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The Interaction of Calmodulin with Human Erythrocyte Spectrin

INHIBITION OF PROTEIN 4.1-STIMULATED ACTIN BINDING*

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The functional significance of calmodulin binding to human erythrocyte spectrin has been investigated under native conditions. Both native calmodulin and calmodulin derivatized with the photoactivable cross-linker methyl 4-azidobezimidate (azidocalmodulin) have been used. When azidocalmodulin is photolyzed in the presence of erythrocyte ghosts, ghost extracts, or purified protein, it cross-links predominately to the \( \beta \) subunit of erythrocyte spectrin. This cross-linking is calcium-dependent, requires photolysis, and is inhibited by 100 \( \mu M \) trifluoperazine or unlabeled calmodulin. Calmodulin labeled spectrin exhibits a specific and non-calcium-dependent inhibition of its ability to bind actin, even in the presence of protein 4.1. Its ability to self-associate or to bind spectrin-depleted membrane vesicles is unperturbed. Native calmodulin also inhibits protein 4.1-stimulated actin binding, but unlike that of covalently bound calmodulin, inhibition by the uncross-linked calmodulin requires calcium. The degree of inhibition of spectrin-actin-4.1 binding induced by native calmodulin is significant since 109 \( \mu M \) calmodulin inhibits over 63% of the spectrin-actin binding induced by 4.5 \( \mu M \) protein 4.1. These results demonstrate a specific effect of calmodulin on erythroid spectrin function and suggest that calmodulin may influence the binding of protein 4.1 and actin to spectrin within the cytoskeleton.

The mammalian erythrocyte contains abundant calmodulin (Jarrett and Penniston, 1978; Penniston et al., 1980). Erythrocytes also undergo shape changes in response to calcium loading or treatment with calmodulin inhibitors (Morrow and Anderson, 1986, and references cited therein). Both strong (\( K_d = 0.0005 \) \( \mu M \)) and weak (\( K_d = 6.7 \) \( \mu M \)) erythrocyte calmodulin-binding sites exist (Agrè et al., 1983; Sobue et al., 1981a; Burns and Gratzer, 1985; Husain et al., 1984), and several calmodulin-binding proteins have been identified (Agrè et al., 1983; Binds and Andreasen, 1981; Gardner and Bennett, 1986). With the exception of the Ca/Mg-ATPase which binds calmodulin with high affinity, all of the calmodulin-binding proteins in the erythrocyte appear to be composed of the cell's membrane skeleton (Bennett, 1985, and references therein).

The major component of the membrane skeleton is spectrin, a heterodimer composed of non-identical \( M \), 240,000 and 220,000 subunits (for reviews see Marchesi, 1985; Cohen, 1983). Proteins similar to erythrocyte spectrin also exist in other cells (for reviews see Morrow, 1984; Goodman and Zagon, 1984). It is clear from several studies that spectrin is a calmodulin-binding protein (Palfrey et al., 1982; Bartelt et al., 1982, 1984; Glenney and Glenney, 1984; Harris et al., 1985, 1986; Husain et al., 1984; Boivin and Galand, 1984; Sobue et al., 1985; Burns and Gratzer, 1985; Sears et al., 1986). However, confusion exists as to the functional significance of this binding and the relationship of the relatively strong calmodulin binding observed in the non-erythroid mammalian spectrin to the much more modest binding demonstrated by mammalian erythrocyte spectrin. The \( M, 240,000 \) or \( \alpha \) subunit of the non-erythrocyte spectrins (fodrin, calsspectrin) binds calmodulin avidly as judged by gel overlay techniques (Palfrey et al., 1982; Bartelt et al., 1982; Harris et al., 1985, 1986) or by its selective retention on calmodulin-Sepharose affinity columns (Kakiuchi et al., 1982; Glenney and Weber, 1985). Similar techniques applied to mammalian erythrocyte spectrin fail to detect binding (Palfrey et al., 1982; Harris et al., 1986), although more sensitive equilibrium methods reveal an interaction characterized by dissociation constants between 6.7 and 25 \( \mu M \) (Burns and Gratzer, 1985; Husain et al., 1984; Berglund et al., 1984). Presumably, it is this interaction with spectrin that accounts for the large number of weak calmodulin-binding sites resident in the membrane skeleton (Sobue et al., 1981a; Agrè et al., 1983). Less clear is the site of interaction within the mammalian erythrocyte protein. While it has been assumed by many authors, based on analogy with fodrin, that the \( \alpha \) subunit is responsible for the weak calmodulin binding in mammalian erythrocyte spectrin, there are no data to support this belief. In fact, the only available data support the notion that mammalian erythrocyte spectrin binds calmodulin on the \( \beta \) subunit. Under denaturing conditions (6 M urea), erythroid spectrin binds to calmodulin-Sepharose affinity columns in a calcium-dependent manner (Sobue et al., 1981a). While both subunits show some tendency to bind (Boivin and Galand, 1984), the \( \beta \) subunit binds with the greatest affinity and in fact such columns can be used to prepare isolated \( \beta \) erythrocyte spectrin subunits (Sears et al., 1986). This is exactly opposite to the result obtained when the non-erythrocyte spectrins are subjected to calmodulin affinity chromatography; for these proteins, only the non-erythroid \( \alpha \) subunit is retained (Kakiuchi et al., 1982). Finally, a specific \( M, 11,000 \) peptide derived from a cyanogen bromide digest of human erythrocyte spectrin has been identified which accounts for the calmodulin binding activity of the parent protein in 6 M urea (Sears et al., 1986). This peptide is derived from a region near the COOH terminus of the \( \beta \) subunit, as

