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ERYTHROCYTE ACTIN AND SPECTRIN

INTERACTIONS WITH MUSCLE CONTRACTILE AND REGULATORY PROTEINS

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Summary

Actin and spectrin were isolated from washed red blood cell membranes. Spectrin bound and polymerized erythrocyte actin in the absence of potassium. Spectrin coated onto polystyrene latex particles bound 8—9 mol of erythrocyte actin per mol of spectrin when actin was in its depolymerized state. Spectrin enhanced the interaction of erythrocyte actin with muscle myosin as manifested by changes in Mg^{2+}-ATPase activity. A similar enhancement also was observed with muscle α-actinin while muscle tropomyosin abolished these effects. The data suggest that spectrin may play the role of polymerizing factor as well as the anchoring site for erythrocyte actin just as α-actinin is the anchoring site for actin filaments in muscle and other non-muscle cells.

Introduction

The protein components of erythrocyte membrane, including the material cytoplasmically adjacent to it, have been the subjects of extensive study [1,2] but the interactivity between these components has not been fully defined. Actin filaments have been found in many cells [3], and in erythrocytes actin is reportedly attached to the cytoplasmic side of the membrane [4]. This position resembles that reported for brain synaptosomal membranes [5, 6] where actin was found associated with a regulatory protein system modulating the release of a neurotransmitter [7].

In the membranes of erythrocytes, brain neuronal endings and other cells, the mode of attachment of actin molecules, monomeric or polymerized, is not understood. In muscle, however, actin filaments anchor to the Z-bands where

available evidence suggests that α-actinin is a major component [8]. α-Actinin-like proteins have been sought in non-muscle cells [9—13], but other proteins have emerged as alternate anchors for actin. These are spectrin in erythrocytes [4] and an actin-binding protein [14]. Spectrin is abundant in erythrocytes [15,16,4] and under certain conditions it is extracted as a complex with erythrocyte actin [17,4].

In this report we describe some properties of the interaction between spectrin and erythrocyte actin requiring no energy release from ATP and the interaction between erythrocyte actin and muscle myosin, accompanied by hydrolysis of ATP, that is modulated by spectrin and α-actinin, and inhibited by tropomyosin.

Experimental procedures

Materials. ATP was purchased from Sigma Co. Bovine serum albumin was purchased from Pentex Co., Ill. All other reagents used were of analytical grade one. [γ-^{32}P]ATP was purchased from New England Nuclear Co. Specific activity was 27 Ci/mmol. Radioactive ATP was used within 2 weeks of delivery. Dithiothreitol was purchased from Sigma Co. Polystyrene (Lytron) particles were obtained from Monsanto [7,18].

Protein preparation. Myosin, actin, tropomyosin, troponin, and α-actinin were prepared from fresh rabbit or dog back muscle following established procedures as previously described [19]. Spectrin was prepared from human erythrocyte membrane according to a method by Marchesi [16]. Erythrocyte actin was prepared from acetone-dried powder of human red blood cell membranes as follows: freshly drawn whole blood collected in citric acid/phosphate buffer and dextrose anti-coagulant (C.P.D. Hayland Laboratory) was centrifuged at 5000 rev./min in a Sorvall RC-3 centrifuge at 4°C. Plasma was discarded and the erythrocytes washed four times with iced 10 mM sodium phosphate in 0.154 M NaCl (pH 7.5) buffer (phosphate/saline buffer). After each wash, the upper white cell buffy coat was discarded. Erythrocytes were hemolyzed with 20—30 vols. of iced 10 mM sodium phosphate buffer containing 1 mM dithiothreitol. Erythrocyte membranes were cold-centrifuged at 40 000 × *g* for 15 min and washed 4—6 times with 10 vols. phosphate buffer containing 30 mM KCl (potassium phosphate buffer) to minimize solubilization of erythrocyte actin. After the fourth or fifth wash, membrane pellets were pink and additional washes did not appreciably clarify the supernatant. These membranes were stirred vigorously twice at 4°C with 3—5 vols. of iced acetone, centrifuging each time in glass Pyrex tubes at 12 000 × *g* for 10 min, and the acetone-treated pellets were spread on glass plates and dried at 4°C for 24—72 h. The dried material was extracted at 4°C for 72 h with 25—30 vols. of 0.05 M Tris-HCl buffer (pH 7.9) containing 0.1 mM ATP and 1 mM dithiothreitol. The suspension was initially dispersed with a Dounce homogenizer and then stired continuously. After the extraction period, the mixture was centrifuged at 35 000 × *g* for 15 min at 4°C. The supernatant was collected, clarified by centrifugation at 100 000 × *g* for 20 min, adjusted initially to 0.1 M KCl and 1 mM magnesium and allowed to stand for 48—72 h. 12 h before the end of the polymerization period, the KCl concentrate was raised to 0.6 M to assure

complete polymerization of actin [20]. After centrifugation at 105 000 × *g* at 4°C for 3 h, translucent pellets were resuspended in 10 vols. of a solution containing 0.5 mM ATP, 0.05 M Tris-HCl (pH 7.5), and 1 mM dithiothreitol. This suspension was dialyzed for 24—48 h against the same solution and further purified by gel filtration through Sephadex G-100 using the same buffer as for dialysis. The actin peak was repolymerized with 0.1 M KCl and 0.1 mM magnesium as mentioned above, depolymerized, and either stored at 4°C for use within 72 h or, after addition of 10% (w/v) crystalline sucrose, stored at —20°C for up to 3 weeks.

Sodium dodecyl sulfate (SDS)-disc acrylamide electrophoresis. Gel electrophoresis was performed at room temperature (Buchler Instruments, Inc., Fort Lee, N.J.) on single, polyacrylamide gels containing 10 mM phosphate buffer (pH 7.0) and 1 g sodium dodecyl sulfate/l. Proteins were dissolved in appropriate buffers containing 0.2% sodium dodecyl sulfate and samples containing 50—125 μg protein were layered on top of acrylamide gels with 1% 2-mercaptoethanol and Bromophenol Blue (tracking dye). A current of 2 mA/gel was applied for 15—30 min to concentrate protein as a thin layer on the gel-entry surface. This was followed by a current of 10 mA/gel until the tracking dye reached the bottom of the gels or was allowed to elute from the gels. Gels were fixed for 120 min at 37°C in 50% methanol, 10% acetic acid and 40% distilled water and stained for 90 min with 0.5% Coomassie Blue dissolved in 5% methanol/7% acetic acid. Gels were destained by diffusion for 72 h in 7% acetic acid and 5% methanol. Stained gels were scanned at 550 nm in a Joel automatic recording densitometer. Sodium dodecyl sulfate-disc acrylamide gel electrophoresis of proteins adsorbed by Lytron particles were processed as indicated elsewhere [19].

