

Erythrocyte Adducin: A Calmodulin-regulated Actin-bundling Protein that Stimulates Spectrin-Actin Binding

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Abstract. Adducin is an erythrocyte membrane skeletal phosphoprotein comprised of two related subunits of 105,000 and 100,000 M_r . These peptides form a functional heterodimer, and the smaller of the two binds calmodulin in a calcium-dependent fashion. Although this protein has been physicochemically characterized, its function remains unknown. We have examined the interaction of human adducin with actin and with human erythrocyte spectrin using sedimentation, electrophoretic, and morphologic techniques. Purified adducin binds actin at physiologic ionic strength and bundles it into arrays of laterally arranged filaments, the adducin forming cross-bridges between the filaments at 35.2 ± 3.8 (2 SD) nm intervals. The stoichiometry of high affinity adducin binding to actin at saturation is 1:7, corresponding to a dimer of addu-

cin for every actin helical unit. Adducin also promotes the binding of spectrin to actin independently of protein 4.1. At saturation, each adducin promotes the association of one spectrin heterodimer. The formation of this ternary spectrin-actin-adducin complex is independent of the assembly path, and the complex exists in a readily reversible equilibrium with the free components. The binding of adducin to actin and its ability to stimulate spectrin-actin binding is down-regulated by calmodulin in a calcium-dependent fashion. These results thus identify a putative role for adducin, and define a calcium- and calmodulin-dependent mechanism whereby higher states of actin association and its interaction with spectrin in the erythrocyte may be controlled.

THE basic structural organization of the erythrocyte cytoskeleton is understood in reasonable detail. The predominant proteins involved, spectrin, protein 4.1, and actin, have been extensively characterized, and all participate in the formation of stable high molecular mass complexes in vitro (for reviews see Cohen, 1983; Bennett, 1985; Marchesi, 1985). The conclusions of studies using in vitro reconstitution have generally been in accord with those using a variety of techniques to directly visualize the erythrocyte membrane skeleton (Cohen et al., 1980; Pinder and Gratzel, 1983; Beaven et al., 1985; Byers and Branton, 1985; Shen et al., 1986; Liu et al., 1987). The consensus that has emerged from these studies is that small, relatively stable complexes containing filamentous actin and protein 4.1 are joined by spectrin tetramers and oligomers to form the anastomosing protein array that lies beneath the erythrocyte membrane.

The roles of several less prominent erythrocyte cytoskeletal proteins remain to be elucidated. One of these is a calmodulin-binding phosphoprotein heterodimer composed of subunits of 105,000 and 100,000 M_r . The presence of this protein in erythrocyte ghosts and in the erythrocyte cytoskeleton appears to have been noted by many workers. Luna et al. (1979) first noted its presence after protease digestion

of erythrocytes, and named it band 3'. Subsequently, it has been identified as band 2.8 and 2.9 (Johnson et al., 1982), CamBP 103/97 (Gardner and Bennett, 1986), PK1 and PK2 (Palfrey and Waseem, 1985), and 115/110 (Wolfe and Sahyoun, 1986). In a recent abstract, Gardner and Bennett (1987) have also called it "adducin" (from the Greek *adducere*, meaning "to pull together"), since they suggested that the protein might play a role in sequentially guiding spectrin to actin. Our studies find no evidence of a pathway-dependent interaction between this protein, spectrin, and actin. However, as reported here, it does "gather" actin filaments to each other and promotes the binding of spectrin to actin. Thus, the name adducin is not inappropriate. While our preliminary reports of this molecule referred to it as p105/100 (Mische et al., 1987), in the interest of clarity and at the risk of propagating a trivial name for a protein whose similarity to other actin-binding proteins is still unknown, we suggest that the name adducin be adopted for this protein. We will refer to it as such in this report.

Adducin has been most extensively characterized by Gardner and Bennett (1986), who established that the protein, as purified from erythrocyte Triton X-100 shells, consists of a heterodimer of related but not identical subunits. These workers established that the smaller subunit bound calmodulin and estimated that $\sim 30,000$ copies of the heterodimer were present in the mature erythrocyte. Adducin has also

A preliminary report of these findings has appeared in abstract form (1987. *J. Cell Biochem.* 11B:154).

been identified as a substrate for several protein kinases, including protein kinase C (Palfrey and Waseem, 1985; Ling et al., 1986; Wolfe and Sahyoun, 1986; and Cohen et al., 1986), calcium-calmodulin-dependent protein kinase (Ling et al., 1986), and cAMP-dependent protein kinase (Cohen and Foley, 1986). The function of adducin, however, is unknown.

In this report we present evidence that adducin can mediate the assembly of actin into discrete bundles of filaments, and that it promotes the association of spectrin with F-actin independently of protein 4.1. We also report that these activities are both down-regulated by calmodulin in a calcium-dependent manner.

Materials and Methods

Membrane Preparation

Erythrocytes were isolated from fresh human whole blood by two sedimentations at 4°C through 0.75% dextran T-500 at unit gravity, in 4 vol of PBS (5 mM sodium phosphate, 135 mM sodium chloride, 1 mM EDTA, pH 7.5) (Bennett, 1983). Membranes were prepared by low ionic strength lysis of the washed erythrocytes as previously described (Morrow and Marchesi, 1981).

Purification of Adducin

Adducin was prepared from white ghosts after extraction with Triton X-100 according to the procedure of Gardner and Bennett (1986) with minor modifications. The ion exchange chromatography step was performed using a 1.2 × 20-cm column of DE-52 cellulose (Whatman Inc., Clifton, NJ) that

was equilibrated in 10 mM Tris, pH 7.5, 50 mM NaCl, 0.25 mM EGTA, 0.1 mM dithiothreitol (DTT), 0.05 mM phenylmethylsulfonyl fluoride (PMSF), and 2 µg/ml leupeptin (equilibration buffer). After sample application, the column was washed with 1 column volume of equilibration buffer, followed by 1 column volume of the same buffer containing 100 mM NaCl. The protein was eluted with a linear salt gradient (5 column volumes) to 300 mM NaCl. Fractions containing adducin were pooled, dialyzed, and stored in 20 mM Tris-HCl, pH 7.4, 60 mM KCl, 10 mM NaCl, 0.25 mM EGTA, 0.1 mM DTT, 5 µg/ml leupeptin.

