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Spectrin Promotes the Association of F-Actin with the Cytoplasmic Surface of the Human Erythrocyte Membrane

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ABSTRACT We have studied the binding of actin to the erythrocyte membrane by a novel application of falling ball viscometry. Our approach is based on the notion that if membranes have multiple binding sites for F-actin they will be able to cross-link and increase the viscosity of actin. Spectrin- and actin-depleted inside-out vesicles reconstituted with purified spectrin dimer or tetramer induce large increases in the viscosity of actin. Comparable concentrations of spectrin alone, inside-out vesicles alone, inside-out vesicles plus heat-denatured spectrin, ghosts, or ghosts plus spectrin have no effect on the viscosity of actin. Centrifugation experiments show that the amount of actin bound to the inside-out vesicles is enhanced in the presence of spectrin. The interactions detected by low-shear viscometry reflect actin interaction with membrane-bound spectrin because (*a*) prior removal of band 4.1 and ankyrin (band 2.1, the high-affinity membrane attachment site for spectrin) reduces both spectrin binding to the inside-out vesicles and their capacity to stimulate increases in viscosity of actin in the presence of spectrin, and (*b*) the increases in viscosity observed with mixtures of inside-out vesicles + spectrin + actin are inhibited by the addition of the water-soluble 72,000-dalton fragment of ankyrin, which is known to inhibit spectrin reassociation to the membrane.

The increases in viscosity of actin induced by inside-out vesicles reconstituted with purified spectrin dimer or tetramer are not observed when samples are incubated at 0°C. This temperature dependence may be related to temperature-dependent associations we observe in solution studies with purified proteins: addition of ankyrin inhibits actin cross-linking by spectrin tetramer plus band 4.1 at 0°C, and enhances it at 32°C.

We conclude (*a*) that falling ball viscometry can be used to assay actin binding to membranes and (*b*) that spectrin is involved in attaching actin filaments or oligomers to the cytoplasmic surface of the erythrocyte membrane.

The shape and deformability of the human erythrocyte (27, 32), the distribution of membrane surface markers (14, 30), and membrane protein mobility (17) are believed to be modulated by a spectrin-actin network that underlies the membrane (6, 18, 19, 27, 32, 34, 36, 37, 41, 44, 48). Although the molecular features of the interaction of spectrin with the cytoplasmic surface of the membrane (in the absence of actin) have been characterized in some detail (1–5, 25, 45, 46, 49), less is known concerning the attachment of actin to the membrane.

Reassociation of monomeric (G) actin with spectrin-actin-depleted vesicles has been measured directly (10, 11). G-actin does not reassociate with these vesicles unless the vesicles are first reconstituted with a high molecular weight complex containing spectrin, actin, band 4.1 and band 4.9.[1] Actin reassociation appears to occur by polymerization of the actin from nucleating sites associated with the reconstituted membranes (11). Thus, the high molecular weight complex itself probably

[1] Nomenclature of erythrocyte membrane proteins is according to Steck (39, 41).

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contains preexisting filamentous (F) actin seeds which serve as nucleating sites for the exogenous actin (7, 16, 23, 33).

The ability of purified spectrin (6, 18, 19),[2] or a spectrin-band 4.1 complex (18, 19, 48),[2] to interact with and cross-link actin in the absence of membranes has led to the idea that actin is associated with the cytoplasmic surface of the membrane as short oligomers cross-linked either directly by spectrin or, alternatively, by a spectrin-band 4.1 complex (7, 18, 19, 28, 48). However, it is also possible that F-actin interacts directly with components on the cytoplasmic surface of the membrane, independent of spectrin or band 4.1.

We decided to study the interaction of actin with the cytoplasmic surface of the erythrocyte membrane by measuring the ability of membrane vesicles to cross-link[3] actin. We reasoned that if membranes have multiple F-actin-binding sites, they should cross-link F-actin just as multivalent actin-binding proteins isolated from a variety of nonmuscle cells (8, 9, 22, 42, 43), including erythrocytes (6, 18, 19, 48),[2] cross-link actin. Because many of the protein associations in the erythrocyte membrane have been studied extensively by other techniques, we have used the erythrocyte to verify this reasoning. The results presented here and in the accompanying paper (26) demonstrate that the low-shear viscometric assay (20, 29), previously used to monitor actin cross-linking (18, 19, 20, 29), can provide useful information about membrane-associated actin-binding sites. We find that reconstitution of spectrin-actin-depleted inside-out membrane vesicles with purified spectrin confers on them the ability to cross-link F-actin. These results show that spectrin promotes the attachment of actin filaments or oligomers to the inner surface of the membrane.