Determination of ATPase activity. ATPase activities of proteins were determined by the release of inorganic phosphate ($^{32}P_i$) from [γ-^{32}P]ATP [19]. 0.5-ml aliquots of the 1-butanol layer were counted in a scintillation spectrophotometer. ATPase assays were performed in volumes of 5 ml. Proteins and reagents were initially mixed in a volume of 0.9 ml, and then incubated at either room temperature or 37.5°C for 15 min when indicated. The amounts of actin from muscle or erythrocytes used were initially 0.5—1 mg/ml, above the critical concentrations required for polymerization. The solution was diluted to 5 ml with 50 mM Tris-HCl (pH 7.5), and then incubated with the mixture of radioactive ATP at 37.5°C for 10 min. For assay purposes, the quantity of proteins used, except where otherwise indicated, were: myosin, 0.01 mg/ml; erythrocyte actin, 0.01—0.02 mg/ml; spectrin, α-actinin and tropomyosin, each 0.01—0.02 mg/ml. The reaction was initiated by addition of 0.1 ml 5 mM [γ-^{32}P]ATP allowed to proceed for 10 min. Aliquots of 1 ml were removed at various time intervals. Reactions were stopped by addition of 0.4 ml 20% trichloroacetic acid. ATPase was estimated as the difference between P_i at zero time and P_i at the time of removal of an aliquot and addition of trichloroacetic acid.

Protein concentration. Protein concentrations were measured by the method of Lowry et al. [21], using bovine serum albumin as the standard.

Coating of Lytron particles with proteins. A general procedure reported previously [18] was used. The polystyrene (Lytron) particles (uniform, spheri-

cal) were prepared commercially by catalytic emulsion polymerization of styrene in the presence of a surface active agent. The dimensions of the particles were 2500 Å diameter and 1964×104 Å^2 surface. There were 1.19×10^{11} Lytron particles in 1 mg of dried material and the surface charge was slightly charged with negative ions provided by carboxyl radicals. The binding kinetics were calculated as previously reported [18]. Protein concentrations were adjusted to 0.75—1.25 mg/ml. Small volumes of a Lytron particle suspension (10 mg/ml) were added to protein solutions in appropriate buffers and these mixtures were gently agitated at room temperature. Formation of the first protein coat was obtained within 3—5 min. A large excess of protein molecules relative to Lytron particles was used to assure complete surface coverage of Lytron particles. Such protein-coated particles were used for complexing with other proteins. Lytron particles were sedimented at $35\,000 \times g$ for 15 min. When complexes with other proteins were sought, the Lytron particles, after centrifugation, were resuspended in the appropriate second protein solution and allowed to react for 30 min. Protein-coated Lytron particles were sedimented as indicated above. Supernatant fluid was decanted and used for protein determination. The amount of protein adsorbed in each instance was estimated from the difference between the initial protein concentration and the remainder in solution after adsorption.

Electron microscope studies. Negative staining: Arrowhead formation on actin filaments was obtained by incubation of a heavy meromyosin preparation from rabbit muscle [4] (0.1—0.2 mg/ml) with an equal amount of actin in 0.1 M KCl, 0.1 mM $MgCl_2$, and 0.05% NaN_3 for 15 min at 37°C and 16 h at 4°C. Spectrin, at equal volumes and concentrations of actin, was reacted with actin for 15 min before addition of heavy meromyosin to the mixture. 10 ml of protein solution to be examined was placed on top of a Formvar carbon-coated grid. The solution was left on the grid for 30—60 s, blotted, and then stained with a drop of 1.0% unbuffered uranyl acetate for 1—2 min. Excess stain was removed with filter paper.

For transmission electron microscopy, erythrocyte ghosts and mixtures of ghosts with actin and heavy meromyosin were used. Arrowhead formation was observed as described above followed by centrifugation of ghosts at $39\,000 \times g$ for 15 min at 4°C. Pellets were fixed with 1.5% gluteraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and post-fixed in 1% OsO_4. Samples were rinsed, stained and embedded in Epon. Thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and viewed under a Joel 100-B electronmicroscope at 80 kV [19].

Results

Erythrocyte actin extraction

Human erythrocyte membranes are easily obtainable after hemolysis of erythrocytes in low ionic strength (10 mM) phosphate buffers. To prevent loss of actin by solubilization in the low ionic strength medium at which the membranes are treated after hemolysis, membranes were washed with phosphate buffers containing 30 mM KCl. A small amount of residual hemoglobin in these membrane preparations was difficult to remove without decreasing the yield of

actin. One unit (450 ml) of whole, human blood yielded approx. 50 g of wet-packed membranes after the final centrifugation. The light brown, dry residue obtained after acetone extraction (1.5—2.5 g/unit whole blood) yielded 15—20 mg of partially purified actin. Erythrocyte actin was allowed to polymerize 72 h at 4°C by adjusting KCl concentrations first to 0.1 M and finally to 0.6 M to assure full polymerization. A translucent, slightly yellow pellet was obtained after high-speed centrifugation. On sodium dodecyl sulfate-disc acrylamide electrophoresis this material contained a protein with a molecular weight of 45 000, a 38 000 molecular weight band and hemoglobin monomers of 14 000 molecular weight (Fig. 1, gel A). Two cycles of polymerization/depolymeriza-