Preparation of Actin

Chicken skeletal muscle actin was prepared from acetone-extracted and -lyophilized chicken breast muscle by the method of Spudich and Watt (1971), and was stored at 4°C under conditions of continuous dialysis versus G buffer (2 mM Tris-HCl, 0.2 mM ATP, 0.2 mM calcium chloride, 0.5 mM DTT, 0.2% sodium azide, pH 8.0). The G-actin was spectrophotometrically determined using an extinction coefficient of 10.9 for a 1% solution.

Preparation of Spectrin

Spectrin was prepared from human erythrocyte ghosts by extraction at 37°C with 0.1 mM EDTA, pH 9.0, followed by separation of the dimer and tetramer forms by gel filtration on Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) in 20 mM Tris-HCl, pH 7.5, 130 mM KCl, 20 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.05 mM PMSF (Morrow and Marchesi, 1981).

Preparation of Protein 4.1 and Calmodulin

Human erythrocyte protein 4.1 was prepared from 1 M KCl extracts of spectrin-depleted erythrocyte vesicles, as described by Tyler et al. (1980). Calmodulin was prepared from frozen bovine brain by ion exchange and

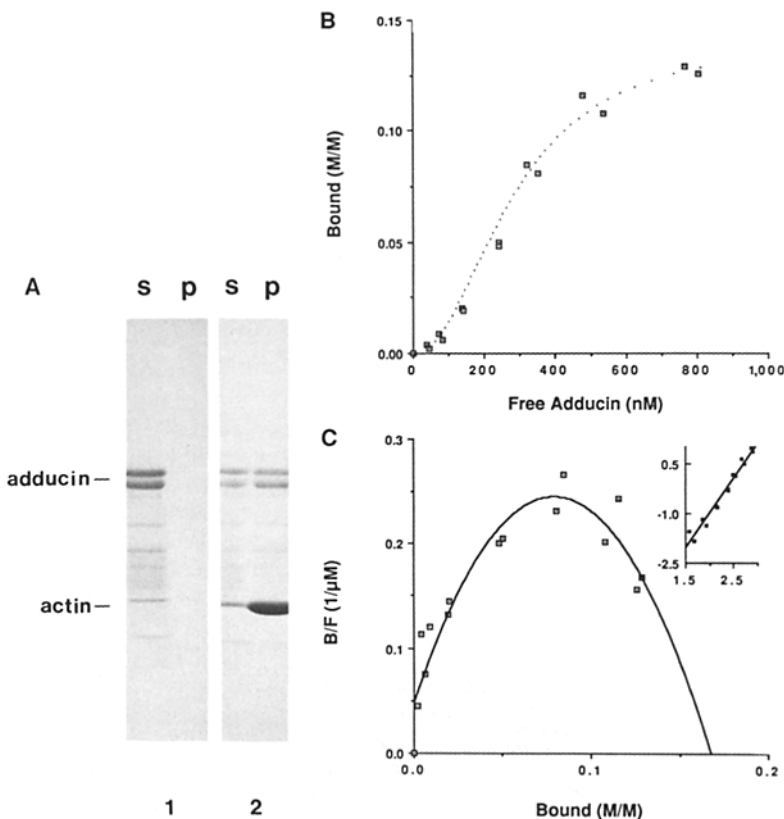


Figure 1. Adducin binds actin. (A) SDS-PAGE analysis of supernatants (lanes *s*) and pellets (lanes *p*) from a 100,000 *g* sedimentation of a solution of 0.3 µM adducin alone (pair 1) or of the same solution of adducin with 5 µM actin (pair 2). The faint additional bands of lower molecular mass (50–74,000 *M_r*) that are evident in the analysis of adducin are proteolytic degradation products which have been identified by others (Gardner and Bennett, 1986). (B) Quantitation of the binding of adducin to actin. The amount of adducin bound to actin was determined by elution of the Coomassie Blue dye from stained SDS gels. The binding is expressed as a ratio of adducin to actin sedimented. The binding conditions were as above, except that the concentration of KCl was 60 mM. The sigmoidal binding curve saturates near 0.143 (*M/M*). The apparent *K_d* for this interaction determined by nonlinear regression analysis is 283 nM. The Hill coefficient assumed for this fit is 2.09, as derived below; a binding ratio of adducin to actin of 1:7 (0.143) was assumed. (C) Scatchard analysis of the adducin-actin binding data presented above. The downward concave curve indicates the positively cooperative nature of this interaction. Extrapolation of the regression curve from this analysis suggests a saturation binding value of 0.16 *M/M*. (Inset) Hill plot of the binding data. The slope of the regression curve is 2.1 ± 0.2 (2 SD), indicating strong cooperativity. The log [*y*/(1 - *y*)] is plotted on the ordinate; log[free (nM)] is plotted on the abscissa, (*y* represents the fractional saturation: bound/maximal binding).

affinity column chromatography as previously described (Burgess et al., 1980).

Cosedimentation Assays

Actin-binding assays were performed by cosedimentation with either copolymerized or preformed F-actin (prepolymerized at 1.2 mg/ml by incubation in 20 mM Tris-HCl, pH 7.4, 120 mM KCl, 10 mM NaCl, 0.1 mM DTT, 2 mM MgCl₂, pH 7.4). Various concentrations of adducin, spectrin, and protein 4.1 were used in 20 mM Tris-HCl, 10 mM NaCl, 0–250 mM KCl, 0–10 mM magnesium chloride, 0.25 mM EGTA, 0.1 mM DTT. Actin (5 μ M) was added at time zero in all experiments. In the copolymerization assays, the samples were gently sheared by pipetting several times during the first 15 min of the incubation period to facilitate nucleation, and then incubated for 30 min.

In experiments containing calcium, all proteins were dialyzed before the experiment into the appropriate calcium-containing buffer in order to assure the proper levels of free calcium. For solutions with calcium concentrations below 10 μ M, 10 mM calcium-EGTA buffers were used to control the free calcium level (Bartfai, 1979).

Samples were cosedimented at either 100,000 g (high speed) for 45 min or at 10,000 g (low speed) for 20 min at 4°C in a rotor (42.2 Ti; Beckman Instruments, Inc., Fullerton, CA). Supernatants and pellets were separated and the pellets were resuspended to their original volume. Identical volumes of each were analyzed by SDS-PAGE (Laemmli, 1970) and visualized by staining with Coomassie Brilliant Blue.