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determined by both amino acid sequencing (Speicher and Marchesi, 1984) and by peptide mapping (Sears et al., 1986; Speicher et al., 1982). Thus, the non-erythroid spectrin binds calmodulin on their α subunit under both native and denaturing conditions. Mammalian erythroid spectrin binds calmodulin at a specific site on its β subunit under denaturing conditions. No information exists as to the site of calmodulin binding to this protein under native conditions.

Little is known concerning the functional significance of calmodulin binding to spectrin. The stomatogenic effects of the calmodulin inhibitors may be unrelated to their action on calmodulin (Morrow and Anderson, 1986, and references therein), and in any event appear to act independently of calcium (Burns and Gratzler, 1985). Calmodulin also appears to have no effect on the phosphorylation-dependent gelation of erythrocyte cytoskeletal extracts (Burns and Gratzler, 1985).

Other studies of the effects of calmodulin on the function of mammalian erythroid spectrin have not been reported, although in other tissues calmodulin has been shown to competitively inhibit binding of F-actin to cytoskeletal proteins such as caldesmon and non-erythroid spectrin (cal-spectrin) in the presence of a third unidentified factor (Sobue et al., 1983).

As an alternative approach to exploring the role of calmodulin in mammalian erythrocyte spectrin function, a photoactivable and 125I-labeled analog of calmodulin (azidocalmodulin, Andreasen et al., 1981) is used here to prepare a covalently coupled complex of human erythrocyte spectrin and bovine brain calmodulin. The composition of this complex is described and its activity compared with that of native spectrin and bovine brain calmodulin. Under native conditions azidocalmodulin specifically labels human erythrocyte spectrin in a calcium-dependent fashion. Both native and covalently bound calmodulin specifically depress the protein 4.1-stimulated actin binding to this protein under native conditions.

**EXPERIMENTAL PROCEDURES**

**Preparation of Calmodulin-cross-linked Spectrin—** Human erythrocyte spectrin was prepared by extraction of freshly mixed blood with equal volumes of 0.1 M EDTA, 0.2 M dithiothreitol, polyethylene glycol 3000, 0.03 M phenylmethylsulfonyl fluoride, and 0.05 M phenylmethylsulfonyl fluoride, pH 8.5, followed by purification on Sepharose CL-4B (Morrow and Marchesi, 1981).

Calmodulin was prepared from calf brain homogenized in 10 mM sodium acetate, 1.0 mM mercaptoethanol, 5.0 mM EGTA, 0.05 M phenylmethylsulfonyl fluoride, pH 7.2. Soluble heat-stable (85°C) proteins precipitating between 50 and 70% saturated ammonium sulfate at 4°C were subjected to affinity chromatography in 50 μM calcium on phenothiazine-Affi-Gel (Bio-Rad) as previously described (Seah et al., 1986). The protein was stored as a lyophilized powder at −20°C after dialysis into water.