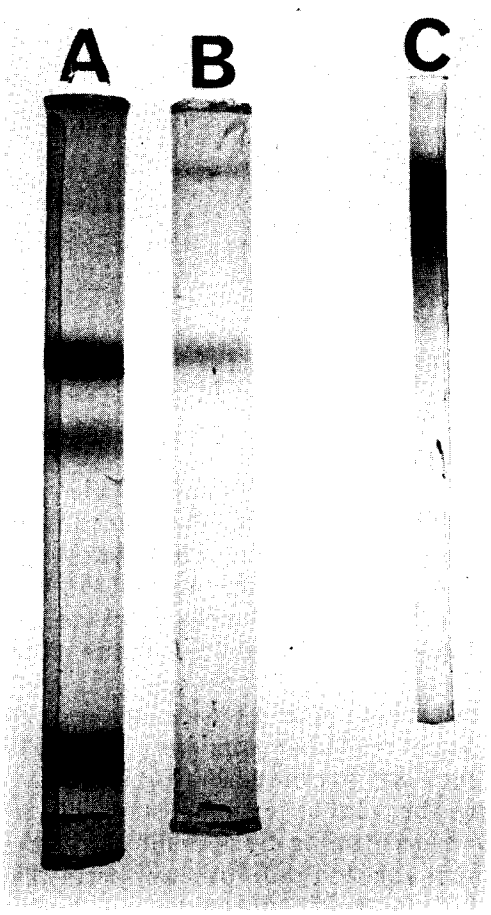
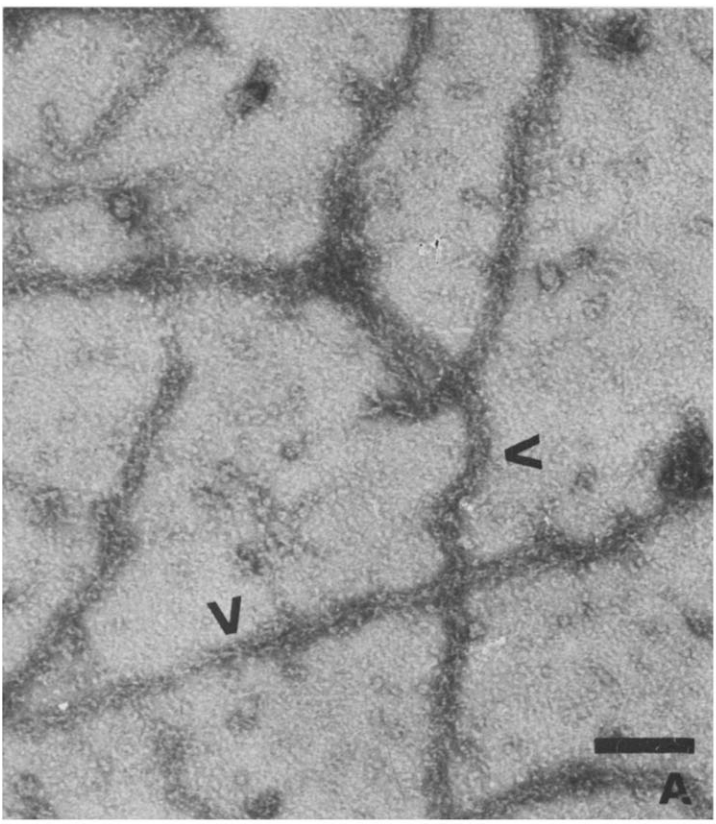


Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of erythrocyte ghost proteins. Electrophoresis performed on single gels of 7.5% polyacrylamide. Gel A is a 15 mm thick gel containing 250 μg of partially purified erythrocyte actin. The 45 000 molecular weight band is actin. The band below is a 38 000 molecular weight component, not yet identified. The lower band is hemoglobin monomer, molecular weight 15 500. Gel B is erythrocyte actin (100 μg) obtained by two cycles of polymerization and depolymerization after gel filtration through Sephadex G-100. Erythrocyte actin is devoid of the 38 000 molecular weight component and hemoglobin. A minor contaminant (=150 000 mol. wt.) faintly present in gel A has been partially concentrated in the final purification step of erythrocyte actin. Gel C is 4% acrylamide gel/0.1% sodium dodecyl sulfate of spectrin (50 μg) prepared as described in the text.



tion after passage of the extract through Sephadex G-100 assured adequate purity (Fig. 1, gel B). The final product shows the 45 000 molecular weight protein and a small amount of another polypeptide of higher molecular weight (approx. 150 000 molecular weight). The yield of erythrocyte actin, low because of actin lost by solubilization during hemolysis, membrane washes and polymerization/depolymerization procedures, was 0.1—0.2% of the total erythrocyte membrane protein.

Ultrastructurally, negatively stained erythrocyte actin preparations showed filaments identical to those of muscle actin. The filaments were of similar width and could be arrowhead-decorated by heavy meromyosin (Fig. 2). That a fraction of erythrocyte actin is lost by the various washing steps of the erythrocyte membranes was verified by concentrating by lyophilization the fluid of a third wash. The resulting protein solution did not show actin filaments when allowed to polymerize with 0.1 mM Mg^{2+}, 0.1 and 0.6 M KCl with or without heavy meromyosin. However, when this solution was incubated with a small amount of highly washed clear membranes of a separate erythrocyte ghost preparation (0.2 ml of packed ghosts/4 ml of 20 times concentrated wash solution) in the presence of 0.1 M KCl and 0.1 mM $MgCl_2$. Microfilaments identified as actin by heavy meromyosin arrowhead decoration (Fig. 3) were sedimented attached to membranes at 39 000 $\times g$ for 15 min. Microfilaments did not form if membranes were left out.