Quantitation of Binding

Two methods were used to quantitate the amount of protein present in each of the Coomassie Blue-stained gel bands. Densitometric determinations were performed directly on gel slabs using a scanning densitometer (model 1650; BioRad Laboratories, Richmond, CA). The areas under the peaks were measured using an electronic planimeter (model 1224 electronic digitizer; Numonics Corp., Lansdale, PA). Alternatively, the stained bands were sliced from the gels and the bound dye eluted with a constant volume of 25% pyridine and quantitated by absorbance at 605 nm (Fenner et al., 1975). Standard curves were obtained for each protein by elution of Coomassie Blue-stained bands from known quantities of protein (spectrin, actin, adducin) analyzed on SDS-PAGE. Protein determinations were carried out by the method of Lowry et al. (1951). The binding data was analyzed by least squares regression analysis (linear and nonlinear).

Electron Microscopy

The complexes of the assembled proteins were viewed on carbon-parlodion grids with a Zeiss 10CA electron microscope after fixation with 0.2% glutaraldehyde and staining with 1% aqueous uranylacetate.

Results

Adducin Binds Actin and Bundles Actin Filaments

Purified adducin was assayed for its ability to bind actin by cosedimentation at 100,000 g. In the absence of actin, no adducin is sedimented, while in the presence of actin, a substantial fraction of the adducin sediments (Fig. 1 A). Increasing amounts of adducin cosediment with F-actin as the free concentration of adducin is increased, until saturation is reached. These results are depicted quantitatively in Fig. 1, B and C. At saturation, one adducin binds an average of six to seven actin monomers. The apparent K_d for this reaction is 283 nM, as judged by nonlinear regression analysis of the binding curve (Fig. 1 B). Scatchard analysis of this binding data demonstrates that the binding of adducin to actin is strongly cooperative (Fig. 1 C), with a Hill coefficient of 2.1 ± 0.2 (2 SD) (Fig. 1 C, inset). The same degree of actin binding is observed when adducin is added to either preformed F-actin or when the actin is polymerized in the presence of adducin.

The stoichiometry and the positive cooperativity of the binding of adducin to actin suggested that it may be an actin-bundling protein (e.g., see references by Korn, 1982; Pollard, 1986). To examine this possibility, cosedimentation assays at 10,000 g (low speed) were performed. Under these conditions, F-actin will not pellet unless cross-linked. Fig. 2 A illustrates the effect of adducin on actin sedimentation. In the absence of adducin, <15% of the F-actin sediments under these conditions (Fig. 2 A, experiment 2). In the presence of 200 nM adducin, 92% of the actin is pelleted (Fig. 2 A, experiment 1). At higher levels of adducin, essentially all of the actin is sedimented (data not shown).

Aliquots of adducin-actin complexes were also examined by electron microscopy, and are shown in Fig. 3. Fixed and negatively stained samples revealed highly ordered linear arrays of actin filaments, with adducin decorating the filaments at periodic intervals of 35.2 ± 3.8 (2 SD) nm (Fig. 3 B, inset). This minimal repeat distance between adducin molecules bound to actin corresponds to the length of the actin filament helical repeat (14 actin monomers) (Huxley and Brown, 1967). Therefore, if one assumes that a dimer of adducin is required for cross-linking, the stoichiometry of adducin binding to actin is 1:7, similar to that determined above by the cosedimentation assays.

It should be noted that brief fixation of the adducin-actin complexes with glutaraldehyde was required in order for the complexes to withstand the conditions required for negative staining. Unfixed preparations retained virtually no adducin-actin complexes (data not shown).

Adducin Promotes Spectrin-Actin Association

At physiologic ionic strength, in the absence of protein 4.1, human erythrocyte spectrin binds actin with low affinity (Fig. 4 A, pair 1). This binding is markedly enhanced in the presence of adducin (0.165 μ M), as shown in Fig. 4 A, pair 2. Time-course studies of this interaction indicate that equilibrium between adducin, actin, and spectrin is achieved rapidly, requiring <5 min at 4°C (data not shown). Separate sedimentation velocity experiments demonstrate no stable direct interaction between spectrin and adducin. The enhanced binding of spectrin to actin that is dependent on adducin saturates at one to one and two-tenths spectrin heterodimers bound for every molecule of adducin in the complex

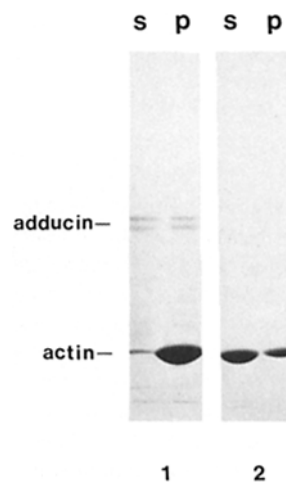


Figure 2. Adducin bundles actin. SDS-PAGE analysis of supernatants (lanes s) and pellets (lanes p) of a low speed sedimentation (10,000 g, 20 min) of a solution containing either 200 nM adducin and 5 μ M actin (pair 1) or actin alone (pair 2). Note that under these conditions most actin did not pellet in the absence of adducin.