Alternatively, calmodulin was prepared by two stages of ion-exchange chromatography using DE52-cellulose (Whatman) and by gel filtration on Sephadex G-100 (Pharmacia), following the procedure described by Burgess et al., (1980). Briefly, after ammonium sulfate precipitation (50%) of a bovine brain extract at pH 7.0, crude calmodulin was precipitated from the remaining supernatant at pH 4.1, and chromatographed on Sephadex G-100 in the above buffer containing calmodulin were pooled, concentrated by precipitation at pH 7.5, and eluted with a gradient of 0.1–0.5 M NaCl. The fractions containing calmodulin were pooled, concentrated by precipitation at pH 4.1, and chromatographed on Sephadex G-100 in the above buffer with 0.1 M NaCl.

Finally, the calmodulin-containing fractions were rechromatographed on DE52 after the addition of calcium chloride to 1.0 mM. This column was washed with 2 mM EDTA followed by elution with a 0.15–0.3 M NaCl gradient. Calmodulin purified in this manner was pure by SDS-PAGE and showed no bands immunoreactive with antibodies to S-100 protein by Western immunoblotting.

Calmodulin was covalently labeled with 125I by incubating 3 mg of calf brain homogenized in 430 μl of buffer containing 0.1 M HEPES, pH 7.2, 0.58 M calcium chloride, 30 mM glucose, 50 μl of freshly hydrated synthetic beads containing immobilized lactoperoxidase and glucose oxidase (Enzymobeads, Bio-Rad), and 2 μl of carrier-free Na125I (Amersham Corp.) for 30 min at room temperature. The reaction was terminated by 0.4 M 2-mercaptoethanol, followed by gel filtration on a 0.7 × 14 CM-Sephadex (Pharmacia) G-25 column. Material inactivated during the iodination was removed by affinity chromatography using phenothiazine-Affi-Gel as above.

Azidocalmodulin was prepared by reacting the iodinated calmodulin with method 4-azido-2-bromobenzamide (Ph-Bz) in 50 mM sodium acetate, 0.2 M calcium chloride, pH 9.5, at room temperature for 2 h, followed by dialysis into 10 mM MOPS, 0.2 mM calcium chloride, 1.0 mM 2-mercaptoethanol, pH 7.1, on Sephadex G-50 (Andreasen et al., 1981; Harris et al., 1986). The biologic activity of the iodinated azidocalmodulin was monitored by its ability to stimulate phosphodiesterase (Fertel and Weiss, 1978). No other proteins crossed this material after SDS-PAGE by either Coomassie Blue staining or by autoradiography. In addition, no proteins immunoreactive with antibodies to S-100 protein (Vector Laboratories) could be detected by immunoblotting.

Photocoupling of azidocalmodulin to spectrin or other proteins was activated by long-wavelength ultraviolet light (Minerlite UVS-2) for 5–10 min at 0°C. Unless otherwise specified in the figure legends, the conditions of labeling were 10 mM MOPS, 1.0 mM 2-mercaptoethanol, pH 7.10, 0.2 mM calcium chloride or 1.0 mM EGTA, and 0–2 μM calmodulin. The nominal concentrations of spectrin (expressed as dimer) ranged between 0.3 and 2 μM; the molar ratio of azidocalmodulin to spectrin was between 0.2 and 0.8 for all experiments. The extent of photolysis of the azido moiety was monitored spectrophotometrically by the loss of optical density at 270 nm (McKee, 1977). After photolysis, residual reactive intermediates were quenched by 2 mM DTT, 2 mM ascorbic acid, and 10 μM ethanolamine. Calmodulin-cross-linked spectrin used in the IOV or F-actin binding studies was separated from free azidocalmodulin by chromatography on Sepharose CL-4B (Pharmacia) in buffer A (130 mM KCl, 20 mM NaCl, 10 mM Tris, pH 7.6) containing 0.1 mM EDTA, 0.1 mM DTT, and 0.05 mM phenylmethylsulfonyl fluoride.

**Protein Immunoprecipitation—** Immunoprecipitation was done after disruption of noncovalent protein associations with SDS (Palfrey et al., 1982). Samples at 0.5–1.0 mg/ml were dissolved in SDS solubilization buffer (Laemmli, 1970). Aliquots (75 μl) were added to 25 μl of NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.02% sodium azide) containing 10% Triton X-100 and 5 mg/ml bovine serum albumin, followed by 20 μl of a 25% suspension of Staphylococcus aureus cells (Pansorbin, Behring Diagnostics). After incubation at 4°C for 10 min the samples were sedimented at 16,000 × g. The supernatants were incubated with 12 μl of affinity-purified rabbit anti-erythrocyte spectrin antibody (Harris et al., 1985) for 20 min at 0°C, followed by a 5-min incubation with 20 μl of S. aureus cells. The S. aureus cell-IgG precipitate was washed once with NET buffer and solubilized in 10% SDS at 100°C. The S. aureus cells were removed by sedimentation, and the supernatant was analyzed by SDS-PAGE.