MATERIALS AND METHODS

Preparations

MEMBRANES: Fresh whole human blood drawn into acid-citrate-dextrose was obtained through the Northeastern Regional Red Cross, and was used within 1 wk of drawing. White erythrocyte ghost membranes (depleted of band 6), spectrin-actin-depleted inside-out vesicles, and inside-out vesicles further depleted of ankyrin and band 4.1 by high-salt extraction (ankyrin-band 4.1-depleted inside-out vesicles) were prepared as described by Hargreaves et al. (21) (Fig. 1, lanes *a–c*). Sodium hydroxide-stripped vesicles were prepared essentially as described by Steck and Yu (40). All of the membrane preparations were given a final wash in 20 mM KCl, 1.0 mM EDTA, 0.2 mM dithiothreitol (DTT), 3.0 mM NaN_3, 1.0 mM sodium phosphate, pH 7.6, and then resuspended in this buffer to a volume equivalent to that of the ghost membranes from which they were derived. Electrophoresis on 5% polyacrylamide gels was performed as described by Fairbanks et al. (15).

SPECTRIN: Spectrin dimers or tetramers were extracted from ghosts into 1.0 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid, pH 8.0 (measured at 0°C), 0.1 mM EDTA, 0.4 mM diisopropyl fluorophosphate, at 37° or 0°C, respectively, and purified by gel filtration over Sepharose 4B (35, 47), in 20 mM KCl, 1.0 mM EDTA, 3.0 mM NaN_3, 1.0 mM sodium phosphate, pH 7.6 (18, 46). Peak fractions containing pure spectrin (Fig. 1, lane *d*) were pooled and stored in this buffer at 0°C without further manipulation.

ACTIN: G-actin was prepared from an acetone powder of rabbit skeletal muscle with a single cycle of polymerization and sedimentation from 0.8 M KCl (38). After subsequent depolymerization and clarification of the G-actin at 100,000 *g* for 3 h at 4°C, the G-actin either was used directly in the viscosity measurements or was stored as a lyophilized powder at −20°C for later resuspension and preparation of G-actin (18). In either case, the G-actin was dialyzed up to 10 d at 0°C against 2.0 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM DTT, 3.0 mM NaN_3. The low-shear viscosity of F-actin varied from batch to batch (18) but remained constant with time within a batch. Protein concentrations of actin and membrane preparations were determined by the method of Lowry et al. (24).

