The Cytoskeletal System of Nucleated Erythrocytes. II. Presence of a High Molecular Weight Calmodulin-binding Protein

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ABSTRACT Calmodulin was detected in dogfish erythrocyte lysates by means of phosphodiesterase activation. Anucleate dogfish erythrocyte cytoskeletons bound calmodulin. Binding of calmodulin was calcium-dependent, concentration-dependent, and saturable. Cytoskeletons consisted of a marginal band of microtubules containing primarily tubulin, and trans-marginal band material containing actin and spectrinlike proteins. Dogfish erythrocyte ghosts and cytosekeletons were found to contain a calcium-dependent calmodulin-binding protein, CBP, by two independent techniques: (*a*) ^{125}I-calmodulin binding to cytoskeletal proteins separated by SDS PAGE, and (*b*) *in situ* azidocalmodulin binding in whole anucleate ghosts and cytoskeletons. CBP, with an apparent molecular weight of 245,000, co-migrated with the upper band of human and dogfish erythrocyte spectrin. CBP was present in anucleate ghosts devoid of marginal bands and absent from isolated marginal bands. CBP therefore appears to be localized in the trans-marginal band material and not in the marginal band. Similarities between CBP and high molecular weight calmodulin-binding proteins from mammalian species are discussed.

Generation and maintenance of correct cytoskeletal organization is a subject of primary interest in cells such as erythrocytes (3) in which specific mechanical properties and asymmetric morphology are vital to cell function. Evidence obtained in recent years implicates calcium ion in the control of such organization as well as many other cellular phenomena. Assembly of both microtubules (29, 36) and microfilaments (18, 38) has been shown to be calcium-mediated in vitro, and localized intracellular calcium levels are believed to regulate formation of at least some microtubule systems in vivo (21, 28). Calmodulin is a known mediator in the control of numerous cellular enzymes by calcium (12, 25) and its interaction with microtubule-associated proteins (MAPs) has been suggested as the mechanism by which calcium causes microtubule disassembly (7).

To elucidate the possible role of calcium and calmodulin in the formation and function of the erythrocyte cytoskeleton, we have examined nonmammalian erythrocytes for calmodulin content and for the presence of calmodulin-binding proteins associated with the cytoskeleton. The nonmammalian erythrocyte was chosen for this study because it is a relatively simple nucleated cell type with characteristic flattened elliptical morphology, and is obtainable in pure populations. Its cytoskeletal system contains components of universal interest, including a marginal band of microtubules (3, 14), a spectrin-containing network of trans-marginal band material (TBM; reference 15), and intermediate filaments attached to the nucleus (37). The cytoskeleton of nonmammalian erythrocytes resembles those of nucleated nonerythroid cells more closely than does the mammalian erythrocyte cytoskeleton and therefore may serve as a more appropriate model system. The dogfish erythrocyte is well suited for this study because it is available in quantity and, due to its relatively large size (15), provides a cytoskeleton readily visualized by phase-contrast microscopy.

MATERIALS AND METHODS

Diisopropyl fluorophosphate (DFP), EGTA, HEPES, PIPES, *p*-tosyl arginine methyl ester (TAMe), and lactoperoxidase were obtained from the Sigma Chemical Co., St. Louis, MO. Na^{125}I, 2 Ci/mmol, was a product of Amersham Corp. (Arlington Heights, IL). Standard molecular weight markers were obtained from

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Bio-Rad Laboratories, Richmond, CA. Leupeptin was a product of Transformation Research Laboratories, Framingham, MA. Trifluoperazine (TFP) was the generous gift of Dr. Carl Kaiser of Smith, Kline and French Laboratories, Philadelphia, PA. Twice-cycled bovine brain tubulin (30) was the gift of Dr. George Langford of the University of North Carolina. Canine cortical postsynaptic densities (PSDs) were prepared as described by Carlin and co-workers (8).

Preparation of Washed Erythrocytes

Blood drawn from smooth dogfish (*Mustelus canis*) was diluted with heparinized Elasmobranch Ringer's solution (11), and leukocytes were removed as described previously (15). The final erythrocyte pellets, with <1% leukocyte contamination, were resuspended in 35 volumes of Ringer's solution.

Human erythrocytes were obtained by diluting a fresh sample of heparinized blood with one volume 20 mM sodium phosphate, 150 mM sodium chloride, pH 7.3 (PBS), and sedimenting the red cells through 25% PBS containing 0.5 M sucrose at 750 *g* for 5 min. The supernate containing suspended leukocytes was removed by aspiration and the erythrocytes were washed in 15 volumes of PBS.