**Binding Studies—** Erythrocyte inside-out vesicle binding was measured by the ability of 125I-labeled azidocalmodulin-spectrin (or directly iodinated control spectrin) to rebind spectrin-depleted erythrocyte membranes, as previously described (Harris et al., 1985). Resealed fresh erythrocyte ghosts served as the control for nonspecific binding. Protein 4.1-stimulated spectrin to F-actin binding was measured by cosedimentation assay (Becker et al., 1983; Coleman et al., 1987). Protein 4.1 (Tyler et al., 1985; Leto and Marchesi, 1984) and rabbit skeletal muscle actin (Gibbs and Watt, 1981) were characterized by standard methods. Freshly prepared actin was stored under conditions of continuous dialysis against depolymerizing buffer (0.2 mM ATP, 0.5 mM DTT, 0.2 mM calcium chloride, 2.0 mM Tris, pH 8.0) at 4°C. Actin was polymerized at 1.2 mg/ml by incubation in either

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1. The abbreviations used are: EGTA; [ethylenebis(oxyethylene-nitrito)]tetraacetate acid; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IOV, inside-out, spectrin-depleted inverted erythrocyte membrane vesicles; MOPS, 3-(N-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.
buffer A (experiments with protein 4.1) or 50 mM KCl, 10 mM Tris, pH 7.4 (experiments with actin alone). In addition, each buffer contained 2.0 mM magnesium chloride, 0.5 mM DTT, and calcium or EGTA as described in the figure legends. Incubation was at room temperature for 45 min. Filament length was limited and nucleation facilitated by mild stirring of the solution every 5 min for the first 15 min. Native spectrin was labeled with \(^{125}\)I by lactoperoxidase-glucose oxidase-catalyzed oxidation as described above for calmodulin. Spectrin and protein 4.1 were dialyzed against the final incubation buffer (with calcium or EGTA) immediately before the binding experiments. F-actin (0-144 μg) was incubated with 188 μg of \(^{125}\)I-spectrin or calmodulin-cross-linked spectrin and various amounts of protein 4.1 in a total volume of 120 μl for 1 h at room temperature and then stored at 0°C until centrifuged. Aliquots (110 μl) were layered over 150 μl of 5% sucrose in incubation buffer and the actin-spectrin complexes sedimented at 20,000 rpm in a JA-20 rotor (25,000 × g) for 2 h.

The experiments measuring the effect of underivatized calmodulin on spectrin-actin binding were done as above, except that actin was polymerized in buffer A containing 0.1 mM 2-mercaptoethanol, pH 7.2, 2.0 mM magnesium chloride, 0.1 mM EGTA, and 25 μl/ml bovine serum albumin, and special precautions were taken to assure that sufficient free calcium concentrations were attained in those experiments requiring calcium. This was assured by dialyzing the spectrin and protein 4.1 exhaustively before the experiment against buffer A containing 100 μM EGTA and 2-mercaptoethanol, and the calmodulin against the same buffer except with 100 μM magnesium chloride in place of the EGTA. The spectrin was then added as described for the protein 8 μg of actin, 10 μg of spectrin, 42 μg of protein 4.1) were mixed along with sufficient amounts of the calmodulin dialysis buffer to compensate for the various amounts of calmodulin. Additional amounts of EGTA or calcium chloride necessary to bring the final solution to 10 mM EGTA (for the calcium-free experiments) or 1.0 mM calcium (for the calcium-requiring experiments) were then added, and the mixture incubated at room temperature for 1 h. Subsequent manipulations were as above. The spectrin used in these experiments was labeled with \(^{125}\)I following the method of Bolton and Hunter (1973). Bolton-Hunter reagent was obtained from Amersham Corp.

