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

DIANA C. BARTELT, RICHARD K. CARLIN, GEORGE A. SCHEELE, and WILLIAM D. COHEN

Department of Biological Sciences, Hunter College of the City University of New York, New York, New York 10021; Marine Biological Laboratory, Woods Hole, Massachusetts 02543; and the Department of Cell Biology, The Rockefeller University, New York, New York 10021

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 cytoskeletons 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$^{131}$I, 2 Ci/mmol, was a product of Amersham Corp. (Arlington Heights, IL). Standard molecular weight markers were obtained from...
Bio-Rad Laboratories, Richmond, CA. Leupeptin was a product of Transfor-
mation 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 postsyn-
aptic 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 heparin-
ized Elasmobranch Ringer's solution (11), and leucocytes were removed as
described previously (15). The final erythrocyte pellets, with <1% leucocyte
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 supernatant containing suspended leucocytes
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 MgCl₂,
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 tetrafenamate (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 MgCl₂,
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 MgCl₂, 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 MgCl₂, 0.1
mM DFP, pH 6.8, and 0.4% Triton X-100, containing 0.15% Triton X-100.
Incubations were carried out in 1 mM EGTA, or 1 mM CaCl₂, or 1 mM CaCl₂
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 autoradi-
ographed 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 µg protein/ml) in the presence of 1 mM CaCl₂ (A), 1 mM EGTA (B), 1 mM CaCl₂ + 40 µM trifluoperazine (TFP; ▲). Activation is calcium-dependent and inhibited by TFP.](image)

![Figure 2: Binding of 125I-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 125I-calmodulin as described in Materials and Methods. Incubations were performed either in
the presence of 1 mM CaCl₂ ([]) or 1 mM EGTA (●).](image)
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 125I-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 125I-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 x 10^-3 of protein standards. Dogfish CBP bound calmodulin in a calcium-dependent manner and co-migrated with dogfish and human S7.
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 125I-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 x 10^-9 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.

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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. 4a, 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. 3a 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. 4a, lane 1) which is greatly decreased after Triton extraction (Fig. 3a, lane 2).

125I-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 125I-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 CaCl2 and 1 mM EGTA. The autoradiograms in Fig. 3b and c correspond to the gel in Fig. 3a, which had been incubated with 125I-calmodulin before
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-125I-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 125I-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 Mr of 57,000 (17). However, it was first characterized in chick embryo fibroblasts as a 52,000 Mr protein (6), which
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 125I-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 $M_c$ 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 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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