CaBP37 on the basis of their M_r values as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration [1].

These proteins have been analyzed for their subcellular distribution in bovine brain by an immunochemical approach [2]. CaBP33 and CaBP37 were found soluble in the cytoplasm as well as associated with membranes. One fraction of membrane-bound CaBP33 and CaBP37 was resistant to EGTA and extractable with Triton X-100, and another fraction was resistant to Triton X-100 and extractable with high concentrations of EGTA/KCl [2]. While the former fraction was recovered in the Triton X-100-extractable material irrespective of the presence or absence of Ca^{2+}, the Triton X-100-resistant fraction required the presence of Ca^{2+} during the homogenization of brain tissue and subsequent steps for it to be recovered [2]. The detergent-resistant fraction of membrane-associated CaBP33 and CaBP37 was considered bound to membrane-skeleton protein in a Ca^{2+}-dependent way [2].

We have undertaken a study aimed at characterizing the binding properties of the CaBP33-CaBP37 mixture to a crude cytoskeleton fraction obtained from bovine brain membranes, in an attempt to identify the molecular target(s) of the two proteins in cytoskeletons.

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2. MATERIALS AND METHODS

The CaBP33-CaBP37 mixture was purified from bovine brain as in [1]. An antiserum against the unfractionated mixture was raised in rabbits and characterized as in [2]. Actin was purified from porcine heart as in [6].

A crude cytoskeleton fraction was obtained from bovine brain as follows. Frozen brain tissue (40 g) was thawed and homogenized in 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 0.25 mM phenylmethylsulfonyl fluoride (buffer A) (1:3, w:v) and centrifuged at 900 × *g* for 10 min in a Kontron (Centrikon H-401) centrifuge to remove nuclei and cell debris. The supernatant was saved and the pellet re-extracted and centrifuged as above. The two supernatants were combined and centrifuged at 30 000 × *g* for 60 min. The pellet was resuspended in buffer A, loaded onto a cushion of 0.8 M sucrose in buffer A and centrifuged at 30 000 g in a Kontron (Centrikon T 1055) centrifuge for 45 min to remove myelin. The pellet was washed once in buffer A containing 1 mM, instead of 5 mM, EGTA (buffer B) and resuspended in 230 ml of buffer B containing 1% (v:v) Triton X-100. After 60 min under agitation, the suspension was centrifuged at 200 000 g for 60 min The pellet was washed twice in buffer B by resuspension and centrifugation, and resuspended in buffer B. All operations were done at 4°C.

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Three procedures were used to monitor the interaction between the CaBP33-CaBP37 mixture with Triton X-100-resistant (cytoskeleton) protein obtained as described above. In one case, the CaBP33-CaBP37 mixture was incubated with the crude preparation of brain cytoskeleton in 0.5 ml of buffer B containing 0.1 M KCl, 1 mM $MgCl_2$ (buffer C) in the absence and presence of $CaCl_2$ for 60 min at room temperature, after which the suspensions were centrifuged at 150000 × *g* for 30 min. The supernatants were saved and the pellets were washed once in the respective incubation media. The final pellets were resuspended in 0.5 ml of buffer B. Equal portions of individual supernatants and pellets were subjected to SDS-PAGE. Gels were stained with Coomassie blue. Destained gels were subjected to densitometry. In some instances, proteins separated by SDS-PAGE were electroblotted onto nitrocellulose paper and analyzed for the presence of the CaBP33-CaBP37 mixture with the aid of the anti-CaBP33-CaBP37 antiserum by the method in [7]. See legends to Figs. 1–4 for further details. In the second set of experiments, the CaBP33-CaBP37 mixture was incubated with the crude preparation of brain cytoskeleton as above in 75 μl of buffer C (final volume) in the absence and presence of different concentrations of $CaCl_2$ for 60 min, after which the bifunctional cross-linker disuccinimidyl suberate (DSS) was added to 0.5 mM for 5 min at room temperature. The reaction was terminated by adding SDS and 2-mercaptoethanol to 2%. Samples were subjected to SDS-PAGE and immunoblotting as described above. In the third set of experiments, samples of the crude preparation of brain cytoskeleton were subjected to SDS-PAGE and electroblotted onto nitrocellulose paper. Membranes were then incubated at 37°C for 60 min in buffer C containing 3% (w:v) bovine serum albumin (BSA) (buffer D) in the absence and presence of $CaCl_2$, and then with 10 μg of the CaBP33-CaBP37 mixture/ml of buffer D in the absence and presence of $CaCl_2$, respectively, for 4 h at room temperature. Membranes were washed 3 times, 5 min each, in the respective incubation buffer minus BSA, and subjected to immunoblotting as above. Washings between steps were done as above.