Polyacrylamide Gel Electrophoresis and Other Procedures—Samples were analyzed in one-dimensional 8% polyacrylamide SDS gels or two-dimensional isoelectric focusing/SDS-PAGE by the methods of Laemmli (1970) and O'Farrell (1975), respectively, using gel conditions previously specified (Speicher et al., 1982). Nondenaturing PAGE was done as described by Morrow and Haigh (1983). Proteins were visualized by Coomassie Brilliant Blue (Bio-Rad) after fixation of the gels in 10% acetic acid, 30% methanol. Gels used for autoradiography were dried between permeable cellulose sheets in 5% glycerc (Morrow and Haigh, 1983). Mapping of chymotryptic peptides was done as described by Speicher et al. (1982). Autoradiograms were exposed at −80°C using XAR-5 (Kodak) film and fluorescent intensifying screens. Protein concentration was measured by the method of Lowry et al. (1951).

RESULTS

Azidocalmodulin Labels Only the β Subunit of Native Spectrin—Photolysis of \(^{125}\)I-labeled azidocalmodulin in the presence of leaky erythrocyte ghosts resulted in the labeling of a fixed subset of ghost proteins, as shown in Fig. 1. No label is incorporated in the absence of photolysis or when prephotolyzed azidocalmodulin is added. Separate measurements indicate that, under the conditions used here, more than 90% of the photolabel is activated (data not shown). Proteins predominately labeled in the ghost are M, 235,000, M, 140,000-160,000 (a diffuse band), M, 117,000, and M, 57,000. These correspond to parent molecular weights (assuming a single molecule of calmodulin bound per protein) of about M, 220,000, 125,000-145,000, 100,000 and 41,000. This labeling pattern is similar to that observed by others (Agre et al., 1983; Hinds and Andreassen, 1981). The three most predominantly labeled proteins are extracted from ghosts by incubation in low ionic strength buffers. The same subset of proteins is also labeled directly when the extract alone is photolyzed in the presence of azidocalmodulin (data not shown). The only major band not extracted is at approximately M, 150,000, corresponding to the membrane Ca/Mg-ATPase (Hinds and Andreassen, 1981). This protein displayed the strongest calmodulin binding, since repetitive washing of the ghosts after incubation with azidocalmodulin but before photolysis reduced the amount of labeling in the extractable proteins but not in the Ca/Mg-ATPase. (After two rounds of washing, the Ca/Mg-ATPase became the most prominently labeled band.)

The smallest calmodulin-protein adduct extracted at low ionic strength (M, 57,000) is similar to the M, 57,000 complex reported by Agre et al. (1983). An additional adduct (M, 120,000) corresponds to calmodulin labeling of the smaller subunit of a M, 105,000 and 100,000 doublet. The protein responsible for this doublet has been extensively characterized (Gardner and Beattie, 1986). The molecular weights cited for the subunits in taat study are slightly smaller, M, 103,000 and 97,000, probably because of differences in gel calibration between the two studies.

The third adduct (M, 235,000) contains spectrin, since it is quantitatively precipitated by anti-erythrocyte spectrin antibodies (Fig. 2). The size of the adduct, intermediate between the α and β subunits of spectrin, is consistent only with cross-linking of a single calmodulin molecule to the β subunit. In some experiments (Fig. 3) a small amount of labeling in a band larger than the spectrin α subunit is observed. This is consistent with either multiple labeling of β spectrin or labeling of α spectrin. In either case the amount of this species is never more than 5% of the β spectrin label. The labeling of β spectrin with azidocalmodulin is also stimulated severalfold by 100 μM calcium (Figs. 1 and 2). Azidocalmodulin is also photocross-linked to purified spectrin, as shown in Fig. 3. This binding is inhibited by underivatized calmodulin and by 100 μM trifluoperazine (Fig. 3). It is reduced but not eliminated by 100 mM KCl. Cross-linking is also not appreciably reduced by the presence of 2-mercaptoethanol, although higher concentrations of this reagent or DTT reduce the efficiency of labeling (Table I).

Oligomer Formation and IOV Binding Are Normal in Spectrin-Azidocalmodulin Complexes—The ability of calmodulin-spectrin to spontaneously self-associate to tetramers and higher molecular weight oligomers is shown in Fig. 4. A purified solution of spectrin was reacted with \(^{125}\)I-azidocalmodulin, and the pattern of incorporation into the dimer, tetramer, and hexamer oligomeric forms measured by nondenaturing PAGE (Morrow and Haigh, 1983). Because the efficiency of labeling is only about 1% (data not shown), most of the spectrin remains unlabeled, although all of the radioactive material represents photocross-linked spectrin-calmodulin (any free calmodulin being removed during electrophoresis). Thus the Coomassie Blue-stained profile (Fig. 4, lane CB) represents an internal control of unlabeled spectrin, while the autoradiogram (Fig. 4, lane AR) represents the pattern of oligomer formation for the calmodulin-spectrin complex. A comparison of the two, expressed as specific activity, is shown on the right in Fig. 4. No differences exist; hence, calmodulin does not affect spectrin oligomerization.