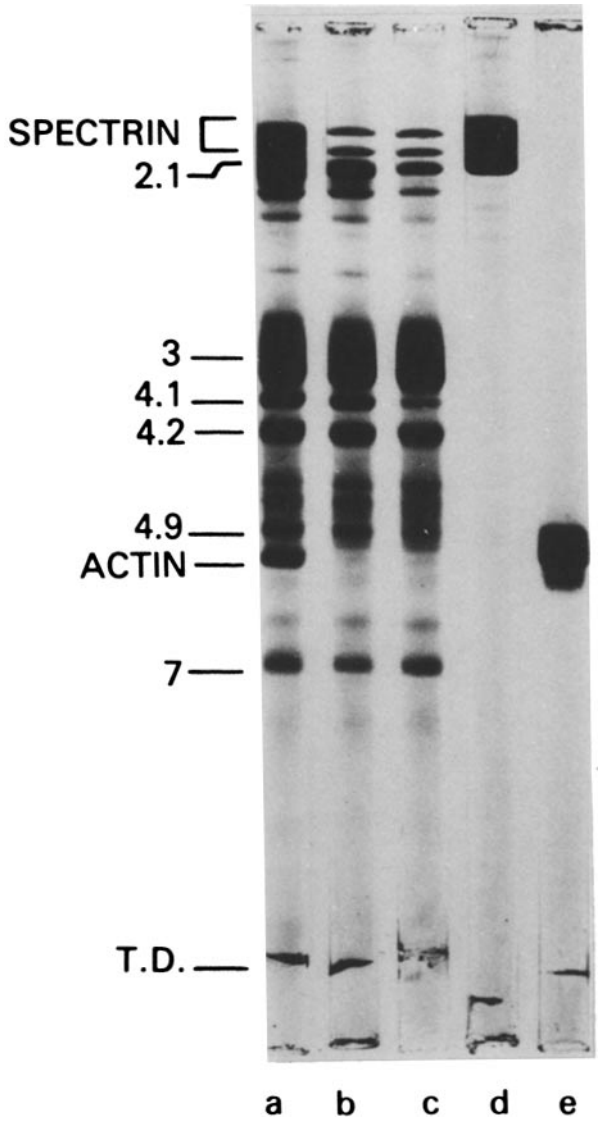

FIGURE 1 SDS polyacrylamide gels of (*a*) human erythrocyte membranes depleted of band 6 (white ghosts), (*b*) spectrin-actin-depleted inside-out vesicles, (*c*) ankyrin(band 2.1)-band 4.1-depleted inside-out vesicles, (*d*) purified spectrin dimer, and (*e*) purified rabbit skeletal muscle actin. Gels *a*, *b*, and *c* were loaded with equal volumes of membrane samples, gel *d* with 10 μg of spectrin, and gel *e* with 11 μg of actin. The spectrin and actin samples were overloaded to demonstrate the purity of the preparations, and the membrane samples were overloaded to show minor bands.

Viscosity Measurements

Viscosity was measured using a low-shear falling ball viscometer (20, 29) as modified by Fowler (19) and Fowler and Taylor (18), in an assay buffer previously found to be optimal for gelation of actin by extracts from erythrocyte membranes (18, 19). The assay buffer contained 50 mM KCl, 20 mM PIPES, pH 7.0, 0.5 mM DTT, 2.0 mM EGTA, 0.1 mM $CaCl_2$, and 1.0 mM MgATP ($[Ca^{++}]_{free}$, $\sim 10^{-8}$ M). (Less than 1.0 mM each of sodium phosphate, EDTA, and Tris was contributed by the addition of membranes, spectrin, and actin to the assay mixture.)

Increasing the free calcium ion concentration from $\sim 1 \times 10^{-8}$ M (free calcium ion concentrations calculated as in reference 18) to $\sim 2 \times 10^{-6}$ M inhibited the increases in viscosity of inside-out vesicle-spectrin-actin mixtures in some exper-

[2] Cohen, C. M., and C. Korsgren. Personal communication.

[3] In this paper, the term "cross-link" refers to interactions of filamentous actin with polypeptides or membranes that contain a multiplicity of actin-binding sites and are therefore multivalent for actin. These interactions give rise to anastomosing networks or gel-like structures in which actin filaments are bound to one another, or "cross-linked" (for a more extended discussion see references 8, 9, 22, 42, and 43).

iments but not in others, in contrast to the consistent inhibitory effect of calcium on the interaction of actin with spectrin- and band 4.1-containing extracts from erythrocyte membranes (18, 19). Interestingly, the inhibitory effect of $\sim 2 \times 10^{-6}$ M free calcium on inside-out vesicle-spectrin-actin interactions was never observed when preformed F-actin rather than G-actin was added to the assay mixture and incubated with inside-out vesicles and spectrin (under polymerizing conditions). Similarly, the inhibitory effect of micromolar free calcium on the increases in viscosity of actin induced by purified spectrin plus band 4.1 (no membranes) was observed only when G-actin and not preformed F-actin was used. The difference in calcium sensitivity between G- and preformed F-actin was not further investigated.

Unless otherwise indicated, rabbit skeletal muscle actin was added to the assay mixture immediately after addition of the purified spectrin to the inside-out vesicles at 0°C. After mixing of all components, samples were vortexed briefly, drawn up into the 100-μl micropipettes, and incubated in a horizontal position (so as to prevent destruction of the gel by rising bubbles) at 32°C for 1 h before the viscosities were measured (18, 19). The viscosities presented are averages of triplicate determinations for each sample; thus, a 0.3-ml sample volume was used to fill three 100-μl micropipettes. Viscosities above ~500 cp (indicated by an asterisk in the figures, where the sample begins to gel) were not measured because our calibration curves do not extend beyond this point (18, 19).