Anucleate Dogfish Erythrocyte Ghosts and Cytoskeletons

Anucleate dogfish erythrocyte ghosts were prepared by hypotonic lysis in 50 mM PIPES at pH 6.8, containing 10 mM TAMe, 2.5 mM EGTA, 0.5 mM $MgCl_2$, and 0.1 mM DFP followed by shearing through a 22-gauge needle as described by Cohen et al (15). Anucleate ghosts and cytoskeletons analyzed by SDS-PAGE (Figs. 3 and 6) were prepared in the presence of the following protease inhibitors: 1 mM sodium tetrathionate (23), 10 μg/ml leupeptin, 10 mM TAMe, and 0.1 mM DFP. Before gel electrophoresis, some ghost preparations were detergent-extracted with 0.5% Triton X-100 in 10 mM TAMe, 5 mM EGTA, 1 mM $MgCl_2$, 0.1 mM DFP, buffered with either 100 mM PIPES or HEPES, pH 6.8. These cytoskeletons were collected by centrifugation and washed twice with medium containing no Triton. Anucleate ghosts devoid of marginal bands were prepared from cells in which microtubules had been disassembled in vivo by incubation at 0 °C for 2 h. For these preparations all manipulations were performed at 4°C. Marginal bands were isolated from anucleate cytoskeletons by elastase digestion of the TBM as described previously for nucleated cytoskeletons (15).

Human Erythrocyte Ghosts

Human erythrocyte ghosts were prepared by hypotonic lysis of washed cells in 10 volumes of the same medium used to prepare anucleate dogfish erythrocyte ghosts. The lysate was centrifuged at 11,000 *g* for 15 min and the sedimented ghosts were washed with 10 pellet volumes of 100 mM PIPES, 10 mM TAMe, 5 mM EGTA, 1 mM $MgCl_2$, 0.1 mM DFP, pH 6.8. The ghosts were collected by centrifugation and resuspended in one volume of this medium. Spectrin and actin were prepared by low ionic strength extraction of human ghosts (34).

Assay of Calmodulin Activity in Dogfish Erythrocyte Lysate

A lysate of dogfish erythrocytes was prepared in 10 volumes of medium consisting of 100 mM PIPES, 10 mM TAMe, 5 mM EGTA, 1 mM $MgCl_2$, 0.1 mM DFP, pH 6.8, and 0.4% Triton X-100. The calcium-dependent activation of partially purified calmodulin-sensitive bovine brain phosphodiesterase (PDE) was used to assay this lysate for the presence of calmodulin. PDE was depleted of calmodulin by chromatography on DEAE Sephadex (26). PDE activity was measured as described by Grab and co-workers (19). Levels of endogenous phosphate and PDE activity were subtracted from the total phosphate released to determine the extent of activation of the bovine brain PDE by calmodulin. Incubations were carried out in 1 mM EGTA, or 1 mM $CaCl_2$, or 1 mM $CaCl_2$ plus 40 μM trifluoperazine. Calmodulin content was estimated by comparing the stimulation of PDE to one-half maximal velocity by the dogfish erythrocyte lysate with that obtained using known amounts of bovine brain calmodulin purified according to Watterson et al. (35). The molarity of the calmodulin standard was determined by amino acid analysis.

^{125}I-Calmodulin Binding to Anucleate Dogfish Erythrocyte Cytoskeletons

Cytoskeletons derived from anucleate ghosts containing 400 μg protein were incubated with 30–2,550 ng (1.8–150 mM) biologically active ^{125}I-calmodulin (128 cpm/ng) in 1.0 ml 20 mM PIPES·KOH, 100 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM DFP, 8 mg/ml BSA, 0.4% Triton X-100, pH 6.8, and either 0.1 mM $CaCl_2$ or 1 mM EGTA for 30 min at 25°C with shaking. Cytoskeletons were collected by centrifugation at 12,500 *g* for 10 min. The supernates were removed by aspiration and the pellets were washed twice with 1 ml of incubation medium. The washed pellets were counted in a Packard Gamma scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). Protein concentrations were estimated by the method of Bradford (5).

^{125}I-Calmodulin Binding to Proteins Separated by SDS PAGE

PAGE was performed as described by Cohen et al. (13). Iodination of calmodulin with $Na^{125}I$ using lactoperoxidase and the binding of ^{125}I-calmodulin to proteins separated by SDS PAGE were carried out as described by Carlin and co-workers (9, 10). Degradation of the high molecular weight calmodulin-binding protein, CBP, was estimated by scanning autoradiograms of dried gels.

Photoaffinity Labeling of Calmodulin Binding Proteins in Erythrocyte Ghosts

Azido-^{125}I-calmodulin was prepared by the method of Andreasen et al. (2). Dogfish and human erythrocyte ghosts (100 μg protein) were incubated for 15 min at 23°C with 480 nM azido-^{125}I-calmodulin in 20 mM Tris·HCl, 100 mM NaCl, pH 7.5, containing either 0.6 mM $CaCl_2$ or 0.2 mM EGTA in the presence or absence of 0.15% Triton X-100. Cross-linking of the photoaffinity label to calmodulin-binding proteins was achieved by UV irradiation (5 min each at 253 and 375 nm) at 23°C. Proteins in the samples were then separated by gradient SDS PAGE. To detect calmodulin-protein complexes, dried gels were autoradiographed at −80°C using a DuPont Cronex Lightning Plus intensifying screen (Dupont Instruments) and Fuji RX x-ray film.