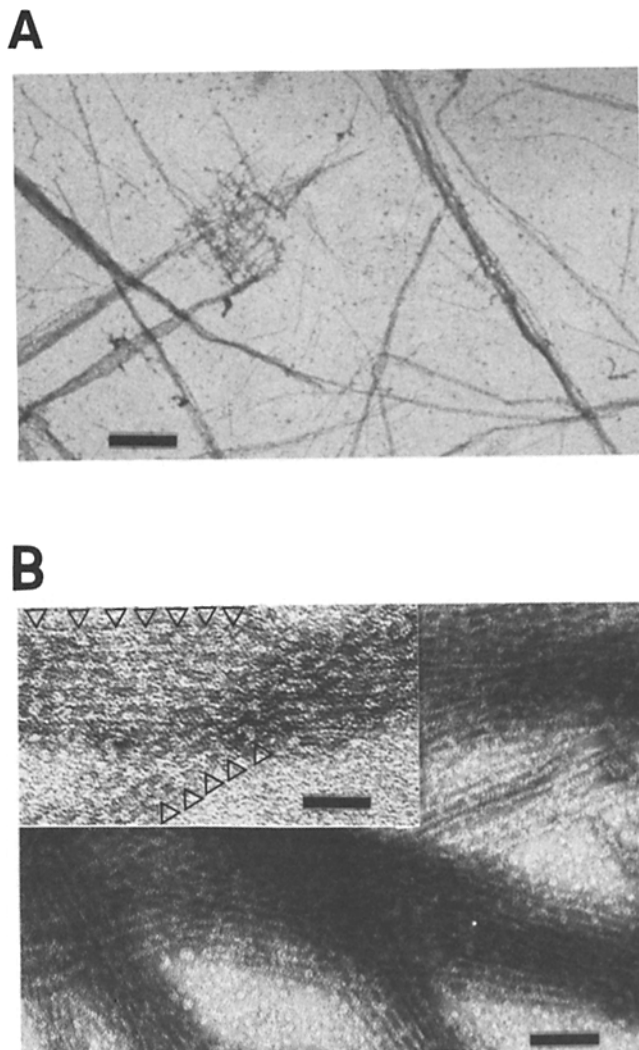


Figure 3. The adducin-actin complexes may be visualized after negative staining. Electron micrographs of the pellet fraction after a 10,000 g sedimentation of a solution of F-actin (4.5 μM) and 0.170 μM adducin. (A) Extensive bundles of actin were formed, accounting for the enhanced sedimentability of the actin in the presence of adducin. (B) Higher magnification view of the sample shown in A, demonstrating the presence of adducin molecules decorating the filaments. (Inset) Adducin decorated the actin bundles at minimum intervals of 35.2 ± 3.8 (2 SD) nm. All samples were prepared by direct dilution into 0.2% glutaraldehyde and were viewed on parlodian-carbon coated grids after staining with 1% uranyl acetate. Fixation of the complexes was necessary for their preservation; rare complexes were observed in the absence of glutaraldehyde treatment. Bars: (A) 0.5 μm ; (B) 0.1 μm ; (inset) 50 nm.

(Fig. 4, B and C). As observed for the binding of adducin to actin, the binding curve for spectrin to the adducin complex is sigmoidal, with an apparent K_d as estimated by regression analysis (dotted curve) of 104 nM. The values of the Hill coefficient and the saturation binding value derived from this analysis were 2.2 and 1.00, respectively. The positive cooperativity displayed by this interaction was also evident in the downward concave nature of the Scatchard analysis (Fig. 4 C). Extrapolation of the nonlinear regression curve from this analysis indicated a saturation value of spec-

trin to adducin of 1.2:1, very similar to the value derived directly from the analysis of the curve in Fig. 4 B. The Hill coefficient estimated directly from the Hill plot (not shown) was 2.1 ± 0.2 , identical to the value determined for the binding of adducin to actin.

Formation of the Ternary Complex of Spectrin-Adducin-Actin Is Independent of the Order of Assembly

Adducin (0.2 μM) and spectrin (0.3 μM) were preincubated separately for 1 h with either G-actin (5.6 μM) or with preformed filamentous actin (6.6 μM). After 1 h, spectrin or adducin was added to the respective preincubated binary complexes and the incubation was continued for an additional 30 min. The supernatants and pellets were analyzed after sedimentation at 100,000 g as in previous experiments. The results for F-actin are shown in Fig. 5. Pairs 1 and 2 show the results of the experiments with the spectrin-actin and the adducin-actin binary complexes, respectively.

Pair 3 demonstrates the ternary complex formed by the addition of adducin to the sample used in pair 1; pair 4 represents the ternary complex formed by the addition of spectrin to the sample used in pair 2. As can be seen, there are no differences in the amount of either adducin or spectrin cosedimented with actin in the two experiments. In addition, no differences were observed when either spectrin dimer or tetramer were used in these experiments (data not shown). These results indicate that the final composition of the complex is independent of the assembly path.

Association of Adducin with F-Actin and with Spectrin-Actin Is Dependent on Ionic Strength

The ionic strength of the buffer chosen for adducin actin binding proved critical. Fig. 6 A shows an SDS-PAGE analysis of the complex formed at 60 and 150 mM ionic strength, with all other conditions remaining constant. As can be seen, less adducin and spectrin binds actin at the higher ionic strength. Increased osmolarity (600 mosM sucrose) does not alter the binding (data not shown). This effect of ionic strength is depicted quantitatively in Fig. 6 B.

The presence of spectrin does not alter the sensitivity of the the adducin-actin complex to ionic strength (Fig. 6 B). In addition, neither calcium alone (10–200 μM) (Fig. 8 A) or magnesium (1–10 mM) (data not shown) has any effect on the binding affinity of adducin for actin. The interaction of adducin with actin is unaffected by variations in pH between 6.8 and 7.6 (Fig. 6 C).

Adducin and Protein 4.1 Can Simultaneously Stimulate Spectrin-Actin Binding

The predominant spectrin-actin stabilizing protein in the erythrocyte is protein 4.1 (Tyler et al., 1980). Protein 4.1 binds spectrin-actin with a K_d of 0.1 μM (Tyler et al., 1980), does not bind actin in the absence of spectrin (Ohanian et al., 1984), and appears to interact predominately with the beta subunit of spectrin (Coleman et al., 1987). The effect of protein 4.1 on the interaction of adducin with spectrin and actin was therefore of interest. Spectrin (0.17 μM), actin (5.0 μM), and adducin (0.3 μM) were incubated for 30 min, after which increasing quantities of protein 4.1 were added (0.5–3.75 μM) and the incubation was continued for an additional