RESULTS

Increases in Viscosity of Actin Induced by Inside-Out Vesicles plus Spectrin

When erythrocyte membranes (ghosts) (Fig. 1, lane *a*) are extracted at 37°C with 0.3 mM sodium phosphate, pH 7.6, spectrin and actin are eluted from the membrane (Fig. 1, lane *b*) and the membranes vesiculate into inside-out vesicles (1, 41). Low concentrations of these spectrin-actin-depleted inside-out vesicles reconstituted with purified spectrin dimer induce large increases in the viscosity of purified rabbit skeletal muscle G-actin under ionic conditions that promote actin polymerization (50 mM KCl, 1 h, 32°C) (Fig. 2). In the absence of membranes, these low concentrations of purified spectrin have no detectable effect on the viscosity of actin (Fig. 2, and see references 18 and 19). The viscosity of comparable concentrations of inside-out vesicles plus actin in the absence of spectrin, or of the inside-out vesicles plus heat-treated spectrin (5 min, 60°C) plus actin, is not appreciably different from that of actin alone (Fig. 2). Neither leaky (Fig. 2) nor resealed (not shown) ghosts cause increases in the viscosity of actin, in the presence or absence of spectrin. (In the absence of actin, all samples have viscosities equivalent to that of the buffer: 1–2 cp).

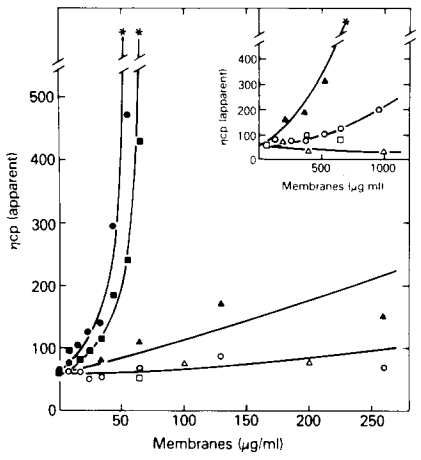

FIGURE 2 Effect of purified spectrin dimer on increases in viscosity of actin induced by spectrin-actin-depleted inside-out vesicles: effect of varying the inside-out vesicle concentration in the presence of (○) 0 μg/ml spectrin, (▲) 2 μg/ml spectrin, (■) 5 μg/ml spectrin, (●) 30 μg/ml spectrin, or (□) 5 μg/ml heat-treated spectrin (5 min, 60°C). (△) White ghosts plus actin in the presence or absence of 30 μg/ml spectrin. Rabbit muscle G-actin (final concentration 0.8 mg/ml) was incubated with the inside-out vesicles (in the presence or absence of spectrin) under polymerizing conditions as described in Materials and Methods. (*Inset*) Increases in viscosity of actin induced by higher concentrations of inside-out vesicles.

The increases in viscosity of the inside-out vesicle-spectrin-actin mixtures depend on both the vesicle concentration and the spectrin concentration (Fig. 2). In the absence of added spectrin, greater than 1 mg/ml of inside-out vesicles (which contain some residual spectrin) is required to induce extensive increases in the viscosity of actin (Fig. 2, *inset*).

Viscosity Increases are Mediated Only by Specifically Bound Spectrin

Four lines of evidence demonstrate that the viscosity changes we measure are caused by bound rather than unbound spectrin. First, inside-out vesicles were preincubated with spectrin but without actin, and then centrifuged to separate the vesicles with bound spectrin from any free spectrin in the supernate. Most of the spectrin binds to the inside-out vesicles (Fig. 3*a*) and thus, as expected, these preincubated and washed vesicles induce increases in the viscosity of actin comparable to an equivalent volume of inside-out vesicles preincubated with spectrin but not washed (Fig. 3*b*). (Washed vesicles are not used routinely because the added steps are cumbersome.)

Second, we extracted the inside-out vesicles with salt or sodium hydroxide to deplete them of the spectrin-binding proteins, ankyrin and band 4.1, and thus reduce spectrin reassociation with the cytoplasmic surface of the membrane (45). The capacity of such ankyrin-band 4.1-depleted inside-out vesicles (Fig. 1, lane *c*) to stimulate increases in the viscosity of actin in the presence of spectrin is reduced in comparison to untreated inside-out vesicles (Fig. 4). In the absence of spectrin, the viscosity of the salt- or NaOH-treated inside-out vesicles plus actin is not appreciably different from that of actin alone, even at inside-out vesicle concentrations >1 mg/ml (not shown).