RESULTS

Presence of Calmodulin in Dogfish Erythrocytes

Dogfish erythrocyte lysate was assayed for calmodulin activity using a preparation of bovine brain PDE as described in Materials and Methods. As shown in Fig. 1, microliter quantities of the lysate were sufficient to activate the PDE in a calcium-dependent manner. This activation was blocked by 40 μM trifluoperazine (TFP), a known inhibitor of calmodulin, in the presence of 1 mM calcium. The level of activity present in

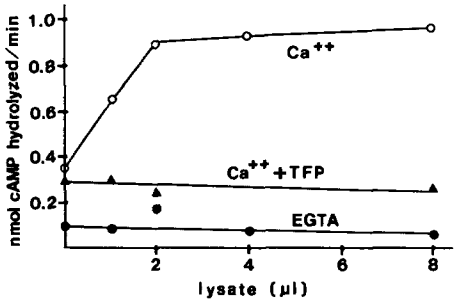

FIGURE 1 Activation of bovine brain PDE by dogfish erythrocyte lysate (58 mg protein/ml) in the presence of 1 mM $CaCl_2$ (○), 1 mM EGTA (●), 1 mM $CaCl_2$ + 40 μM trifluoperazine (TFP; ▲). Activation is calcium-dependent and inhibited by TFP.

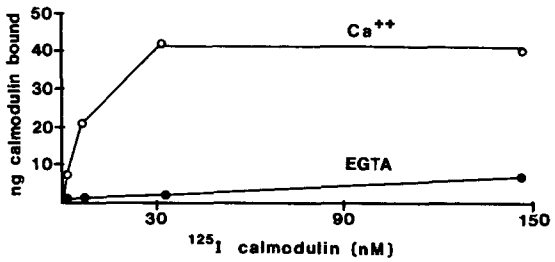

FIGURE 2 Binding of ^{125}I-calmodulin in vitro to whole anucleate dogfish erythrocyte cytoskeletons. Cytoskeletons derived from anucleate ghosts containing 400 μg protein were incubated for 30 min at 25°C with biologically-active ^{125}I-calmodulin as described in Materials and Methods. Incubations were performed either in the presence of 1 mM $CaCl_2$ (○) or 1 mM EGTA (●).

the lysate was equivalent to 34 μg calmodulin/ml of packed erythrocytes, or ~2 μM.

In Vitro Binding of Calmodulin to Dogfish Erythrocyte Cytoskeletons

Anucleate dogfish erythrocyte cytoskeletons bound ^{125}I-calmodulin in a calcium-dependent manner (Fig. 2). The binding was concentration-dependent, with saturation achieved above 30 nM.

Protein Constituents of Anucleate Dogfish Erythrocyte Ghosts and Cytoskeletons

Identification of the major dogfish erythrocyte cytoskeletal proteins was the first step in determining which proteins were responsible for the binding of calmodulin to anucleate cytoskeletons. The components of dogfish erythrocyte ghosts and cytoskeletons were compared with known cytoskeletal proteins by gradient SDS PAGE (Figs. 3 *a* and 4 *a*). Identification of the two spectrin bands and the actin band was made on the