Spectrin is also able to bind the cytoplasmic face of erythrocyte membranes (IOVs) via an interaction with erythrocyte ankyrin (Bennett and Branton, 1977). Fig. 5 shows a comparison of the IOV binding activity of radiolabeled calmodulin-spectrin versus purified spectrin which was directly labeled with \(^{125}\)I. Both calmodulin-spectrin and spectrin alone demonstrate high affinity binding and are indistinguishable in this assay (Fig. 5A). There was no calcium dependence to this binding.
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4.1-actin

Fig. 1. Azidocalmodulin labels a fixed subset of erythrocyte ghost proteins. Coomassie Blue staining patterns (lanes A, B, E, F, I, and J) and corresponding autoradiographs (lanes C, D, G, H, K, and L) of erythrocyte ghosts (lanes A–D), IOVs (lanes E–H), and low ionic strength-extracted proteins (lanes I–L) labeled with 125I-azidocalmodulin are shown. Labeling was performed in 10 mM sodium phosphate, 0.2 mM calcium chloride, 0.1 mM magnesium chloride, 1.0 mM mercaptoethanol, pH 7.0, in the absence (lanes A, C, E, G, I, and K) or presence (lanes B, D, F, H, J, and L) of 4.0 mM EGTA. Samples contained either 1.1 mg/ml (ghosts), 0.9 mg/ml (IOV), or 0.3 mg/ml (extracts) of erythrocyte protein and 0.15 pM azidocalmodulin.

Fig. 2. The high molecular weight peptide labeled by azidocalmodulin is precipitated by anti-spectrin antibodies. Shown are the Coomassie Blue-stained (CB) SDS gels (A–D) and their corresponding autoradiographs (AR) (E–H) of low ionic strength-extracted proteins, labeled with azidocalmodulin as described in the legend to Fig. 1, and then precipitated with either rabbit anti-spectrin antibodies (RAS) (lanes A, B, E, and F) or with preimmune nonreactive serum (NRS) (lanes C, D, G, and H). Samples were labeled in the absence (−) (A, C, E, and G) or presence (+) (B, D, F, and H) of unchelated calcium.

Although unlikely, it is conceivable that the strong binding of the cross-linked calmodulin-spectrin complex is partially due to binding of calmodulin to a high affinity site on the membrane Ca/Mg-ATPase. This possibility is excluded by two additional binding inhibition experiments. Purified unlabeled spectrin competes effectively for the binding of calmodulin-spectrin (Fig. 5B), with an inhibition constant ($K_i = 0.1 \mu M$) identical to that for unmodified spectrin (Harris et al., 1986). Similarly, unlabeled calmodulin is without effect on the binding of the calmodulin-spectrin complex (Fig. 5B, inset). Thus photocross-linked calmodulin-spectrin is fully

Fig. 3. Azidocalmodulin labeling is inhibited by phenothiazines or underivatized calmodulin. A, Coomassie Blue-stained SDS gels (lanes 1, 2) and their corresponding autoradiographs (lanes 3, 4) are shown of spectrin (1.9 μM) labeled with 125I-azidocalmodulin (1.5 μM) in 10 mM MOPS, 0.2 mM calcium chloride, 1.0 mM mercaptoethanol, pH 7.1, in the presence (lanes 2, 4) or absence (lanes 1, 3) of 100 μM trifluoperazine. α and β spectrin subunits are indicated by α and β, respectively. B, upper panel, autoradiograph of spectrin (1.0 μM) labeled with 125I-azidocalmodulin (0.7 μM) in the presence of 0.0 μM (lane 1), 25 μM (lane 2), 51 μM (lane 3), or 101 μM (lanes 4, 5) underivatized calmodulin. The conditions were as described for A. except the mercaptoethanol was omitted. Lower panel, Quantitation of azidocalmodulin labeling by scanning densitometry of the autoradiogram shown in B. Nearly complete inhibition (89%) is achieved by 101 μM calmodulin.
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Table 1