SDS-PAGE (10% acrylamide) was done as in [8]. Protein was measured as in [9]. The immune reaction on nitrocellulose paper was evidenced by 0.3 mg of diamine benzidine/ml of 50 mM Tris-HCl, pH 7.5, containing 0.02% (v:v) H_2O_2.

3. RESULTS AND DISCUSSION

The binding of the CaBP33-CaBP37 mixture to the crude cytoskeleton preparation obtained as described in section 2 was studied by a sedimentation assay, followed by SDS-PAGE of supernatants and pellets. This procedure could be employed since we verified that the crude cytoskeleton preparation did not contain endogenous CaBP33 and CaBP37 (EGTA was present throughout), as investigated by both SDS-PAGE and immunoblotting (see Fig. 1, lane 4, and Fig. 5, lanes 7 and 8), in accordance with previous observations [2].

No cosedimentation of the CaBP33-CaBP37 mixture with cytoskeleton protein was detected in the absence of Ca^{2+}, whereas significant cosedimentation of the two proteins was observed in the presence of 1 mM free Ca^{2+} (Fig. 1). About 5% of the CaBP33-CaBP37 mixture sedimented in the presence of 1 mM free Ca^{2+} and absence of cytoskeletons (Fig. 1). No such Ca^{2+}-dependent, cytoskeleton-independent sedimentation of the two proteins was seen in 100 μM free Ca^{2+} (not shown). Cosedimentation, i.e. interaction, of the two proteins with cytoskeletons was abolished when EGTA had been added to samples of cytoskeletons previously incubated with the CaBP33-CaBP37 mixture in the

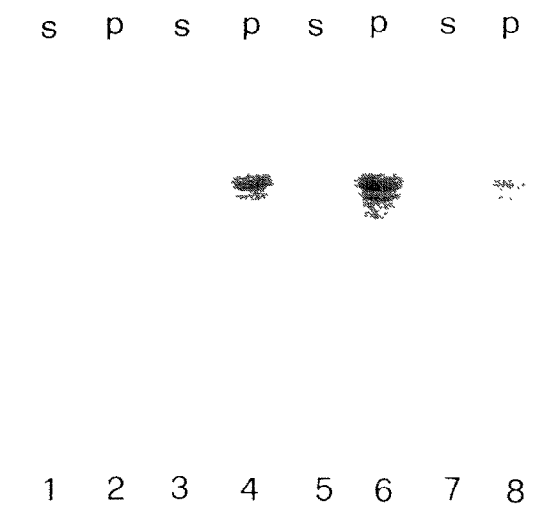

Fig. 1 Ca^{2+}-dependent interaction of the CaBP33-CaBP37 mixture with bovine brain cytoskeletons. Cytoskeleton protein (150 μg) was incubated with 10 μg of the CaBP33-CaBP37 mixture in 0.5 ml of 20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 1 mM $MgCl_2$, 1 mM EGTA in the absence and presence of ~ 1 mM free Ca^{2+} and further processed as described in section 2. Identical vols of the supernatants (s) and pellets (p) were subjected to SDS-PAGE. (Lanes 1,2) The CaBP33-CaBP37 mixture was incubated in the presence of Ca^{2+} and absence of cytoskeleton protein. (3–6) The CaBP33-CaBP37 mixture was incubated with cytoskeleton protein in the absence (lanes 3,4) and presence (lanes 5,6) of Ca^{2+}. (7,8) Conditions were as in lanes 5,6, except that the incubation mixture received EGTA to 5 mM after 60 min of incubation. Note that the two annexins cosediment, i.e. interact, with cytoskeleton protein in a Ca^{2+}-dependent way and that the binding is fully reversed by the final addition of EGTA