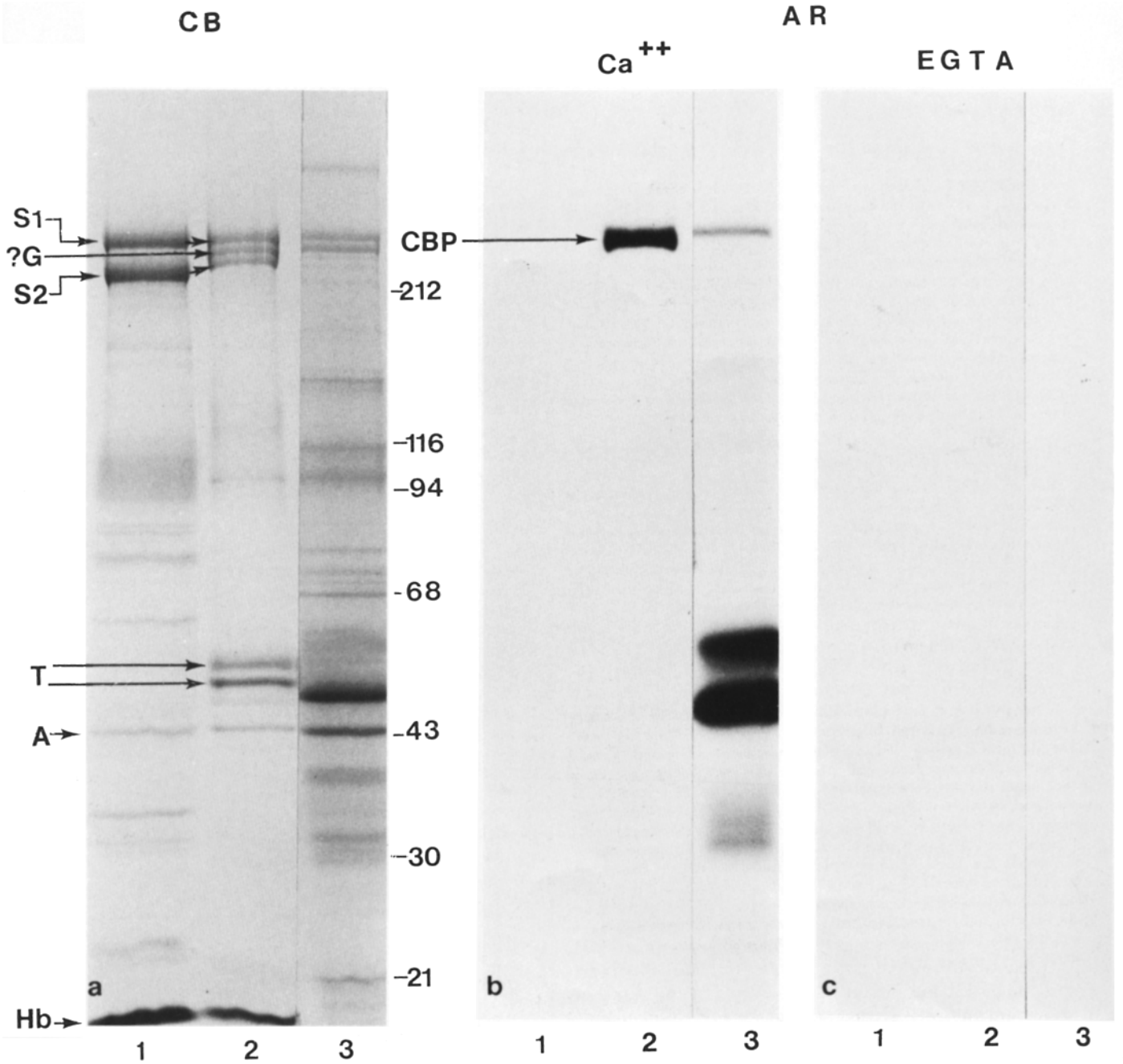

FIGURE 3 Detection of a calmodulin-binding protein (*CBP*) associated with the dogfish erythrocyte cytoskeleton. (*a*) Coomassie-Blue staining pattern (*CB*) of cytoskeletal proteins separated in a 5–15% gradient gel by SDS PAGE. (*b*) and (*c*) Autoradiograms (*AR*) resulting from incubation of gels in ^{125}I-calmodulin in the presence of 1 mM calcium (*b*) or 1 mM EGTA (*c*). Lane *1*: human erythrocyte ghosts; lane *2*: anucleate dogfish erythrocyte cytoskeletons; lane *3*: canine cortical postsynaptic densities (included as an internal standard for calmodulin binding). *S1*, and *S2*, upper and lower bands of spectrin; *G*, goblin (identification tentative); *T*, tubulin; *A*, actin; *Hb*, hemoglobin. Numbers indicate molecular weights × 10^{-3} of protein standards. Dogfish *CBP* bound calmodulin in a calcium-dependent manner and co-migrated with dogfish and human *S1*.