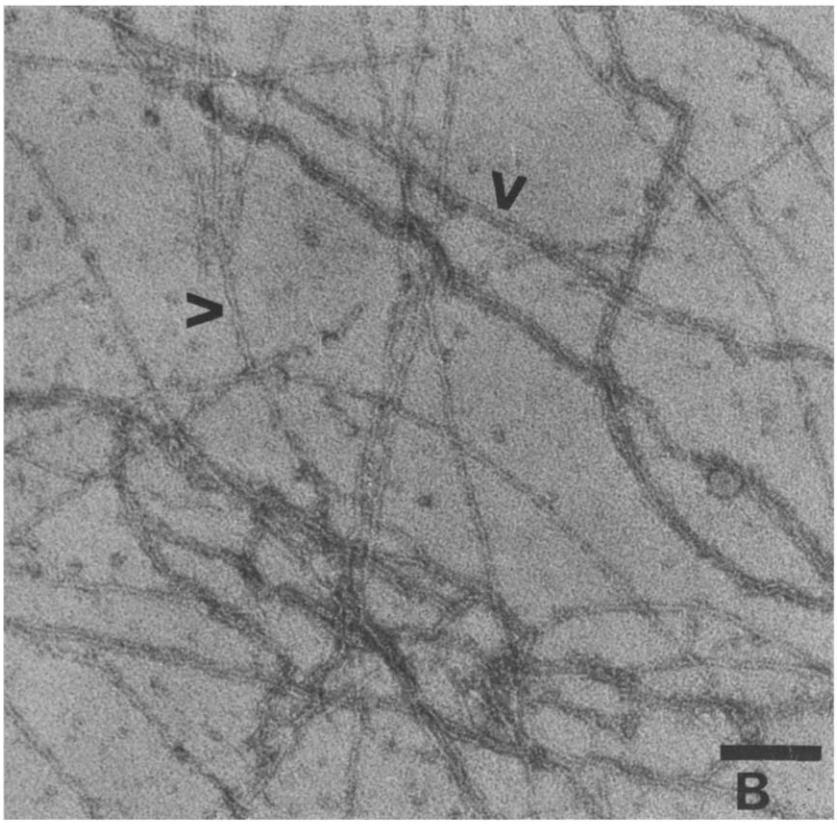


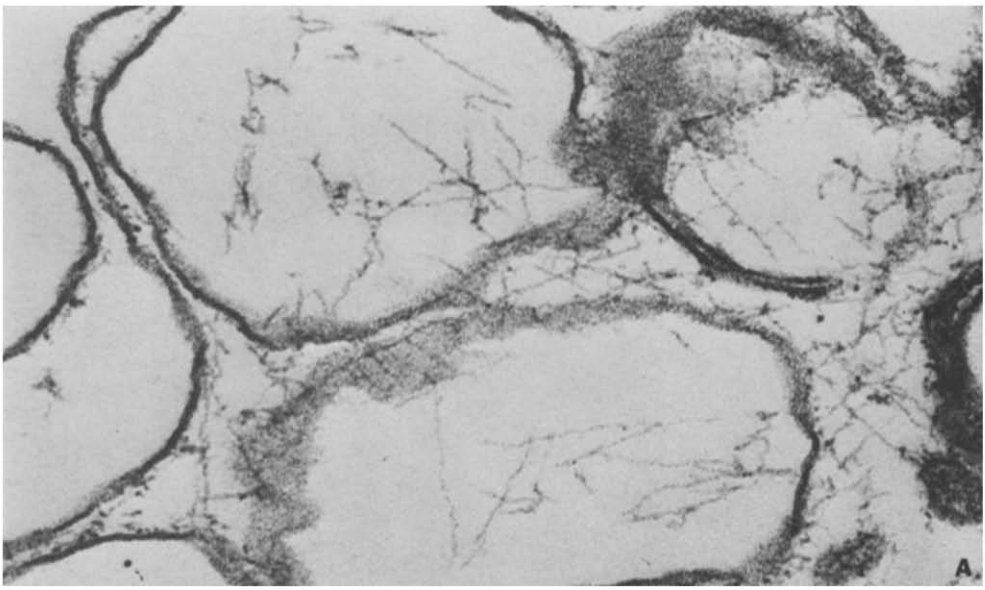
Fig. 2. Negative staining of erythrocyte proteins. (A) Heavy meromyosin-decorated erythrocyte actin filaments. The erythrocyte actin was allowed to react with heavy meromyosin and characteristic arrowhead decorations were obtained (120 000X). (B) Filaments of erythrocyte actin obtained after the first cycle of extraction from acetone powder dry ghosts (120 000X). Solid bar = 0.1 μm.

Myosin-ATPase stimulation by erythrocyte actin

Skeletal rabbit muscle myosin was stimulated by erythrocyte actin. Preincubation of erythrocyte actin with 0.1 M KCl for 15 min at room temperature before incorporation of myosin into the system was found necessary to render ATPase activation by magnesium. ATPase activity was linear up to 10 min of incubation (Fig. 4). Myosin activation varied slightly with different erythrocyte actin preparations. The preparation of erythrocyte actin displayed myosin activation for up to 3—4 days of storage at 4°C in 1 mM dithiothreitol and for at least 8 weeks when stored frozen at −20°C in 10% sucrose. The K_{app} of myosin-Mg^{2+}-ATPase activation was determined as $0.66 \cdot 10^{-6}$ M. Using heavy meromyosin, a similar value was recently reported by Sheetz et al. [17].