<table>
<thead>
<tr>
<th>Condition</th>
<th>Labeling yield</th>
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<tr>
<td>Control (no additions)</td>
<td>100</td>
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<tr>
<td>0.1 mM 2-mercaptoethanol</td>
<td>110</td>
</tr>
<tr>
<td>1.0 mM 2-mercaptoethanol</td>
<td>77</td>
</tr>
<tr>
<td>1.0 mM dithiothreitol</td>
<td>72</td>
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**FIG. 4.** Spectrin oligomerization does not perturb its labeling by azidocalmodulin. The specific activities of 125I-calmodulin cross-linked spectrin dimer, tetramer, and hexamer are identical. The Coomassie Blue-staining pattern (CB) and autoradiograph (AR) of spectrin labeled with 125I-azidocalmodulin and analyzed by nondenaturing PAGE is shown (DIMER, dimer; TETR, tetramer; HEX, hexamer). Also shown are the specific activities of each species, calculated by excising each band and measuring the protein content by extraction of the Coomassie Blue dye and the radioactivity by γ counting. Since all species are labeled to the same specific activity, spectrin oligomerization and calmodulin photolabeling must be independent events. The calcium concentration was 200 μM for this experiment. Error bars indicate 1 standard deviation, n = 3.

Active with respect to IOV binding, presumably a measure of its ankyrin binding, and also with respect to its ability to self-associate into tetramers and higher oligomers.

Calmodulin Inhibits Spectrin-Actin Binding—In contrast to the lack of effect of cross-linked calmodulin on the spectrin functions described above, calmodulin-spectrin complexes demonstrate markedly reduced F-actin binding ability. At physiologic ionic strength, this is most apparent in the presence of protein 4.1 (Fig. 6A). At moderate concentrations of protein 4.1 (up to 3 mol per mole of spectrin dimer), calmodulin-spectrin binds only half as well as spectrin alone. This effect is probably not due to a subpopulation of inactive spectrin molecules, since the inhibition is progressively overcome by increasing amounts of protein 4.1 (Fig. 6A). The addition of 200 μM calcium to the medium mildly depresses the binding of calmodulin labeled spectrin (Fig. 6A); however, there is also a slight depression of binding by calcium in the absence of calmodulin, and the differences are not significant. Uncross-linked calmodulin does not bind to actin in the presence or absence of protein 4.1 with or without calcium.

The binding of spectrin to actin under low ionic strength conditions was explored to determine whether calmodulin would affect actin binding alone. These results are shown in Fig. 6B. Under these (nonphysiologic) conditions the normally weak binding of spectrin is almost completely eliminated in a calcium-independent fashion by the cross-linked calmodulin.

Spectrin binding to actin is also depressed by underivatized calmodulin. These results are shown in Fig. 7. Under the conditions chosen for this assay, (4.5 μM protein 4.1), approximately 55% of the spectrin is precipitated. Separate experiments (Fig. 7A and data not shown) indicate that 100 μM calcium alone will only mildly suppress the protein 4.1-stimulated co-sedimentation of spectrin and actin, consistent with the results of an earlier study (Fowler and Taylor, 1980). However, in the presence of calcium, the inclusion of increasing amounts of calmodulin in the assay markedly reduces the amount of spectrin precipitated, a result analogous to that obtained with the cross-linked calmodulin-spectrin. At a nominal calmodulin concentration of about 50 μM, the amount of spectrin binding F-actin is reduced to less than half of its calmodulin-free control value. However, in contrast to the situation with the cross-linked calmodulin-spectrin, the effect of calmodulin on spectrin-protein 4.1-actin interactions is entirely calcium-dependent, since no inhibition is observed even with 109 μM calmodulin in the presence of 10 mM EGTA (Fig. 7B).
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**FIG. 6.** A, Cross-linked calmodulin suppresses the protein 4.1-stimulated actin binding ability of spectrin. $^{125}$I-calmodulin-spectrin (0.43 μM) (A) does not bind F-actin (0.49 mg/ml) as well as does an equivalent amount of $^{125}$I-spectrin (B). Conditions used were buffer A plus 2.0 mM magnesium chloride, 0.5 mM DTT, and either 0.20 mM calcium chloride (open symbols) or 0.904 mM EGTA, 0.019 mM calcium chloride (closed symbols). B, cross-linked calmodulin also suppresses the ability of spectrin to bind to actin alone. $^{125}$I-calmodulin-spectrin (0.38 μM) (A) shows almost no direct actin binding ability when compared to directly labeled $^{125}$I-spectrin (0.31 μM) (B) when incubated with the indicated concentrations of F-actin in 23 mM potassium chloride, 14 mM Tris, 1.8 mM magnesium chloride, 0.5 mM DTT, 0.05 μg/ml leupeptin, and either 0.199 mM calcium chloride (open symbols) or 0.454 mM EGTA, 0.018 mM calcium chloride (closed symbols).