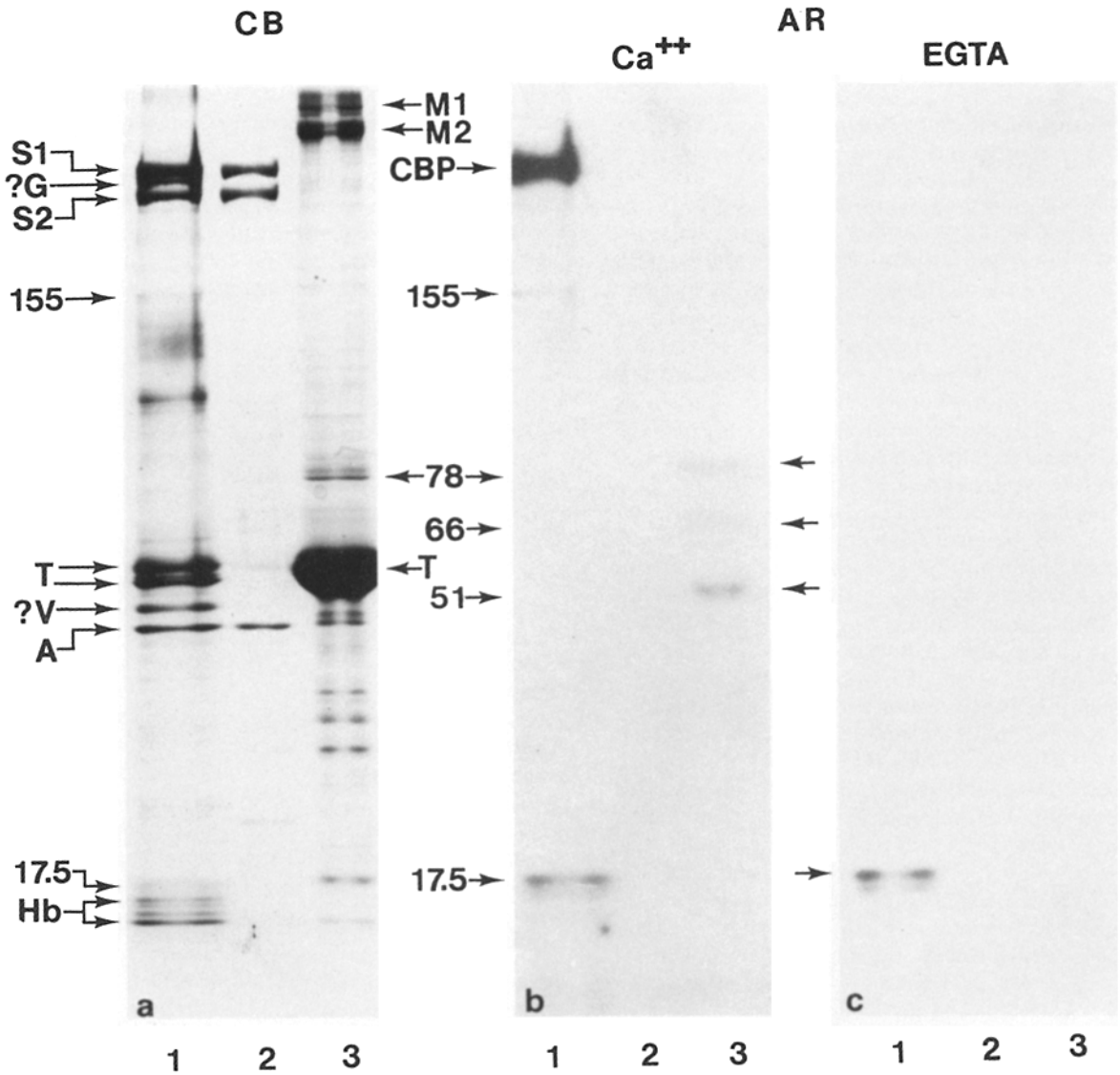

FIGURE 4 Comparison of calmodulin binding to proteins of dogfish erythrocyte ghosts and microtubule proteins. (*a*) Coomassie-Blue staining pattern (*CB*) of proteins separated in a 5–15% gradient gel by SDS PAGE. (*b*) and (*c*) Autoradiograms (*AR*) resulting from incubation of gels in ^{125}I-calmodulin in the presence of 1 mM calcium (*b*) or 1 mM EGTA (*c*). Lane *1*: anucleate dogfish erythrocyte ghosts; lane *2*: human erythrocyte spectrin and actin standards; lane *3*: bovine brain microtubule proteins. *M1*, MAP 1; *M2*, MAP 2; *V*, vimentin (identification tentative). Numbers indicate molecular weights × 10^{-3} of calmodulin-binding proteins and arrows show their location on the gel. All other symbols are as in the legend to Fig. 3. In addition to *CBP* two minor calmodulin-binding proteins were detected. Calmodulin binding is not observed for MAPs 1 and 2 or for human spectrin.

basis of their co-migration with human erythrocyte spectrin and actin and by comparison with electrophoretic patterns reported for proteins of other nonmammalian erythrocytes (4, 31). The tubulin bands were assigned on the basis of co-migration with brain tubulin standards (Fig. 4 *a*, lane *3*; 15), and the presence of microtubules in the anucleate ghosts and cytoskeletons. Goblin was tentatively identified by comparison with the electrophoretic patterns published for turkey and frog erythrocyte plasma membranes (4, 27). Vimentin, an intermediate filament protein, was tentatively identified by its mobility relative to tubulin and actin (6), and by its presence in intermediate filaments associated with the nucleus of chicken erythrocytes (37). Hemoglobin, a contaminant of the preparations, was identified in Fig. 3 *a* by co-migration with human hemoglobin. No proteins co-migrating with bovine brain high molecular weight MAPs 1 or 2 were detected in the anucleate dogfish erythrocyte ghosts or cytoskeletons. The only discernible difference between the Coomassie-Blue staining patterns for ghosts and cytoskeletons was the presence of a broad band with an apparent molecular weight of 110–130,000 in the ghosts (Fig. 4 *a*, lane *1*) which is greatly decreased after Triton extraction (Fig. 3 *a*, lane *2*).