Effect of temperature

The optimal temperature for erythrocyte actin activation of muscle myosin-ATPase was 37.5°C (Fig. 5). At 40, 25 and 12.5°C, ATPase activity was reduced. When a rabbit muscle protein system of myosin and actin was tested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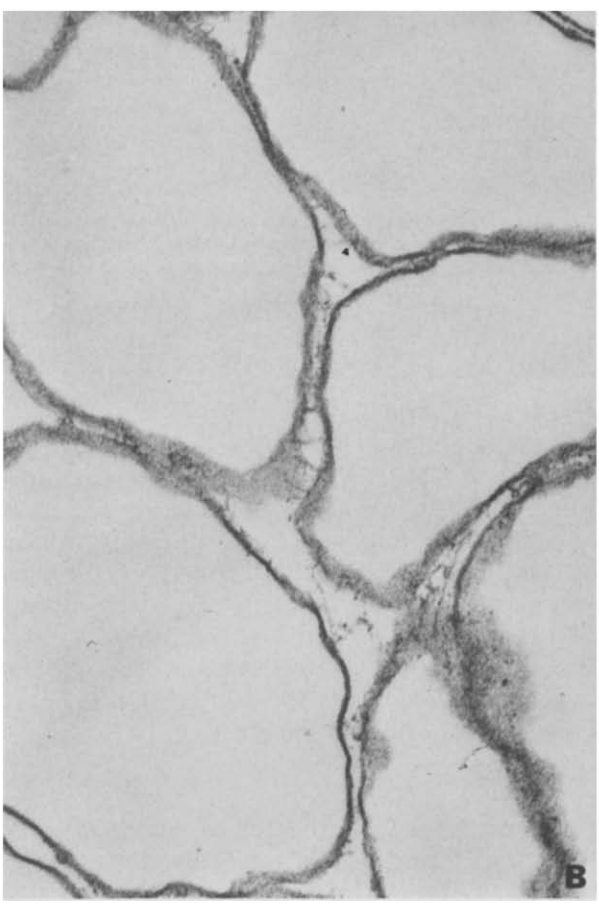
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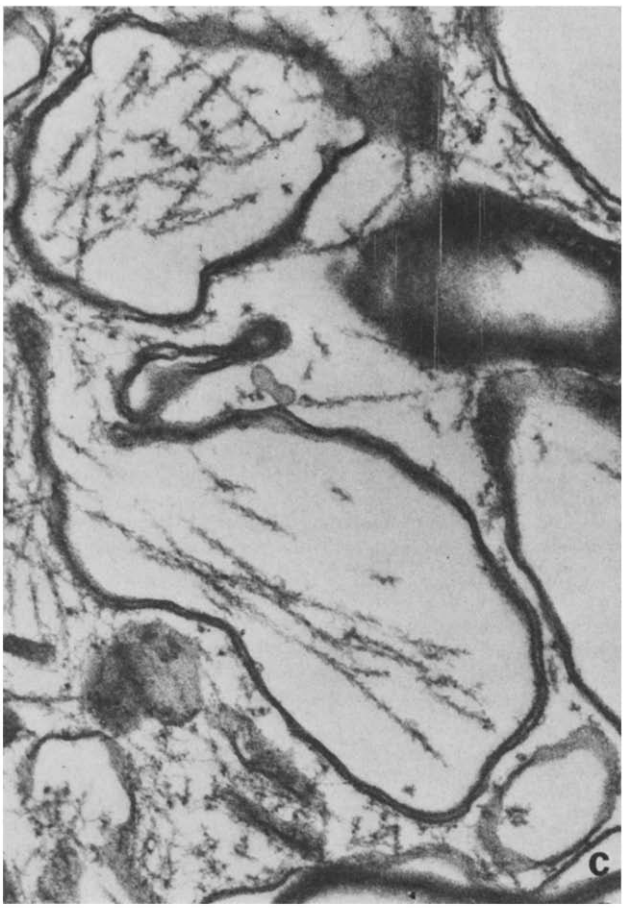
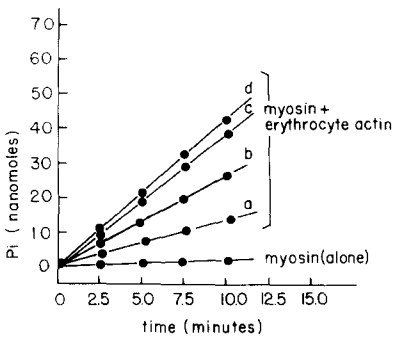


Fig. 3. Transmission electron micrographs of: (A) An erythrocyte membrane preparation incubated for 15 min at 37.5°C and then 16 h at 4°C with a concentrated third-wash solution of erythrocyte membranes in the presence of 0.1 M KCl, 0.1 mM Mg^{2+}, and 0.1 mM ATP (pH 7.5). Actin solubilized in the wash solution is polymerized and attached to segments of membranes showing loss of membrane bilayer integrity. The filaments are short and not well preserved due to the fixation techniques and to the absence of protective agents such as tropomyosin or heavy meromyosin (75 000X). (B) An erythrocyte membrane preparation control processed as in A, but in the absence of concentrated third-wash solution erythrocyte membranes (76 000X). (C) Same preparation as described in A, but with the erythrocyte actin filaments decorated with heavy meromyosin. Heavy meromyosin-decorated microfilaments are seen to have acquired linear rigidity and seemed better preserved than those shown in A. They are attached to multiple membrane sites, especially in areas where the membrane integrity is not well preserved (80 000X).

for comparison, maximum activation by muscle actin was observed at 25°C and no significant difference was noted when the temperature was raised to 37°C (Fig. 5). In all instances, actin from either muscle or erythrocytes was allowed to polymerize for 15 min at 37°C before incorporation of myosin into the medium and adjustment to the various temperatures tested for enzymatic determinations.



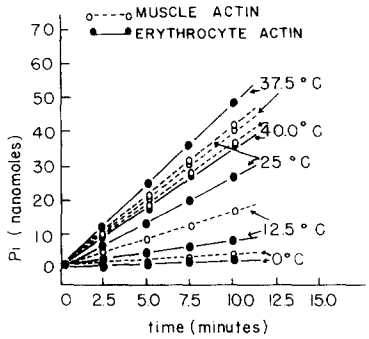


Fig. 4. Erythrocyte actin activation of muscle myosin-Mg^{2+}-ATPase. All contractile or structural proteins in solution were treated in identical fashion and Mg^{2+}-ATPase activity was assayed in volumes of 5 ml. Proteins and reagents were initially mixed in a volume of 0.9 ml, incubated at 37°C for 15 min and then diluted to 5 ml with 50 mM Tris-HCl (pH 7.5) and 0.1 M KCl. At zero time, 0.1 ml of 5 mM [γ-^{32}P]ATP and 10 mM Mg^{2+} were added and mixture was incubated at 37°C for 10 min. Aliquots were removed at periods indicated. Activation was linear with time up to 10 min incubation. Actin/myosin molar ratios: a, 0.1; b, 0.5; c, 1; d, 2. The double reciprocal plot of rabbit muscle myosin-Mg^{2+}-ATPase activation by actin was constructed from data shown in this figure. The K_{app} of activation of erythrocyte actin on muscle myosin was calculated as $0.66 \cdot 10^{-6}$ M.

Fig. 5. Temperature dependence of activation of muscle myosin-Mg^{2+}-ATPase by erythrocyte and muscle actin. Maximum activation for erythrocyte and muscle actin was obtained at 37.5 and 25°C, respectively. In all instances, actins were allowed to polymerize for 15 min at 37°C before addition to myosin and ATP to the medium and adjustment of the temperature for enzymatic hydrolysis. Ionic conditions are the same as shown in the legend for Fig. 4.