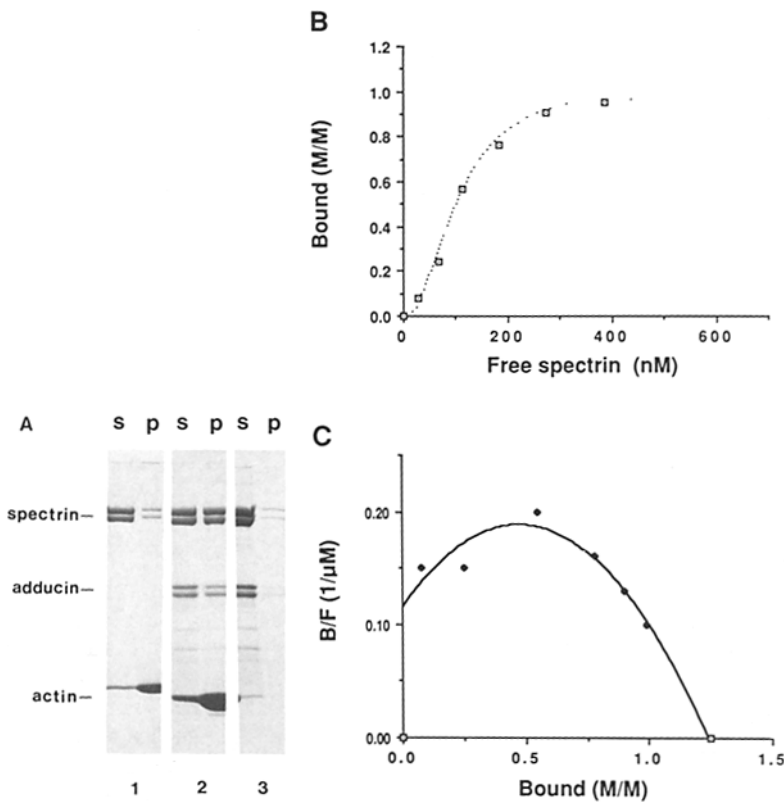


Figure 4. Adducin stimulates spectrin-actin binding. (A) Coomassie Blue-stained SDS gels of the supernatants (lanes *s*) and pellets (lanes *p*) resulting from a 100,000 *g* sedimentation of solutions containing spectrin (0.2 μ M) and actin (5.0 μ M) (pair 1); or of spectrin, actin, and 0.16 μ M adducin (pair 2). Buffer conditions were the same as in Fig. 1 A. Pair 3 shows the results when spectrin and adducin were sedimented under these conditions. (B) Quantitation of spectrin binding to adducin-actin. The conditions of this experiment were the same as in Fig. 1 B. The binding is expressed as the ratio of spectrin sedimenting to the amount of adducin sedimenting (*M/M*). Spectrin binding to adducin-actin saturated near a 1:1 molar ratio. The apparent K_d estimated from the regression analysis (dotted curve) for spectrin binding to the adducin-actin complex is 104 nM. The saturation value estimated from this curve is 1.00. (C) Scatchard analysis of the data shown in B. The stoichiometry of spectrin to adducin in the spectrin-actin-adducin complex estimated from this curve is 1.2:1. An estimation of the Hill coefficient from a Hill plot (not shown) yielded a value of 2.1 ± 0.2 (2 SD), identical to the value determined for the binding of adducin to actin (Fig. 1 C).

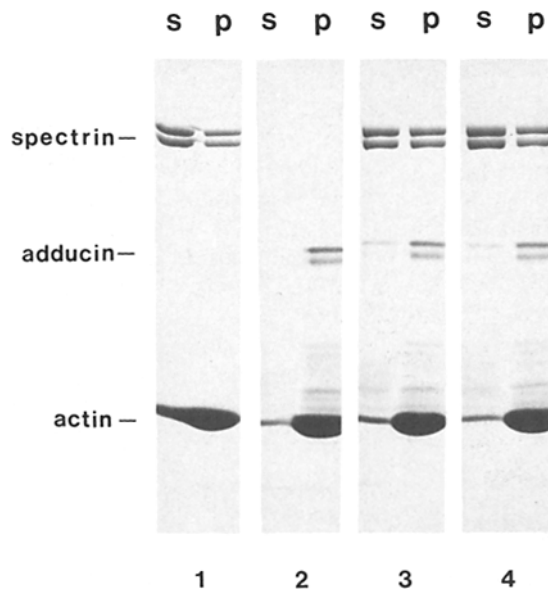


Figure 5. The formation of the spectrin-actin-adducin ternary complex is independent of the assembly path. Supernatants (lanes *s*) and pellets (lanes *p*) of a 100,000 *g* sedimentation are shown after SDS-PAGE analysis. In pair 1, spectrin (0.3 μ M) and actin were incubated for 1 h at room temperature under buffer conditions described in Fig. 1 B. In pair 2, adducin (0.2 μ M) and actin were incubated for a similar period of time. In pairs 3 and 4, adducin and spectrin were added respectively to samples from pairs 1 and 2, so that the final composition of the solutions in pairs 3 and 4 were identical. These samples were then incubated for an additional 30 min, after which they were analyzed. The final actin concentration was 5 μ M. Note that there was no discernable difference in the amount of spectrin, actin, or adducin sedimenting in pairs 3 and 4, even

30 min. These results are presented in Fig. 7. With increasing concentrations of protein 4.1, there is increased binding of spectrin to actin, but the amount of adducin bound to actin remains constant (Fig. 7 A, lanes 2–5). Even with a substantial molar excess of protein 4.1, there is no significant change in the amount of adducin sedimenting with the spectrin-actin-4.1 complex. These results are presented quantitatively in Fig. 7 B.

Similarly, when adducin is added to preformed spectrin-actin-protein 4.1 complexes, there is no significant change in the amount of adducin bound when compared with its binding to similar complexes which lack protein 4.1 (cf. Fig. 7 A, pair 6 with pair 1 or with Fig. 5). Correspondingly, there is also no significant reduction in the amount of protein 4.1 bound to the complex, even in the presence of significant excess adducin (Fig. 7 A, pair 7). Therefore, protein 4.1 and adducin do not compete directly for the same binding sites within the spectrin-actin complex, and their effect on spectrin-actin binding is likely to be additive.

Calmodulin Down-regulates the Ability of Adducin to Bind Actin and to Stimulate Spectrin-Actin Binding

Adducin has been shown to bind calmodulin in a calcium-dependent manner with moderate affinity (Gardner and Bennett, 1986; Anderson and Morrow, 1987). The effect of calmodulin (5–20 μ M) in the presence of 200 μ M calcium on the binding of adducin to actin and on its ability to stimulate

though in the former experiment adducin was added to preformed spectrin-actin complexes, while in the latter experiment, spectrin was added to preformed adducin-actin complexes.