^{125}I-Calmodulin Binding to Proteins in Anucleate Erythrocyte Ghosts and Cytoskeletons

Calmodulin-binding proteins present in anucleate dogfish erythrocyte ghosts and cytoskeletons were detected using a recently developed procedure in which proteins separated by SDS PAGE were incubated with ^{125}I-calmodulin after removal of the detergent and renaturation in the gel (9). To determine whether the binding was calcium-dependent, duplicate gels were incubated in 1 mM $CaCl_2$ and 1 mM EGTA. The autoradiograms in Fig. 3 *b* and *c* correspond to the gel in Fig. 3 *a*, which had been incubated with ^{125}I-calmodulin before

Coomassie-Blue staining. Human erythrocyte ghost proteins (lane *1*) served as standards for protein identification, and canine cortical PSD proteins (lane *3*) were used as an internal standard for calmodulin binding (10). Anucleate cytoskeletons of the dogfish erythrocyte contain one major calcium-dependent calmodulin-binding protein (CBP; Fig. 3 *b*, lane *2*). Binding of ^{125}I-calmodulin to CBP was inhibited by an excess of unlabeled calmodulin in a competition experiment (data not shown). CBP co-migrated with the upper band (*S1*) of human and dogfish spectrin and had an apparent molecular weight of 245,000. Neither band of human spectrin bound calmodulin (Fig. 3 *b*, lane *1*). CBP also co-migrated with a calmodulin-binding protein present in canine cerebral cortical postsynaptic densities (Fig. 3 *b*, lane *3*). The calmodulin-binding pattern for the PSD sample was consistent with results previously obtained using this technique (10). No calmodulin-binding was detected for any sample in the presence of EGTA (Fig. 3 *c*).

Because MAP 2 had been reported to bind calmodulin (7) and to be present in marginal bands of amphibian erythrocytes (31), calmodulin binding by dogfish anucleate ghost proteins was compared with that of bovine brain microtubule proteins containing MAPS 1 and 2 (Fig. *4*, lanes *1* vs. *3*). Unlike CBP, neither MAP 1 nor MAP 2 bound calmodulin using this method. Calmodulin did bind in a calcium-dependent manner to three minor proteins present in a microtubule preparation (Fig. 4 *b*, lane *3*). These proteins had apparent molecular weights of 78,000, 66,000, and 51,000.

Two minor calmodulin-binding proteins were also detected when the loading of dogfish erythrocyte ghost or cytoskeletal protein was heavier than that shown in Fig. 3. As can be seen for ghosts in Fig. 4, one of these had an apparent molecular weight of 155,000, and bound camodulin in a calcium-dependent manner. This protein is thought to be a proteolytic fragment of CBP, since it increased in amount relative to CBP in successive experiments as judged by scanning of autoradiograms. The 155,000 M_r band also co-migrated with a calmodulin-binding protein present in canine PSDs (data not shown). The second minor calmodulin-binding protein (Fig. 4, lane *1*) had a molecular weight of 175,000 and bound calmodulin both in the presence and absence of calcium. This protein was not present in all preparations and may be a contaminant.

Photoaffinity Labeling of Calmodulin-binding Proteins in Erythrocyte Ghosts

As an independent test of the validity of the above results for calmodulin-binding to ghost (Fig. 4, lane *1*) and cytoskeletal proteins (Fig. 3, lane *2*), and as a means of determining whether CBP would bind calmodulin *in situ*, anucleate dogfish and human erythrocyte ghosts were reacted with azido-^{125}I-calmodulin (2). The proteins to which calmodulin was covalently linked were detected by autoradiography of gels after SDS PAGE (Fig. 5). Calcium-dependent binding of calmodulin to CBP in both ghosts and cytoskeletons was indicated by the appearance of a band of reactivity of molecular weight higher than S1, corresponding to a CBP-calmodulin complex (Fig. 5 *a*, lanes *1* and *3*). The binding of calmodulin to CBP in the absence of Triton is presumably due to permeabilization of the ghosts by freezing and thawing before their use. No binding was observed for spectrin in human ghosts (Fig. 5 *b*), consistent with results obtained by ^{125}I-calmodulin binding to proteins separated by SDS PAGE (Fig. 3, lane *1*, and Fig. 4, lane *2*).

Location of CBP Within the Anucleate Cytoskeleton

To determine whether CBP was associated with the marginal band or was a component of the TBM, anucleate ghosts were prepared from cells incubated at 0°C so that they were devoid of marginal bands (15) and greatly depleted of tubulin (Fig. 6 *a*, lane *1*). The amount of CBP present in these ghosts was comparable to that in anucleate ghosts or cytoskeletons containing marginal bands (Fig. 6 *b*, lane *1*, vs. Fig. 3 *b*, lane *2*, or Fig. 4 *b*, lane *1*), indicating that CBP was in the TBM. Localization of CBP to the TBM was further supported by the absence of calmodulin-binding to proteins of marginal bands isolated by treatment of anucleate ghosts with elastase (Fig. 6 *b*, lane *2*). Tubulin was the primary component of such preparations (Fig. 6 *a*, lane *2*).