Spectrin properties

On 4% polyacrylamide-SDS electrophoresis, spectrin isolated from erythrocyte membranes showed two major polypeptide bands of high molecular weights (Fig. 1, gel C). A small amount of contaminating proteins also was observed. Densitometric scans indicated that the contaminant protein did not exceed 5% of the total protein content. It was not determined if the contaminating proteins were components of the membrane protein population or fragments from spectrin, resulting from proteolytic activity. Spectrin had no detectable ATPase activity. Although lack of ATPase activity could be attributable to denaturation during its purification, precautions were taken to: (a) use fresh erythrocytes; (b) isolate spectrin at low temperature throughout the procedure; (c) incorporate 5 mM dithiothreitol at all steps; and (d) test spectrin's biological activity immediately after the final purification step.

Adsorption of spectrin by Lytron particles

Spectrin in solution was allowed to bind to increasing amounts of Lytron particles. Adsorption was carried out in the presence of 0.02 M Tris-HCl buffer (pH 7.5). The maximum quantity of spectrin bound by 1 mg Lytron particles was 0.075—0.081 mg (Table I). Assuming $1.19 \cdot 10^{11}$ particles/mg Lytron and an average molecular weight for spectrin of 450 000, approx. 750 molecules could be bound by each Lytron particle. Erythrocyte-actin binding to Lytron particles was similar to that reported previously for muscle actin [18]. Binding of spectrin to Lytron particles allowed evaluation of the interaction of spectrin

TABLE I

SEQUENTIAL BINDING OF SPECTRIN AND ERYTHROCYTE ACTIN BY LYTRON PARTICLES

Protein concentrations were adjusted to 0.75—1.25 mg per ml. Small volumes of Lytron particles were added to solutions of protein, appropriately buffered and the mixtures were gently agitated at room temperature for the formation of the first protein coat. Lytron particles were sedimented by centrifugation and the pellets were resuspended in appropriate second protein solution for 30 min. Protein-coated Lytron particles were sedimented as indicated in text. The amount of protein adsorbed in every instance was estimated from the difference between the initial protein concentration and the remainder in solution after adsorption. In all instances, care was taken to produce Lytron particles with the first coat of protein completely saturating the surfaces of Lytron particles. (See Experimental Procedures, and ref. 19 for further details).

1st Protein coat (mg protein bound/mg Lytron particle)	2nd Protein coat (mg protein bound/mg Lytron particle)	Molecular ratio of erythrocyte actin/spectrin
0.075 spectrin	0.065 erythrocyte G-actin	8.6
0.081 spectrin	0.073 erythrocyte G-actin	9.0
0.068 spectrin	0.060 erythrocyte G-actin	8.8
0.085 erythrocyte G-actin	0.006 spectrin	—
0.075 erythrocyte G-actin	0.008 spectrin	—
0.090 erythrocyte G-actin	0.007 spectrin	—
0.212 erythrocyte F-actin	0.009 spectrin	—
0.255 erythrocyte F-actin	0.010 spectrin	—
0.075 spectrin	0.005 erythrocyte F-actin	—
0.081 spectrin	0.009 erythrocyte F-actin	—
0.068 spectrin	0.008 erythrocyte F-actin	—

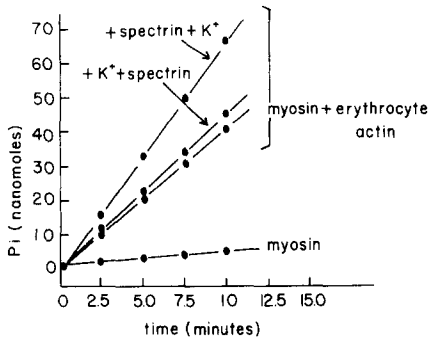
with erythrocyte actin in the globular or fibrillar state. Binding was observed between Lytron-bound spectrin and erythrocyte G-actin (Table I). Between 8 and 9 mol of actin (calculated as the 45 000 monomer) were bound by spectrin. Lytron-bound spectrin did not bind erythrocyte actin when actin was in polymerized form (Table I). Furthermore, little or no binding of spectrin from solution occurred when Lytron-bound erythrocyte G- or F-actin was used. Under similar experimental conditions myosin has been reported to bind F-actin and vice versa [18]. Various proteins forming monolayers have been found to bind specifically proteins from solution forming complexes known to exist naturally when these proteins are extracted from their various sources [7].

Enzymatic determinations

Spectrin enhanced the activation of myosin-Mg^{2+}-ATPase by erythrocyte actin (Fig. 6). For these determinations, spectrin was allowed to react with erythrocyte actin for 15 min in 0.1 M KCl. Spectrin did not influence the activating effect of erythrocyte actin on myosin when added after erythrocyte actin had been in a medium of 0.1 M KCl for 15 min. Spectrin showed its effect over a wide molar ratio of spectrin/actin that ranged from 0.1 to 2.0. For most determinations, a molar ratio of spectrin/actin of 0.2 was used.

Electronmicroscopy

Ultrastructurally, spectrin appeared as elongated, irregularly curled or twisted, asymmetrical molecules and under conditions that favoured polymeri-



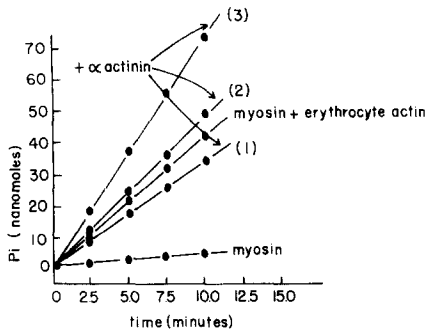


Fig. 6. Effect of spectrin on the ATPase activity of myosin activated by erythrocyte actin. The ionic conditions are the same as those described in the legend for Fig. 4. Spectrin, as indicated in the figure, was added before and after erythrocyte actin had been allowed to react with myosin in the presence of 0.1 M KCl. The effect of spectrin was evident 2.5 min after addition of Mg^{2+}-ATP to the medium and it was linear up to 10 min of incubation.