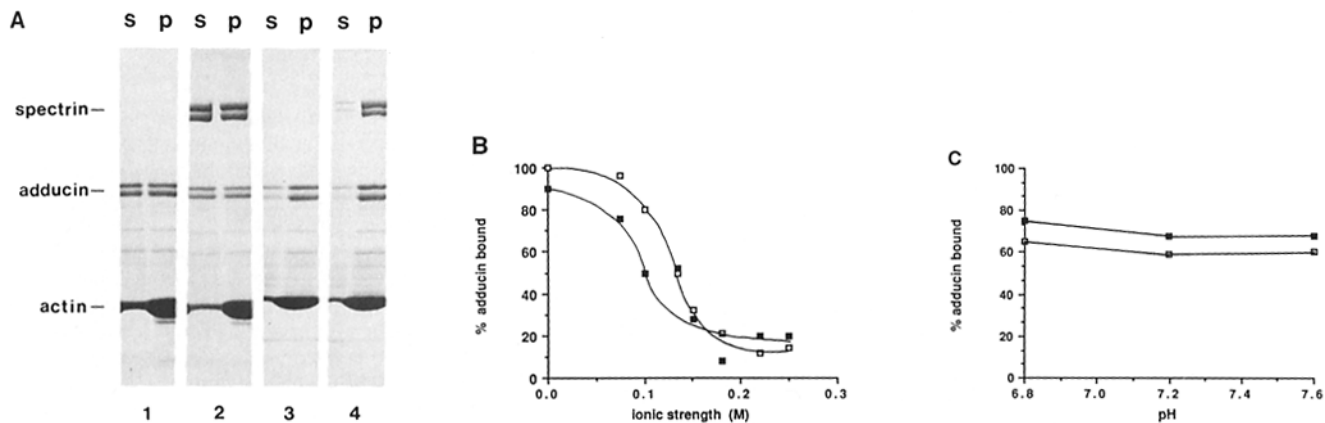


Figure 6. Adducin-actin binding is ionic strength dependent. (A) The effects of ionic strength on the adducin-actin interaction were determined as above. The buffer conditions of this experiment were as described in Fig. 1 A, except that the ionic strength of the solution was varied by changing the concentration of KCl. Pairs 1 and 3 contain adducin and actin. Pairs 2 and 4 contain spectrin, adducin, and actin. Pairs 1 and 2 contain 150 mM ionic strength; pairs 3 and 4, 60 mM ionic strength. (B) Quantitation of the amount of adducin sedimenting with actin. The binding of adducin alone to actin (*open symbols*) was strongly ionic strength dependent. Adducin binding in the presence of spectrin (*solid symbols*) showed the same dependency. The stimulation of spectrin binding to actin by adducin paralleled these curves (data not shown). (C) In contrast to the strong ionic strength dependence, the binding of adducin to actin was insensitive to pH variations within the physiologic range. Symbols and experimental conditions were as described in A and B; the ionic strength of this experiment was 80 mM.

spectrin-actin binding are shown in Fig. 8. As indicated above, calcium alone has no effect on the binding of adducin to actin (Fig. 8 A, pairs 1 and 2). With the addition of 10 μ M calmodulin, there is a marked inhibition of binding (Fig. 8 A, pairs 3 and 4). The addition of spectrin had no effect on this inhibition.

The effect of calmodulin and calcium on the binding of adducin to actin is presented quantitatively in Fig. 8 B. Half-maximal inhibition occurs near 4 μ M calmodulin, a value

similar to the K_d determined for the binding of calmodulin to adducin (Gardner and Bennett, 1986), and well within the physiologic range of the erythrocyte calmodulin concentration. The ability of adducin to stimulate spectrin-actin binding is also down-regulated by calcium and calmodulin (Fig. 8 B). The inhibition of spectrin binding is more pronounced than that for the adducin alone, presumably due to the direct inhibitory action of calmodulin on spectrin-actin interactions (Anderson and Morrow, 1987).

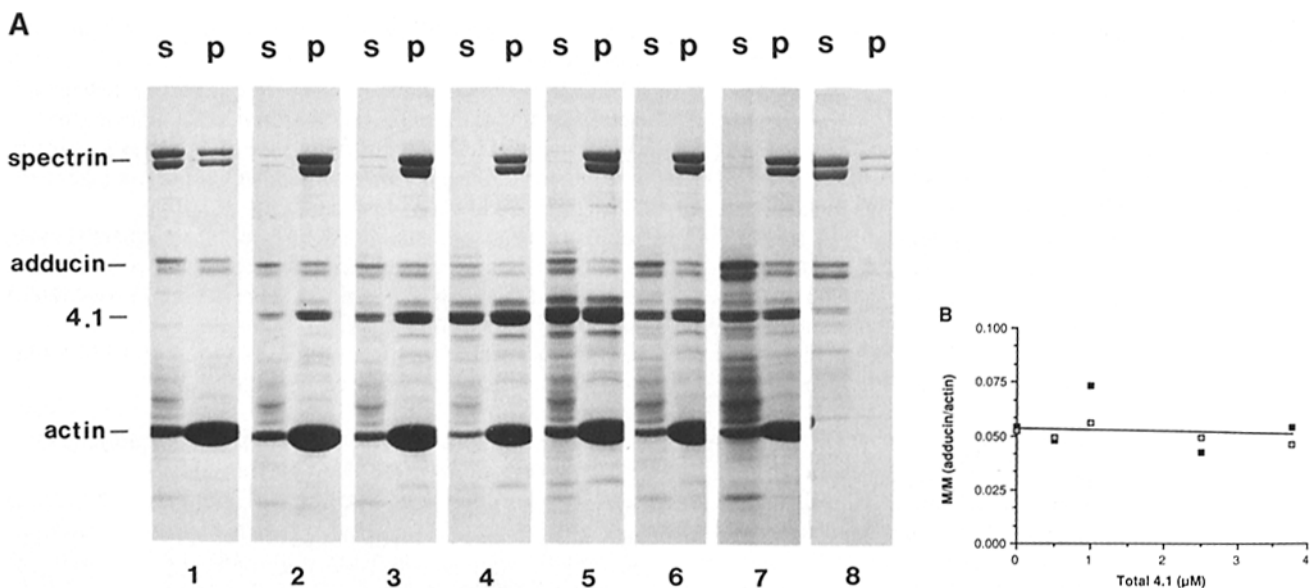


Figure 7. Protein 4.1 and adducin simultaneously augment spectrin-actin binding. (A) The effects of increasing amounts of protein 4.1 on the 100,000 g sedimentation of a solution of spectrin (0.17 μ M), adducin (0.3 μ M), and actin (5 μ M) are shown in pairs 1-5. Other conditions were as described in Fig. 1 A. The amount of protein 4.1 added to pairs 1-5 was: 0.0, 0.5, 1.0, 2.5, and 3.75 μ M, respectively (final concentration). In pairs 6 and 7, protein 4.1 (0.5 μ M) was preincubated for 30 min with spectrin and actin, after which adducin (0.6 and 1.8 μ M, respectively) was added. Note the augmentation of spectrin binding by protein 4.1 without the loss of adducin from the complex. (B) Quantitation of the effects of protein 4.1 on the amount of adducin that sediments with actin. Open and solid symbols are for two independent experiments. Note that there is no significant effect of protein 4.1 on the amount of adducin sedimenting with actin.