DISCUSSION

Cytoskeletons prepared from anucleate dogfish erythrocyte ghosts consist of a marginal band of microtubules enclosed within a network or layer of material (TBM; reference 15). Their major protein components appear to be spectrin, actin, tubulin, goblin, and vimentin, with the last two assignments more tentative than the others. Goblin has been found in two species of nonmammalian erythrocytes (4, 27) and has been shown to be phosphorylated when sodium and potassium transport across the turkey erythrocyte membrane is stimulated by catecholamines (1, 4). Vimentin of intermediate filaments in chicken erythrocytes (37) and mammalian cells has an apparent M_r of 57,000 (17). However, it was first characterized in chick embryo fibroblasts as a 52,000 M_r protein (6), which

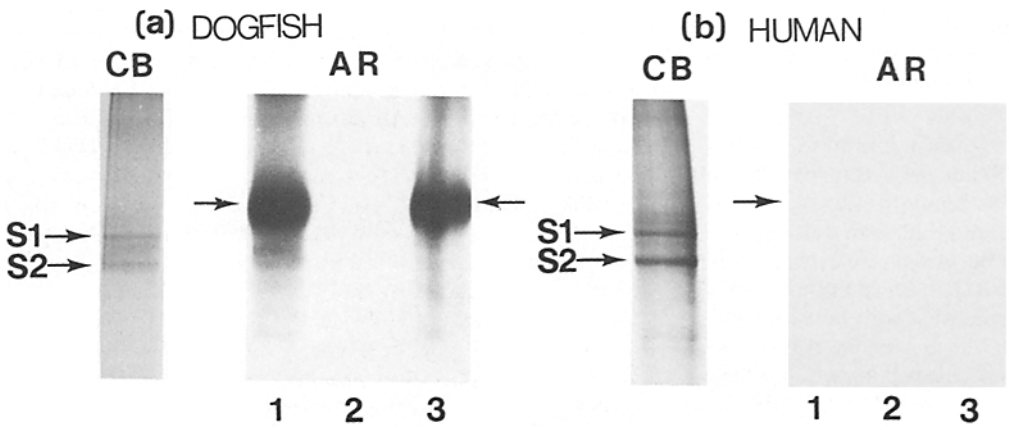

FIGURE 5 Cross-linking of azido-^{125}I-calmodulin to proteins in erythrocyte ghosts. Freeze-thawed ghost preparations were photolyzed in the presence of 0.6 mM $CaCl_2$ (lane *1*); 0.2 mM EGTA (lane *2*); and 0.6 mM $CaCl_2$, + 0.15% Triton (lane *3*). *CB*, Coomassie-Blue staining pattern; *AR*, autoradiogram. Arrows indicate position of CBP-calmodulin complex. All other symbols are as in the legend to Fig. 3. CBP binds calmodulin in whole anucleate dogfish erythrocyte ghosts.

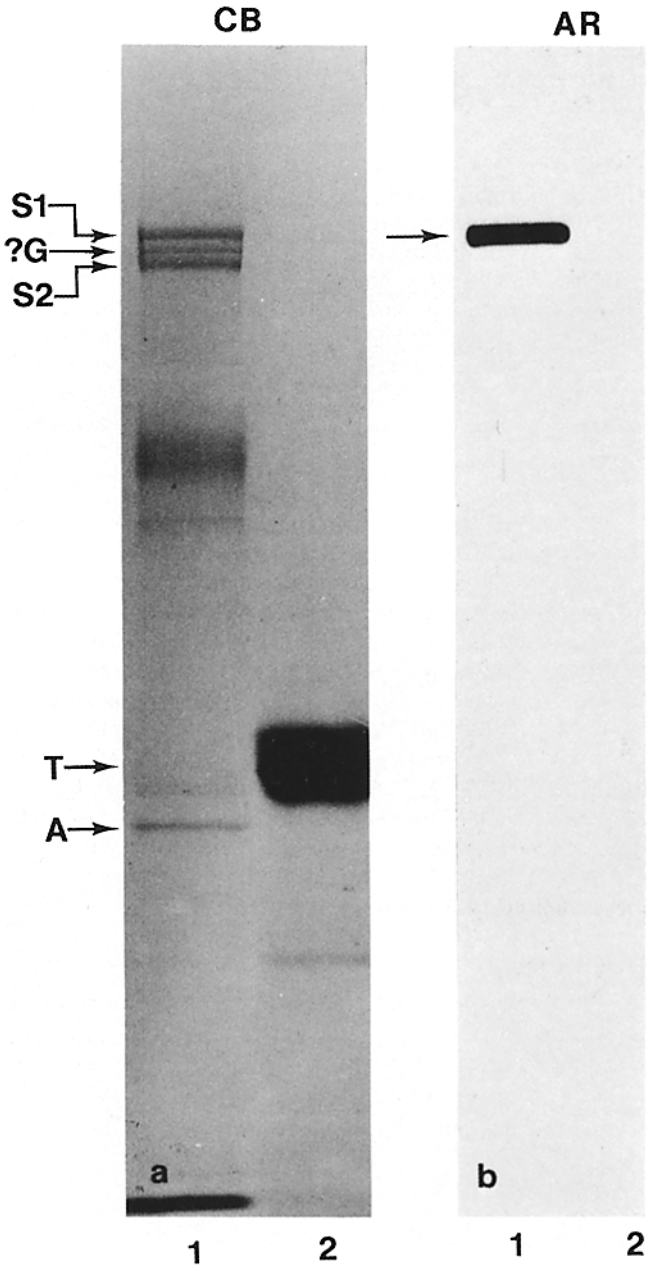

FIGURE 6 Localization of CBP. (*a*) Coomassie-Blue staining pattern (*CB*) of proteins separated in a 5–15% gradient gel by SDS PAGE. (*b*) Autoradiogram (*AR*) resulting from incubation of gels in ^{125}I-calmodulin in the presence of 1 mM calcium. Lane *1*: anucleate ghosts devoid of marginal bands and tubulin; lane *2*: marginal bands isolated from anucleate ghosts. All symbols are as in the legend to Fig. 3. CBP is present in anucleate ghosts lacking marginal bands but is absent from isolated marginal bands.