presence of Ca^{2+} (Fig. 1). These data indicated that the two proteins bind to brain cytoskeletons in a Ca^{2+}-dependent way and that the binding is rapidly reversed by Ca^{2+}-chelators. The extent of CaBP33 and CaBP37 binding to cytoskeletons in the presence of Ca^{2+} increased with increasing concentrations of cytoskeleton protein (Fig. 2). At 1 mM free Ca^{2+}, the binding of the CaBP33-CaBP37 mixture as a function of the CaBP33-CaBP37 concentration was maximal at ~ 30 μg/ml and half-maximal at ~ 15 μg/ml (Fig. 3). By densitometry, the CaBP33-CaBP37 mixture contained nearly equal amounts of individual proteins. This was also true for cytoskeleton-bound CaBP33-CaBP37 in the present assay. On the basis of these observations, assuming an average M_r value of 35 kDa for the mixture, half-maximal binding occurred at ~ 0.45 μM, that represents an approximate measure of the binding affinity. No attempt was done to construct a Scatchard plot of these data, since no CaBP33-CaBP37 mixture could be seen in SDS gels of the supernatants relative to the first four experimental points, i.e. from 2 to 8 μg of the mixture/ml (Fig. 3). In other words, no precise measurement of the unbound protein could be done as far as the first four experimental points were concerned, which hampered a correct calculation of the bound/free ratios. Nevertheless, it is

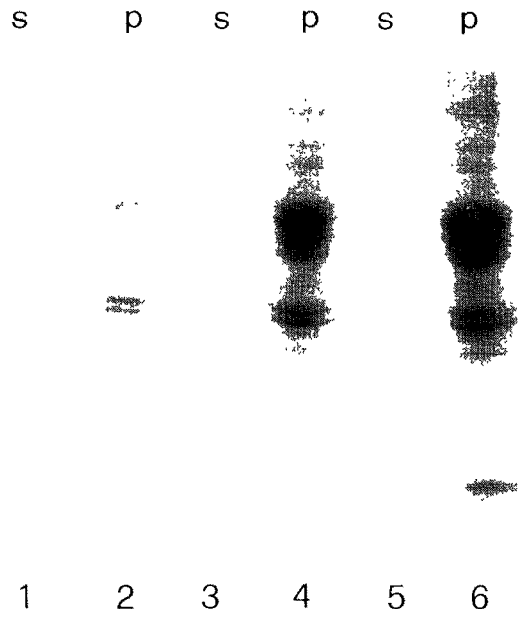

Fig. 2. Binding of the CaBP33-CaBP37 mixture to brain cytoskeleton protein as a function of cytoskeleton protein concentration. Increasing amounts of cytoskeleton protein were incubated with 15 μg of the CaBP33-CaBP37 mixture in the presence of ~1 mM free Ca^{2+} under the condition detailed in Fig. 1. Individual supernatants (s) and pellets (p) were analyzed by SDS-PAGE. Cytoskeleton protein was 100 μg (lanes 1,2), 200 μg (3,4) and 400 μg (5,6).