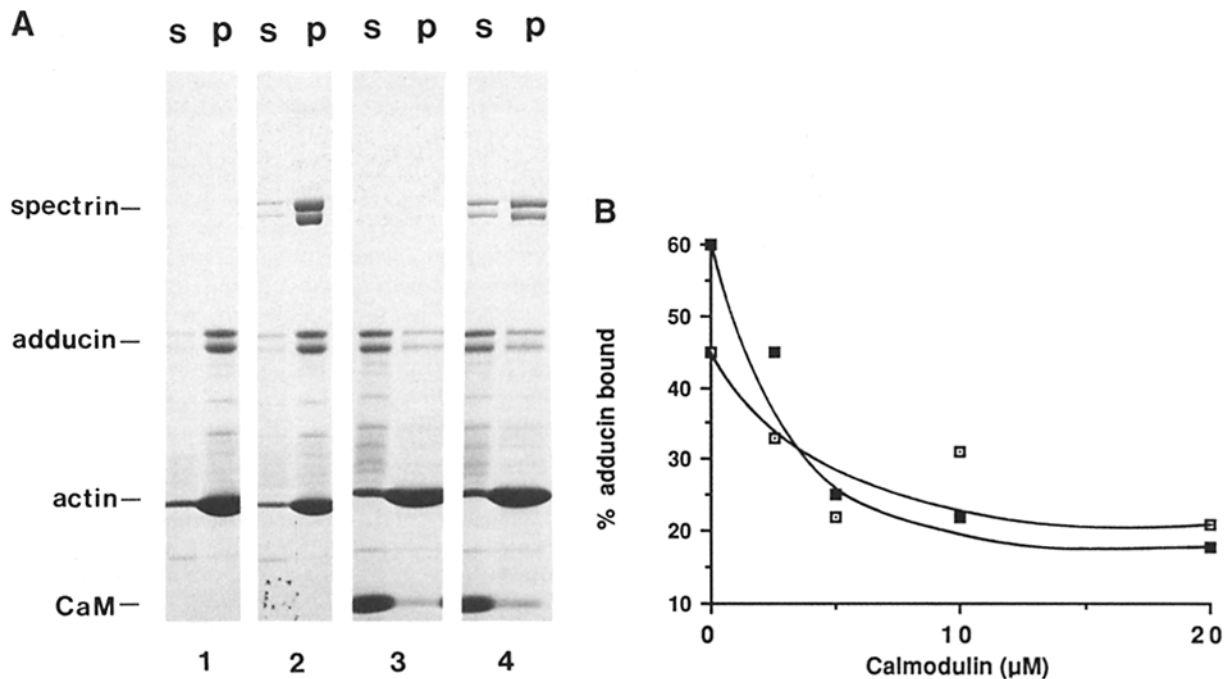


Figure 8 Calmodulin down-regulates adducin binding to actin in a calcium-dependent manner. The effect of increasing amounts of calmodulin on the ability of adducin to bind actin was examined. In the absence of free calcium, calmodulin was without effect (data not shown). The conditions of this experiment were as described in Fig. 1 B. (A) Representative SDS-PAGE analysis of cosedimentation assays in the absence (pairs 1 and 2) or the presence (pairs 3 and 4) of 10 μM calmodulin and 200 μM calcium. Pairs 1 and 3 contained 0.5 μM adducin and 5 μM actin; pairs 2 and 4 also contained 0.25 μM spectrin. (B) Quantitation of the effect of calmodulin on the binding of adducin to actin. Open symbols are from experiments with adducin and actin only. The solid symbols represent experiments with adducin, actin, and spectrin. The calmodulin concentration (final) is as indicated. Note that half-maximal inhibition is achieved near 4 μM calmodulin.

Discussion

This report establishes that adducin, an erythrocyte calmodulin-binding protein: (a) is an actin-binding protein that bundles actin *in vitro* into discrete, ordered arrays of filaments; (b) stimulates spectrin-actin binding independently of protein 4.1; (c) forms a stoichiometric and reversible ternary complex with actin and spectrin; and, (d) forms complexes with actin and actin-spectrin that are down-regulated by calmodulin in a calcium-dependent manner. We also find that the final composition of the ternary complex of adducin, actin, and spectrin is independent of the sequence of assembly of the components; and that protein 4.1, while augmenting the amount of spectrin bound to actin, does not inhibit the interaction of adducin with the actin-spectrin complexes. Adducin is thus similar to other calmodulin-binding molecules, such as caldesmon, that form a class of “flip-flop” proteins that bind actin in a calcium-dependent manner (Sobue et al., 1983).

The literature of actin-binding proteins (e.g., see reviews by Pollard and Cooper, 1986; Korn, 1982; Stossel et al., 1985) describe several macromolecules that will bind actin filaments *in vitro*, but which do not appear to interact with actin *in vivo*. For the most part these proteins typically have basic isoelectric points, and are thought to bind electrostatically and nonspecifically to the acidic actin. Of greater significance are acidic or neutral proteins that bind actin. The isoelectric point of adducin is between 5.8 and 6.3 (pI₀₅ = 5.8–6.1 and pI₀₀ = 6.0–6.3) (data not shown), and it binds actin strongly ($K_d = 283$ nM) in a regulatable fashion. Adducin thus appears to be a bonafide actin-binding protein.

Adducin saturably binds actin at a molar ratio of one adducin to seven actin monomers. Negatively stained complexes examined by electron microscopy reveal that adducin bundles actin into ordered linear arrays with a minimal distance along the filament of 35 nm. This periodicity corresponds almost precisely to the 36-nm repeat of the alpha helical turn of the actin filament (Huxley and Brown, 1967). Since each actin repeat contains 14 monomer units, it is likely that the divalency required for actin bundling is achieved by dimerization of the adducin heterodimer molecule. It is unknown whether the positive cooperativity observed for this binding arises from an allosteric transition within the protein, or is a consequence of enhanced ligand presentation once filament bundling begins. Additional experiments will be required to address these questions.