Third, the increases in viscosity of actin induced by both native and ankyrin-band 4.1-depleted inside-out vesicles appear to be saturable for spectrin (Fig. 4*b*), just as the binding of spectrin to inside-out vesicles is saturable, both at 0°C (1, 45) and also under our assay conditions (50 mM KCl, pH 7.0, 32°C, 1 h; not shown). Although we have observed a significant amount of nonsaturable binding of ^{125}I-labeled spectrin to sodium hydroxide-stripped inside-out vesicles (not shown), this apparently nonspecific association of spectrin with the membrane does not result in increases in viscosity of actin (Fig. 4*b*). Similarly, increased nonspecific binding of heat-denatured ^{125}I-labeled spectrin to inside-out vesicles observed at 32°C (not shown) in comparison to 0°C (1, 45) does not confer actin cross-linking activity on the inside-out vesicles (Fig. 2, open squares).

Fourth, the increases in viscosity observed with mixtures of inside-out vesicles, spectrin, and actin are inhibited by the addition of the 72,000-dalton fragment of ankyrin which inhibits the reassociation of spectrin with spectrin-actin-depleted inside-out vesicles (2) (Fig. 5). The inhibition cannot be the result of proteolysis because SDS polyacrylamide gels of inside-out vesicles plus spectrin appear identical by Coomassie Blue staining in the presence or absence of the 72,000-dalton poly-

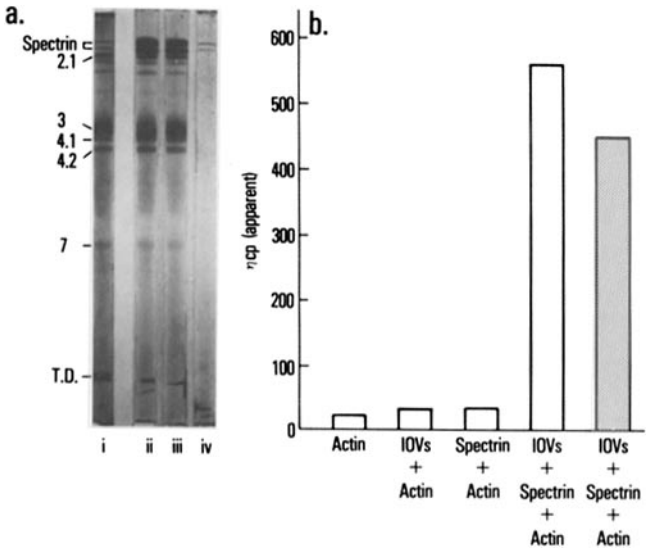

FIGURE 3 (*a*) SDS polyacrylamide gels of (*i*) inside-out vesicles without added spectrin, (*ii*) inside-out vesicles preincubated with spectrin, (*iii*) inside-out vesicles preincubated with spectrin and washed, (*iv*) supernate obtained after pelleting of inside-out vesicles preincubated with spectrin. Equal volumes of all samples were electrophoresed. (*b*) Effect of removing unbound spectrin on the ability of spectrin-actin-depleted inside-out vesicles reconstituted with purified spectrin dimer to induce increases in the viscosity of actin. (Blank bars) inside-out vesicles (IOVs) alone, spectrin alone, or inside-out vesicles plus spectrin preincubated but not washed before mixing with actin. (Dotted bar) inside-out vesicles preincubated with spectrin, then washed before mixing with actin. Purified spectrin dimer and inside-out vesicles (final concentrations 130 and 500 μg/ml, respectively), spectrin alone, or inside-out vesicles alone were preincubated without actin under standard assay conditions for 1 h at 32°C. Samples were then left on ice (blank bars), or centrifuged to separate membrane-bound from free spectrin (1) (dotted bar) before mixing with actin. The membrane pellet was resuspended to the initial volume in the assay buffer and parallel samples (30 μl) of the resuspended pellet and the preincubated but not centrifuged samples were then mixed with G-actin and incubated as specified in Materials and Methods.

peptide (except for a reduction in the amount of spectrin pelleting with the membranes).

Inside-Out Vesicles Reconstituted with Purified Spectrin Cross-link Preformed F-Actin as well as G-Actin under Polymerizing Conditions

Under the conditions used in the experiments presented in Figs. 2–5, the G-actin added to the assay mixture would be expected to polymerize. Inside-out vesicles plus spectrin can also induce increases in the viscosity of preformed F-actin (Fig. 6). The different viscosities achieved in experiments that start with preformed F-actin and those that start with G-actin may reflect the fact that the viscous actin-containing solutions are only partially thixotropic, that is, the final viscosity is not completely recovered after the solution is subjected to mechanical disruption and reincubated (Fig. 6, triangles). It should be remembered that the assay requires us to vortex and pipette, and thus disrupt (8)[4] the preformed F-actin to place it in the micropipettes used to measure viscosity.