Fig. 3. Binding of the CaBP33-CaBP37 mixture to brain cytoskeleton protein as a function of CaBP33-CaBP37 concentration. Cytoskeleton protein (150 μg) was incubated in the presence of ~1 mM free Ca^{2+} with 1, 2, 3, 4, 5, 7.5, 10, 15, 20, 30 μg of the CaBP33-CaBP37 mixture (lanes 1–10, respectively) under the conditions described in Fig. 1. Individual supernatants (s) and pellets (p) were analyzed by SDS-PAGE.

clearly evident from data in Fig. 3 that a very large proportion of the CaBP33-CaBP37 mixture had cosedimented with cytoskeletons at the very low CaBP33-CaBP37 concentrations, strongly suggesting that the binding affinity of the mixture to cytoskeletons is much higher than 0.45 μM, and that individual CaBP33 and CaBP37 bind to cytoskeletons with at least two different affinities. This would anticipate that more than one target exists for individual CaBP33 and CaBP37 in cytoskeletons. Of course, a precise analysis of this point could be done by performing experiments of binding of radiolabeled individual CaBP33 and CaBP37 to cytoskeletons.

The binding of the CaBP33-CaBP37 mixture to brain cytoskeletons was also studied as a function of free Ca^{2+} concentration. The extent of binding was biphasic (Fig. 4). A first component was saturated at ~ 100 μM free Ca^{2+} and half-maximal at ~ 50 μM free Ca^{2+}, and a second component was maximal at ~ 1 mM free Ca^{2+}.

On the basis of the findings illustrated so far, and in consideration that some members of the annexin superfamily, e.g. the heavy chain (p36) of the cytoskeletal protein complex calpactin I and a 73 kDa annexin found in human lymphocytes, monocytes, liver, and placenta and in rat adrenal medulla [10,11] bind to F-actin, we sought to determine whether or not the CaBP33-CaBP37 mixture was capable of binding to F-actin. By a sedimentation assay, no binding of the mixture was observed to F-actin irrespective of the presence (Fig. 5, lanes 1 and 2) or the absence (not shown) of Ca^{2+}. Thus these two annexins are not 'calpactins'.

To have preliminary information on the molecular target(s) of the CaBP33-CaBP37 mixture in brain cytoskeletons, cross-linking experiments were performed as described in section 2. Complex formation between the CaBP33-CaBP37 mixture and cytoskeleton protein was investigated by immunoblotting. In the absence of Ca^{2+}, irrespective of the presence (Fig. 5, lane 3) or absence (not shown) of cytoskeleton protein, a broad CaBP33-CaBP37 immunoreactive species was seen at ~ 70 kDa, besides the expected immunoreactive species at 33 and 37 kDa, suggesting that DSS had cross-linked CaBP33 and/or CaBP37 molecules to one another to some extent. When the binding reaction was done in the presence of 1 mM free Ca^{2+}, a broad immunoreactive species was seen at 85–90 kDa (Fig. 5, lane 4). Only a slight immunoreactivity was observed at the ~ 70 kDa level under these conditions. The formation of the 85–90 kDa immunoreactive species was thus dependent on Ca^{2+}, as also indicated by the fact that the final addition of EGTA to 5 mM to samples of cytoskeleton protein incubated with the CaBP33-CaBP37 mixture in the presence of 1 mM free Ca^{2+} pro-

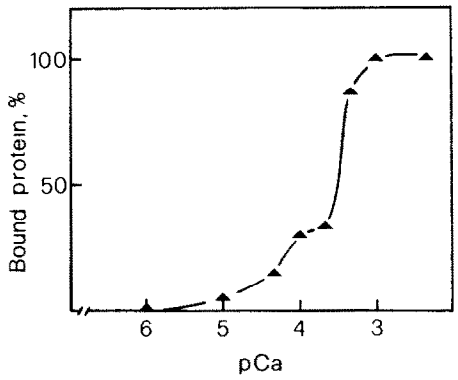

Fig. 4. Binding of the CaBP33-CaBP37 mixture to brain cytoskeleton protein as a function of the free Ca^{2+} concentration. Cytoskeleton protein (150 μg) was incubated with 15 μg of the CaBP33-CaBP37 mixture under the conditions described in Fig. 1 in the presence of increasing concentrations of free Ca^{2+} as indicated. Individual pellets were subjected to SDS-PAGE. Destained gels were analyzed by densitometry. The peak areas of the CaBP33-CaBP37 mixture in pellets were plotted as a function of the free Ca^{2+} concentration (pCa). The peak area at ~1 mM free Ca^{2+} was taken as 100%.