It is also clear that while adducin can promote actin filament bundling, this action is not required for its stimulation of spectrin-actin binding. Time-course experiments have shown that spectrin binding to adducin-actin complexes remains constant regardless of the degree of bundling (unpublished observations). This would be unlikely if spectrin merely preferred bundled actin as a substrate. In addition, the amount of spectrin that is stimulated to bind to actin by adducin saturates at a unit stoichiometric ratio to adducin. Thus, it appears that adducin has two complementary actions: the cross-linking of actin filaments, and the stimulation of spectrin-actin binding.

Adducin stimulation of spectrin-actin binding is probably fundamentally different from the action of protein 4.1. Protein 4.1 binds most strongly to spectrin and actin together,

although it will bind to spectrin alone but not to actin alone (Tyler et al., 1980; Ohanian et al., 1984; Cohen and Langley, 1984). Conversely, adducin binds strongly to actin, and both adducin and protein 4.1 can simultaneously participate in complex formation. Therefore, it is clear that the two molecules act at different sites on spectrin and actin. We postulate that adducin creates a new or altered binding site on the actin filament, which has enhanced affinity for spectrin, while protein 4.1 may most directly regulate the affinity of the spectrin heterodimer for actin. Additional experiments will be required to test these hypotheses, and to exclude the alternative possibility that both protein 4.1 and adducin simply cross-link spectrin to actin.

These results may have significant implications for our understanding of the dynamics of the erythrocyte membrane skeleton. Previously, it has been assumed that protein 4.1 must mediate all of the spectrin to actin binding in the erythrocyte, since the affinity of spectrin alone for actin at physiologic ionic strength is low. This assumption should now be reexamined, as adducin provides an alternative mechanism by which the spectrin-actin linkage may be modulated. By inference, at least four levels of spectrin-actin stabilization may be envisioned: (a) maximally stabilized by both adducin and protein 4.1, (b) strongly stabilized by protein 4.1 alone, (c) moderately stabilized by adducin alone, and (d) unstabilized. In addition, the moderate binding affinity of adducin for actin under physiologic conditions and its ability to be regulated by calcium, calmodulin, and possibly covalent phosphorylation, suggests that this protein may play a pivotal role in the regulation of cytoskeletal interactions. Such putative heterogeneity in the stability of the membrane skeleton may underlie the observation that the extractability of spectrin can vary significantly between different preparations of erythrocyte membranes (Lux and Gratzer, 1985).

The actin-bundling action of adducin also introduces the potential for additional activities that may be regulated in the erythrocyte membrane skeleton. The ability to bundle actin filaments in a calcium and calmodulin regulated way under physiologic conditions has not previously been recognized in any of the other erythrocyte cytoskeletal proteins, although an actin-bundling role has been ascribed to protein 4.9 (Siegel and Branton, 1985). Bundles of actin filaments are not generally thought to exist in the mature erythrocyte. However, the exact state of actin in this cell remains somewhat controversial. Most studies have estimated that actin is found predominantly in the form of short filaments of ~26–41 nm, composed of ~10–17 actin monomers (Lin and Lin, 1979; Pinder and Gratzer, 1983; Byers and Branton, 1985; Shen et al., 1986). Based on these determinations, it has been estimated that there are 30,000 such short actin filaments per cell. The abundance of adducin has been estimated at 30,000 copies per cell (Gardner and Bennett, 1986). Thus, on the average, each protomeric actin filament contains enough adducin to promote filament cross-linking. Under certain conditions actin filaments larger than these protofilaments have been observed in erythrocytes (Shen et al., 1986; Atkinson et al., 1982; Weinstein et al., 1986; Schanus et al., 1985; Liu et al., 1987). Isolated oligomeric complexes from the membrane skeleton contain thicker actin filaments than those extracted with 150 mM KCl (Shen et al., 1986), and the ability of these complexes to support further actin polymerization is eliminated at KCl concentrations >50 mM (Fowler and

Taylor, 1980). These salt conditions correspond to those that reduce the affinity of adducin for actin (Fig. 8), but not the ability of proteins 4.1 and 4.9 to interact with spectrin and actin (Cohen and Langley, 1984; Siegel and Branton, 1985). In addition, the most recent studies of stretched membrane skeletal preparations have also noted a heterogeneity in the size and shape of the actin-containing junctional complexes (Liu et al., 1987). Thus, while the evidence remains circumstantial, the existence of tightly linked actin complexes in the native erythrocyte cytoskeleton cannot be excluded.

Other roles for adducin are possible. It may simply enhance the stability of the actin filaments within the spectrin-actin complex. It may serve as a membrane anchor for actin, since adducin has been shown to bind phosphatidylserine. Interestingly, this binding appears to be under protein phosphorylation control (Wolfe and Sayhoun, 1986). Such an actin-membrane linking role may also serve to recruit filamentous actin or spectrin-actin complexes to the membrane during erythroid development. In this regard it is interesting that the reticulocytes of acetylphenylhydrazine-treated rats have larger membrane cytoskeletal complexes than mature erythrocytes (80–140 vs. 38–50 nm) (Liu et al., 1987). The answers to these questions must await further investigation. However, it is likely that whatever the actual role of adducin, it will be of general importance since analogues of adducin exist in other tissues (Palfrey and Waseem, 1985; Gardner and Bennett, 1986; Wolfe and Sayhoun, 1986; Mische, S. M., and J. S. Morrow, unpublished observations).

It should also be noted that an abstract by Gardner and Bennett (1987) describing the calmodulin-dependent ability of adducin to stimulate spectrin-actin binding appeared coincident with our own abstract describing this study (Mische et al., 1987). Their independent results are in basic agreement with the findings reported here with respect to the ability of adducin to stimulate spectrin-actin binding. However, they find no direct interaction between actin and adducin, and suggest that the binding of adducin to spectrin-actin requires preformed spectrin-actin complexes. They also find a direct competition between protein 4.1 and adducin. We do not understand the reasons for these discrepancies, but note that they used porcine spectrin in their study.

We thank Ms. Carol Cianci for expert technical assistance.

This work was supported by grants HL28560 (J. S. Morrow) and AM25387 (M. S. Mooseker) from the National Institutes of Health, and by a graduate student fellowship (S. M. Mische) from the Joshua Macy Foundation.

Received for publication 18 May 1987 and in revised form 18 July 1987.

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