[4] Taylor, D. L., J. Reidler, J. A. Spudich, and L. Stryer. The detection and measurement of actin assembly by fluorescence energy transfer. Manuscript submitted for publication.

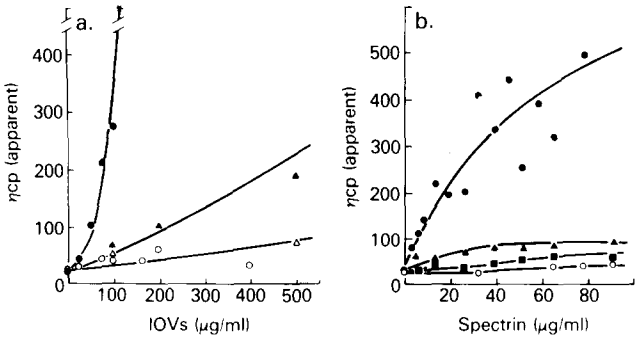

FIGURE 4 (*a*) Comparison of the increases in viscosity of actin induced by spectrin-actin-depleted inside-out vesicles (*IOVs*) in the presence (●) or absence (○) of 5 μg/ml purified spectrin dimer with the increases in viscosity of actin induced by ankyrin-band 4.1-depleted inside-out vesicles in the presence (▲) or absence (△) of 5 μg/ml purified spectrin dimer: effect of varying the inside-out vesicle concentration. Increasing concentrations of sodium hydroxide-stripped vesicles had very little effect on the viscosity of actin in the presence or absence of spectrin (not shown). (*b*) Influence of increasing concentrations of purified spectrin dimer on increases in viscosity of actin induced by (●) spectrin-actin-depleted inside-out vesicles, (▲) ankyrin-band 4.1-depleted inside-out vesicles, (■) sodium hydroxide-stripped inside-out vesicles, or (○) no membranes. Rabbit muscle G-actin (final concentration 0.8 mg/ml) was incubated under polymerizing conditions with equivalent volumes of inside-out vesicles, ankyrin-band 4.1-depleted inside-out vesicles, or sodium hydroxide-stripped inside-out vesicles (final concentrations of 113, 102, and 33 μg/ml, respectively) in the presence of the indicated concentrations of purified spectrin dimer.

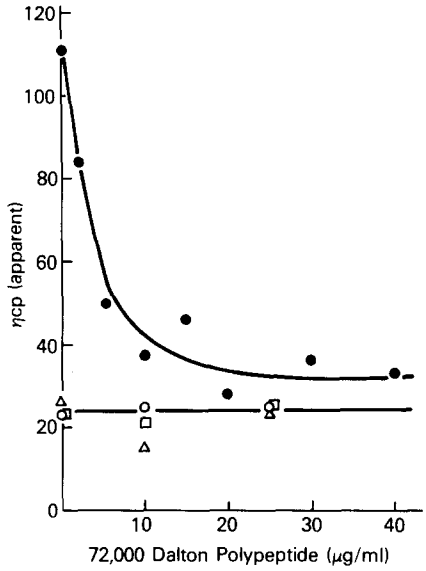

FIGURE 5 Effect of increasing concentrations of the 72,000-dalton polypeptide on the increases in viscosity of actin induced by spectrin-actin-depleted inside-out vesicles in the presence of purified spectrin dimer. (●) 72,000-dalton polypeptide, inside-out vesicles, spectrin, and actin. (○) 72,000-dalton polypeptide, inside-out vesicles, and actin. (□) 72,000-dalton polypeptide, spectrin and actin. (△) 72,000-dalton polypeptide and actin. The 72,000-dalton polypeptide was prepared by chymotryptic digestion of spectrin-actin-depleted inside-out vesicles and purified by ion-exchange chromatography over DEAE cellulose (2). Components were added to the assay mixture in the following order at the indicated final concentrations: inside-out vesicles (100 μg/ml), 72,000-dalton polypeptide (see figure), spectrin (5 μg/ml), G-actin (0.8 mg/ml), and incubated as specified in Materials and Methods.