is similar to the estimated M_r of the dogfish protein (51,000). The presence of intermediate filaments in the dogfish erythrocyte cytoskeleton remains to be demonstrated.

Dogfish erythrocyte spectrin and actin are believed to be localized in the TBM, a cell-surface-associated cytoskeleton as in the mammalian erythrocyte (15). These assignments are supported by the presence of spectrin and actin in anucleate cytoskeletons that lack marginal bands and by their absence from isolated marginal bands in which tubulin is the major component (Fig. 6).

The calmodulin content of dogfish erythrocytes (~2 μM) is comparable to the level in human erythrocytes (~2.5 μM; reference 33). This is well above the concentration required for in vitro saturation of the calcium-dependent calmodulin binding sites of anucleate cytoskeletons (30 nM; Fig. 2). As determined by two independent methods, anucleate dogfish erythrocyte ghosts and cytoskeletons contain one major calcium-dependent calmodulin-binding protein (CBP). These results are consistent with the possibility that calmodulin mediates the functional effects of calcium on the nonmammalian erythrocyte cytoskeleton.

CBP, with an apparent molecular weight of 245,000, always co-migrated with the upper band of dogfish erythrocyte spectrin (S1), raising the possibility that CBP is dogfish S1. Like spectrin, CBP was localized to the TBM, based upon its absence from isolated marginal bands and presence in anucleate ghosts lacking marginal bands. If dogfish S1 is CBP, then it differs from human S1 in that no calmodulin binding to human spectrin was detected either in gels (Figs. 3 and 4) or in situ (Fig. 5), whereas CBP was shown to bind calmodulin by both of these techniques.

Lack of calmodulin binding to human spectrin confirms the results of Hinds and Andreasen (22), who found no binding of azidocalmodulin to spectrin in human erythrocyte ghosts. Sobue and co-workers (32) have recently reported the binding of calmodulin to both bands of human spectrin in the presence of 6 M urea. Urea may be required to expose latent calmodulin binding sites that are not normally accessible. These sites would not be detected in whole ghosts or after renaturation of spectrin in gels.

MAP 2 has been reported to be associated with nonmammalian erythrocyte marginal bands (31) and binds calmodulin in a calcium-dependent manner under certain ionic conditions (7). No proteins co-migrating with MAP 2 were observed in any dogfish cytoskeletal preparations. Moreover, no calmodulin binding was observed for bovine brain MAP 2 after SDS PAGE (Fig. 4). Therefore, dogfish erythrocyte CBP does not appear to be MAP 2. Work is in progress to further characterize and identify CBP using antibodies to various cytoskeletal proteins.

That no calmodulin binding was observed for MAP 2 or for human erythrocyte Ca^{++}-Mg^{++} ATPase (20) after SDS PAGE demonstrates that this assay does not detect all calmodulin-binding proteins present. This may be due to differences in binding conditions or to the failure of some proteins to renature after SDS and heat denaturation. Nevertheless, this technique can be a valuable tool for the direct identification of calmodulin-binding proteins present in complex systems (10, 18).

Dogfish erythrocyte CBP may be related to certain calmodulin-binding proteins recently found in mammalian systems. CBP co-migrated with a calmodulin-binding protein present in canine cortical PSDs (Fig. 3), which in turn co-migrated with a calmodulin-binding protein, CBP I, previously found in bovine brain (16, 24). All three of these proteins are believed to undergo proteolytic breakdown to fragments of 150,000 mol wt that also bind calmodulin in a calcium-dependent manner (Carlin, R. K., D. Grab, and P. Siekevitz, unpublished observation). CBP-I from bovine brain co-migrates with the upper band of human spectrin and binds actin in vitro, but does not react with antibodies to the actin-binding proteins filamin, myosin, or spectrin (16). These results suggest the existence of a class of high molecular weight cytoskeletal proteins, present in both mammalian and nonmammalian cells, that exhibit calcium-dependent binding of calmodulin and are also capable of binding actin.

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Note Added in Proof: We have subsequently characterized dogfish erythrocyte CBP as the upper band of fodrin (Levine, J., and M. Willard, 1981. *J. Cell Biol.* 90:631–643). This is consistent with recent reports from several other laboratories concerning the relationship between non-mammalian erythrocyte "spectrin" and fodrin.

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