Fig. 7. Effect of α-actinin on the activation by erythrocyte actin of myosin-Mg^{2+}-ATPase. All contractile proteins in solution were treated in identical fashion as that described in the legend for Fig. 4. α-Actinin was added to erythrocyte actin 15 min prior to the addition of myosin and Mg^{2+}-ATP. The effect of α-actinin was evident as early as 2.5 min of incubation and was linear with time up to 10 min of incubation. α-Actinin/actin ratio: 1, 0.1—0.5; 2, 2.0; 3, 1.0.

zation [17], no aggregates of filamentous polymers were obtained. However, in the presence of erythrocyte G-actin, spectrin induced actin polymerization even in the absence of added potassium (Fig. 3). A similar effect of spectrin, but on muscle actin, has been reported recently [22].

Effect of muscle α-actinin on erythrocyte actin-spectrin interaction

Purified α-actinin preparations were characterized as reported previously [11,19]. The preparations of α-actinin were free of either actin or myosin and showed neither calcium nor magnesium ATPase activity at low or high concentrations of potassium. Although α-actinin did not alter myosin Ca^{2+}-ATPase activity, it displayed an effect on the Mg^{2+}-ATPase when tested with erythrocyte actin. At a molecular ratio of α-actinin to actin of one, α-actinin sharply potentiated Mg^{2+}-ATPase activity. However, at ratios higher or lower than one, Mg^{2+}-ATPase activity was not significantly affected (Fig. 7). The effect of α-actinin was observed as early as 2.5 min after initiation of ATP hydrolysis and this effect was proportionately larger at longer periods of incubation.

Effect of tropomyosin and/or troponin on the interaction of erythrocyte actin with muscle myosin

The addition of a complex of troponin and tropomyosin resulted in a modulation of the interaction of erythrocyte actin with myosin. When the troponin · tropomyosin complex was allowed to react with polymerizing erythrocyte actin, the Mg^{2+}-ATPase of myosin became Ca^{2+} sensitive. Addition of ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA) to this system resulted in a decreased Mg^{2+}-ATPase. Calcium sensitivity conferred by troponin · tropomyosin was 65%. In the absence of troponin · tropomyosin, erythrocyte actin-myosin-Mg^{2+}-ATPase was Ca^{2+} insensitive (Table II). Eryth-

TABLE II

Ca^{2+} SENSITIVITY CONFERRED BY A MUSCLE TROPOMYOSIN · TROPONIN COMPLEX ON THE Mg^{2+}-ATPase OF MYOSIN ACTIVATED BY ERYTHROCYTE ACTIN

All contractile proteins in solution were treated in identical fashion, and Mg^{2+}-ATPase activity was assayed in volumes of 5 ml. Proteins and reagents were initially mixed in a volume of 0.9 ml, incubated at room temperature for 10 min and diluted to 5 ml with 50 mM Tris-HCl (pH 7.5) and 0.1 M KCl. At zero time, 0.1 ml of 5 mM [γ-^{32}P]ATP/10 mM Mg^{2+} was added and mixtures were incubated at 37°C for 10 min. The quantities of protein are indicated in text. Values shown are the mean ± S.D. of triplicate determinations for a given protein preparation.

Protein	Mg^{2+}-ATPase activity (μmol/mg protein per min)	
	— EGTA	+ EGTA
Erythrocyte actin	—	—
Muscle myosin	0.01 ± 0.005	0.01 ± 0.005
Muscle myosin + erythrocyte actin	0.360 ± 0.020	0.340 ± 0.019
Muscle myosin + erythrocyte actin + tropomyosin · troponin	0.342 ± 0.021	0.120 ± 0.030

rocyte actin at the final step of the fractionation procedure showed no protein bands with mobilities similar to those of troponin from muscle, brain [7] or platelets [23,24].

Tropomyosin itself abolished both spectrin and α-actinin effects on the interaction of erythrocyte actin with muscle myosin. The inhibitory effects were maximal at the molar ratio of tropomyosin/actin of one. Tropomyosin was effective when added even 1—2 min before ATP, that is, 12—14 min after erythrocyte actin had been allowed to polymerize in the presence of 0.1 M KCl and 0.1 mM $MgCl_2$ (Fig. 8).

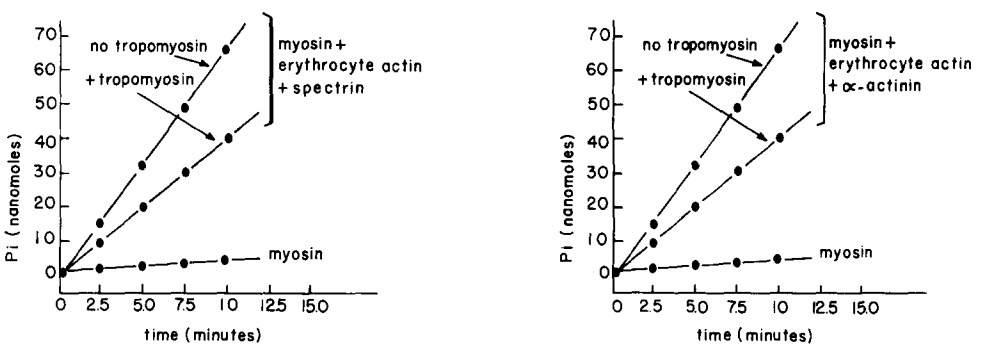


Fig. 8. Effect of tropomyosin on the activation by spectrin (A), and α-actinin (B) of the interaction of erythrocyte actin and muscle myosin. Ionic conditions were the same as those described in Fig. 4. Tropomyosin at equimolar concentrations to that of actin, was added after actin had been allowed to interact with myosin in the presence of spectrin (A), or α-actinin (B), and allowed to react for 5—10 min prior to addition of ATP to the medium. Tropomyosin in both instances abolished the activating effect of spectrin or α-actinin on the interaction of erythrocyte actin with muscle myosin. The effect was linear up to 10 min of incubation.