duced the disappearence of the 85-90 kDa species (Fig. 5, lane 5). Results identical to those illustrated in Fig. 5, lane 4, were obtained when the binding reaction had occurred at 0.1 mM free Ca^{2+} (Fig. 5, lane 6). When either pure G-actin or F-actin was used in cross-linking experiments, no specific complex formation was evidenced (not shown), in accordance with the lack of cosedimentation of the CaBP33-CaBP37 mixture with F-actin documented in Fig. 5, lanes 1 and 2.

Direct information on the molecular species in brain cytoskeletons with which the CaBP33-CaBP37 mixture interacted was obtained by an overlay procedure. In this case, in the presence (Fig. 5, lane 7), but not in the absence (Fig. 5, lane 8) of Ca^{2+}, CaBP33-CaBP37 immune reactivity was seen at the level of 4 polypeptides of M_r values ranging from ~ 10 to ~ 20 kDa and, to a much smaller extent, at the level of an ~ 110 kDa polypeptide. We cannot exclude the possibility that other CaBP33-CaBP37-binding polypeptides in the brain cytoskeleton preparation used in the present study

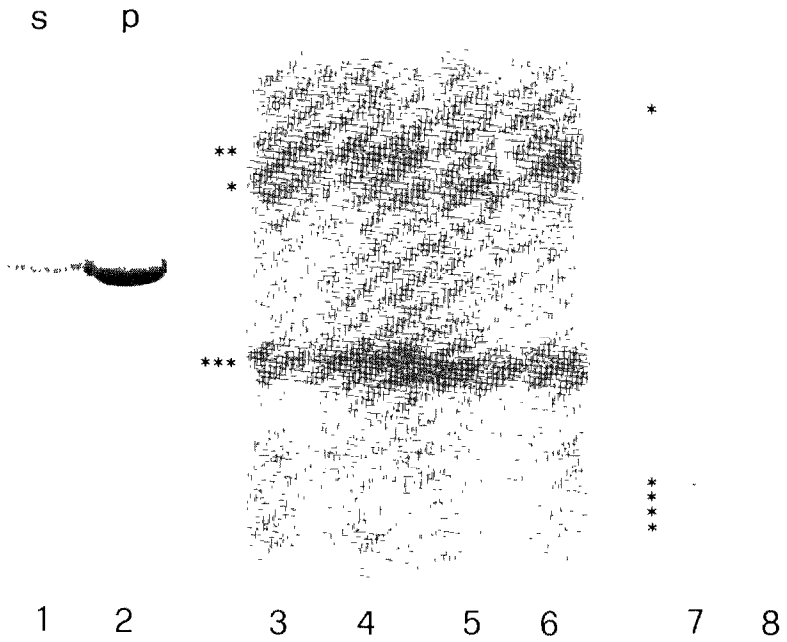

Fig. 5. Analysis of the molecular targets of the CaBP33-CaBP37 mixture in brain cytoskeletons. (Lanes 1,2) Porcine heart actin (200 μg) was polymerized in 0.5 ml of 20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 1 mM $MgCl_2$, 0.2 mM ATP, 1 mM EGTA, 1 mM free Ca^{2+}, 10 μg of the CaBP33-CaBP37 mixture at room temperature for 60 min, and centrifuged at 100000 × g for 60 min. The supernatant was saved. The pellet was washed once in the polymerization buffer and resuspended in 0.5 ml of buffer B. Identical vols of the supernatant (s) and pellet (p) were analyzed by SDS-PAGE. Note that the two annexins do not cosediment with F-actin. Identical results were obtained in the absence of Ca^{2+} (not shown). Also, increasing the G-actin concentration to 4 mg/ml did not result in cosedimentation of the two annexins with F-actin (not shown). No effect of the two annexins was seen on the extent of actin polymerization (not shown). (3-6) Cross-linking experiments. The CaBP33-CaBP37 mixture (10 μg) was incubated with 150 μg of cytoskeleton protein in 75 μl of the incubation buffer described in section 2 in the absence of $CaCl_2$ (3), in the presence of ~1 mM free Ca^{2+} (4), in the presence of ~1 mM free Ca^{2+} followed by the addition of 5 mM EGTA (5), and in the presence of ~0.1 mM free Ca^{2+} (6) for 60 min at room temperature. Samples then received DSS to 0.5 mM for 5 min, followed by SDS and 2-mercaptoethanol to 2% before SDS-PAGE. Proteins so separated were electroblotted onto nitrocellulose paper and subjected to immunoblotting using the rabbit anti-CaBP33-CaBP37 antiserum [2]. The triple asterisk points to the position of the uncomplexed annexins, the double asterisk to the position of the annexins cross-linked to cytoskeleton polypeptides, and the single asterisk to the position of the two annexins cross-linked to one another. No cross-linking of the two annexins to one another was seen in the absence of DSS (not shown). (7,8) Overlay experiments. Cytoskeleton protein (230 μg) was subjected to SDS-PAGE. Separated proteins were electroblotted onto nitrocellulose paper and processed as described in Section 2 in the presence of ~1 mM free Ca^{2+} (7) and in the absence of Ca^{2+} (8). The CaBP33-CaBP37 mixture bound to cytoskeleton polypeptides was evidenced with the aid of the anti-CaBP33-CaBP37 antiserum. Asterisks point to the positions of cytoskeleton polypeptides with ability to bind the CaBP33-CaBP37 mixture.

might have escaped detection because of dissociation of the two annexins from these binding sites as a consequence of the numerous passages and washings inherent to the overall procedure employed. If so, then the conclusion could be reasonably drawn that the binding of the CaBP33-CaBP37 mixture to the 10–20 kDa and to the 110 kDa polypeptides is of relatively high affinity and/or capacity, also in consideration of the concentration ($\sim 0.3\ \mu M$) of the annexin's mixture in the binding assay. Moreover, it is possible that other CaBP33-CaBP37-binding polypeptides have not been detected because of non-renaturation after transfer and immobilization onto the nitrocellulose membrane. Analyses of the CaBP33-CaBP37-binding proteins in cytoskeletons by affinity chromatography will hopefully elucidate these points. On the basis of the data in Fig. 5, lane 7, the 85-90 kDa complex detected by immunoblotting in cross-linking experiments probably results from binding of one or both annexins to the low M_r polypeptides shown to bind the CaBP33-CaBP37 mixture in the overlay experiments.

Altogether, the present data suggest that CaBP33 and CaBP37 bind to a set of cytoskeleton proteins Ca^{2+}-dependently with a relatively high affinity and, hence, that membrane-associated proteins, besides acidic phospholipids in the membrane bilayer, could be targets of these two annexins. Within the limits of the techniques used in this and the previous [2] studies, it seems that the CaBP33-CaBP37 mixture would preferentially bind to the lipid bilayer at the free Ca^{2+} concentrations found within cells upon appropriate activation, since half-maximal binding to acidic phopholipids occurs at $\sim$ 10 μM [2], whereas half-maximal binding to cytoskeleton proteins occurs at significantly larger free Ca^{2+} concentrations, in vitro. However, it is possible that some unknown factor(s) might stabilize the interactions of these two annexins with the cytoskeleton and/or stimulate these interactions at physiological free Ca^{2+} concentrations. Preliminary data indicate that in rat cerebellum and sciatic nerves, where only CaBP33 is expressed [2], CaBP33 is restricted to glial cells (in preparation). Future studies using cultured glial cells will help to elucidate both the identity of cytoskeleton proteins that are targets of these two annexins and the nature of factors, including the free Ca^{2+} concentration, that regulate the association of CaBP33 and CaBP37 with membranes.

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