**DISCUSSION**

The data presented here demonstrate that azidocalmodulin photolabels a single specific site in the β subunit of human erythroid spectrin under physiologic conditions. Such labeling, which is calcium-dependent, suppresses the protein 4.1-stimulated actin binding ability of spectrin, but has no effect on spectrin's other functions as measured in vitro. Underivatized calmodulin also displays a similar inhibitory action on protein 4.1-stimulated spectrin-actin binding, although, unlike the suppression induced by covalently bound calmodulin, the effect with native calmodulin is strongly calcium-dependent. Taken together, these data indicate that calcium and calmodulin regulate spectrin-actin interactions via a calcium-dependent binding of calmodulin to spectrin. It is unlikely that any direct interaction between protein 4.1 and calmodulin is involved, since no photolabeling of protein 4.1 by azidocalmodulin could be detected, and since covalent labeling of spectrin alone with calmodulin was sufficient to down-regulate its affinity for actin.

Because the efficiency of azidocalmodulin cross-linking to spectrin is not accurately known, these data do not allow determination of a dissociation constant. However, the fact that azidocalmodulin could be largely removed from the ghosts before photolysis by one or two cycles of centrifugation and resuspension suggests that the binding is readily reversible and is consistent with the binding affinities measured by others ($K_d = 6.7–25$ μM) (Husain et al., 1984; Berglund et al., 1984; Burns and Gratzer, 1985). The rapid dissociation of calmodulin from native erythroid spectrin explains the diffi-
The suppression of spectrin-actin binding is not due simply to injury of the spectrin during the photolabeling. Oxidative damage (Becker et al., 1986), is unlikely, since abundant DTT is present at all stages in the isolation of the cross-linked protein and during the actin binding studies. In addition, the 2-nitro-5-thiocyanobenzoic acid and tryptic two-dimensional isoelectric focusing/SDS-PAGE maps of the cross-linked protein are identical to controls, while separate studies indicate that such maps change significantly with even mild protein oxidation. Also, no other functions of spectrin are disturbed by the cross-linking, so it has not experienced global damage. Analogous experiments in other systems, such as with insulin and its receptor (Brandenburg et al., 1980; Wang et al., 1982), also yield cross-linked protein complexes that demonstrate permanent activation of a native activity. And finally, the suppressive effect of cross-linked calmodulin on spectrin-actin binding can be demonstrated with native undervatized calmodulin. It is unlikely that this inhibition is due to an additional effect of calmodulin on actin or protein 4.1, since calmodulin does not interact with actin (Sobue et al., 1981b; Howe et al., 1980), and does not cross-link to protein 4.1 (cf. Fig. 1).

The inhibition of spectrin-actin-protein 4.1 binding following cross-linking of calmodulin to spectrin is unaffected by calcium, probably because cross-linking prevents the dissociation of calmodulin. The inhibition by uncross-linked calmodulin requires calcium. Thus, calmodulin confers a calcium dependence on the interaction of spectrin with actin and protein 4.1. Less clear is the actual mechanism by which calmodulin exerts this effect. It is the β subunit of spectrin that determines whether protein 4.1 is required to stimulate its interactions with actin (Coleman et al., 1987). As demonstrated here, the β subunit of human erythrocyte spectrin also contains the calmodulin-binding site. However, it is unlikely that the antagonistic effects of protein 4.1 and calmodulin are simply due to a competition for a single binding site. The suppression of protein 4.1-stimulated actin binding caused by the cross-linked calmodulin is not complete and can be overcome by increasing the amount of protein 4.1 in the assay. This would not be possible if they were competing for mutually exclusive sites, since the calmodulin is covalently attached. Calmodulin also suppresses the protein 4.1-independent binding of spectrin to F-actin. Thus, it appears that calmodulin down-regulates the binding of F-actin to spectrin in a fashion antagonistic to the stimulation caused by protein 4.1. This effect is similar to that of calmodulin on other proteins such as caldesmon (Sobue et al., 1983).

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