Discussion

By adapting procedures used for the extraction of actin from muscle, a protein resembling actin from muscle is extracted from erythrocyte membranes [19,17,4]. This protein-activated myosin-Mg^{2+}-ATPase interacted with troponin and tropomyosin to confer Ca^{2+} sensitivity to erythrocyte actin-myosin-Mg^{2+}-ATPase; it formed arrowheads with heavy meromyosin; it was bound by spectrin; it interacted with α-actinin and tropomyosin; and it had a molecular weight similar to other actins. Different from muscle actin was the temperature of activation of muscle myosin-ATPase. While muscle actin activated Mg^{2+}-ATPase of myosin optimally at 25° C, erythrocyte actin required 37° C.

The experimental yield obtained by the extraction procedure is, in our hands, 5—10% of the theoretical estimated by the relative concentrations of actin with respect to the other proteins present in the membranes. The low yield value could result from a loss by solubilization and/or from an unknown amount of non-polymerizable actin in erythrocytes. Actins with varied polymerizing properties exist in brain cells [25], blood platelets [26], slime mold [27], and sperm [28]. When an aliquot of the third wash solution from erythrocyte membranes was dialyzed, lyophilized and reconstituted in a buffer containing K^+-Mg^{2+}-ATP, it showed no microfilament formation. However, when added to an extensively washed erythrocyte ghost suspension, filaments were formed that were identified as actin by arrowhead decoration with heavy meromyosin. Thus an uncertain amount of erythrocyte actin is lost while separating hemoglobin from red cell membranes. The actin lost during membrane washes seems to require a factor or factors still present in membranes after several washes and one of these factors might well be spectrin.

The search for an anchoring material to actin in the membrane of non-muscle cells is important to understand the manner by which actin participates in membrane physiology. Adjacent to the cytoplasmic side of the membrane, actin may represent a large reservoir for nucleotide binding since non-muscle actins bind 1 mol of nucleotide per mol of protein [3,5]; 1 mol of a divalent cation per mol of protein [3]; 1 mol of phosphorous release per mol of actin per one-half cycle of transformation (polymerization) [5]; and 1 mol of nucleotide exchange per mol of protein per one-half cycle of transformation (depolymerization) [5]. Biophysically, the plasticity of the erythrocyte membrane may be altered to a large extent by the viscoelastic properties conferred by anchoring or regulatory proteins on actin when the equilibrium constant shifts from the globular (dispersed) state to the fibrillar state (filamentous or reticular) or vice versa. These considerations can apply also to other cell membranes.

Our results indicate that spectrin interacts with erythrocyte G-actin, binding it, possibly activating it by shifting its monomeric state to the F-actin filamentous state. When this occurs, spectrin probably remains bound to erythrocyte actin. The ionic requirements and the biochemical changes occurring in actin during the protein state change are under study. The apparent contradiction that exists between published reports that postulate a shift by spectrin on actin to the G-state [4] or to the F-state [22] may be due to intrinsic ionic requirements of each protein to exist in one or more states of aggregation for optimal or minimal spectrin-actin interactions [29,30].

In our hands, Lytron particles, when coated with a given protein, allowed ionic strength conditions to be of lesser importance in the interaction of one protein in solution (actin) with the other protein immobilized on the surfaces of the Lytron particles (spectrin) or vice versa. The binding kinetics of spectrin itself on Lytron particles indicated formation of a monolayer. Under this condition, it was found that spectrin could bind 8—9 mol of erythrocyte actin when originally its presence was as G-actin and in solution. Little erythrocytic actin was bound from solution in the F-form. Erythrocyte G-actin bound by Lytron could not bind spectrin. This seems to indicate that spectrin binds to actin and holds it bound by altering the state of polymerization of actin. When erythrocyte G-actin is bound first by the Lytron surfaces, it cannot polymerize as when it is in solution, and consequently is unable to bind or to retain bound spectrin. Polymerization of erythrocyte actin by spectrin was observed electron microscopically. α-Actinin was also reported to organize actin by producing large arrays of laterally cross-linked actin filaments [10,31]. In our preparations, spectrin was not observed cross-linking actin filaments as reported for α-actinin. Similar results were reported by Sheetz et al. [17], who also could not polymerize spectrin as linear aggregates.

The modulation by spectrin of erythrocyte actin-muscle myosin interaction deserves further comment. It is apparent that spectrin, muscle myosin, muscle α-actinin and muscle tropomyosin all have the capacity to interact with erythrocyte actin with varied specificities. With myosin in particular, the interaction with erythrocyte actin is manifested by Mg^{2+}-ATPase activation. α-Actinin and spectrin, by altering the degree of actin organization, may facilitate erythrocyte actin-myosin interaction. Little or no effect is obtained by spectrin or α-actinin on polymerized erythrocyte F-actin-stimulated myosin-Mg^{2+}-ATPase. Tropomyosin, however, may alter the conformations of erythrocyte F-actin into a refractory state to spectrin or α-actinin, most likely by attaching to actin filaments as reported for muscle.

Our preparations of spectrin did not show cation-stimulated ATPase activities [32], although a highly sensitive, γ-labeled, phosphorous-release method from ATP was used. If spectrin indeed is an ATPase below our level of detection, it would contribute little to the other ATPases of the erythrocyte membrane in utilizing energy from the hydrolysis of ATP. On the other hand, it is possible that other proteins may exist in red blood cells to act as cofactors for spectrin expression of ATPase activity.

The reported antigenic similarities of spectrin with myosin from uterus are intriguing [17]. Myosins from smooth muscle and non-muscle tissues possess differences and similarities to spectrin both biochemically and ultrastructurally. Non-muscle myosins are mostly cytoplasmic [20], but also anchored to membranes by their rod segments [33,13,34]. Spectrin is known to be attached to the cytoplasmic side of the erythrocyte membrane [4]. Spectrin and myosin both have two heavy polypeptides of similar molecular weights, but both differ in their ATPase activities. Molecules of spectrin, when isolated by published procedures, lack light chains, while myosins have several. The related immunogenicities reported by Sheetz et al. [17] suggest that spectrin and myosin(s) share ultrastructurally a certain degree of conformational identity. The postulate that spectrin forms part of erythrocytes' contractile or cytoskeletal struc-

ture in association with red cell actin seems, at present, substantiated by the results now reported. Further study is needed to elucidate its functions.

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