Effect of Spectrin on Actin Binding to Membranes

The increases in viscosity of actin induced by inside-out vesicles reconstituted with spectrin are paralleled by an increase in the amount of actin bound to the inside-out vesicles in the presence of spectrin (Fig. 7). Although some actin pellets with the inside-out vesicles even in the absence of added spectrin (compare to Fig. 1, lane *b* and to Fig. 3 *a*, lane *a*), the inside-out vesicle concentration used here (200 μg/ml) does not induce detectable increases in the viscosity of actin in the absence of added spectrin (see Fig. 2). The presence of spectrin has no effect on the amounts of actin pelleting with right-side-out ghost membranes (Fig. 7). However, some of the exogenous G-actin, but not preformed F-actin, which is incubated with the ghosts under conditions that promote actin polymerization appears to pellet with the ghosts (compare Fig. 7 *a* and *b*, and see reference 26). This is consistent with previous reports that indicate that G-actin polymerizes from preexisting seeds within ghosts (10, 11).

Effect of Temperature on the Increases in Viscosity of Inside-Out Vesicles + Spectrin + Actin

The increases in viscosity of actin induced by inside-out vesicles reconstituted with purified spectrin dimer are not observed if samples are incubated at 0°C (Fig. 8 *a* and *b*). This result may explain why Cohen and Branton (11) did not initially observe significant spectrin-enhanced reassociation of G-actin with inside-out vesicles at 0°C. The lack of inside-out vesicle-spectrin-actin cross-linking at 0°C is not caused by the inability of the spectrin dimers to associate to form tetramers at 0°C (35, 47) because similar results are obtained for inside-out vesicles reconstituted with purified spectrin tetramer instead of spectrin dimer (Fig. 8 *c* and *d*). Because 0°C incubation does not inhibit actin cross-linking by spectrin tetramer plus band 4.1 in the absence of membranes (18, 19, 48)[2, 5] we considered the possibility that a temperature-sensitive interaction of spectrin with its high-affinity membrane attachment site, ankyrin, might influence the interaction of spectrin with actin. We tested this idea in a preliminary fashion by looking at the effect of purified ankyrin on interactions between spectrin and actin in the absence of membranes. Indeed, at 0°C, purified ankyrin inhibits the increases in viscosity induced by

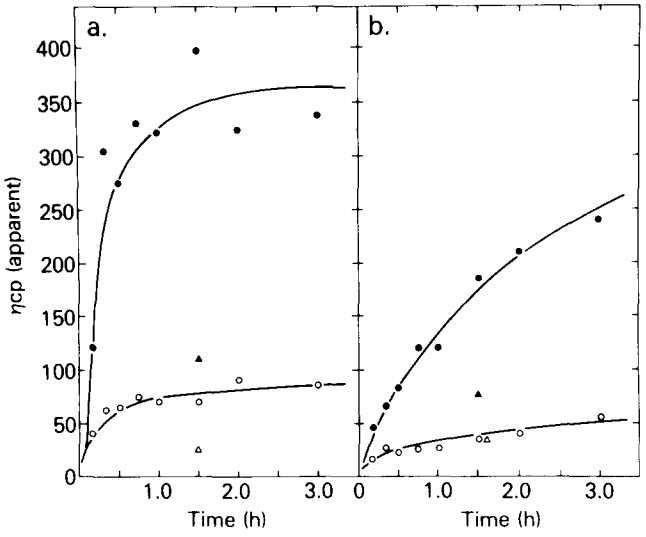

FIGURE 6 Increases in viscosity of actin with time induced by spectrin-actin-depleted inside-out vesicles reconstituted with purified spectrin dimer. (●) Actin, inside-out vesicles, and spectrin. (○) Actin alone. (▲) Actin, inside-out vesicles, and spectrin preincubated for 90 min, 32°C in a test tube, vortexed, and then incubated for 90 min in the micropipettes before measuring the viscosity. (△) Actin alone treated similarly. Rabbit muscle (*a*) G-actin or (*b*) F-actin (polymerized in 0.1 M KCl, 2.0 mM $MgCl_2$, 30 min, 32°C) was mixed with inside-out vesicles and spectrin and incubated under polymerizing conditions as described in Materials and Methods. Additional $MgCl_2$ was added to the samples containing G-actin so that the final [$MgCl_2$] would be equivalent to those containing F-actin (~0.2 mM). Final concentrations of the actin, inside-out vesicles, and spectrin were 0.8 mg/ml, 54 μg/ml, and 5 μg/ml, respectively. Samples were incubated in the micropipettes at 32°C for the indicated times before the viscosities were measured.

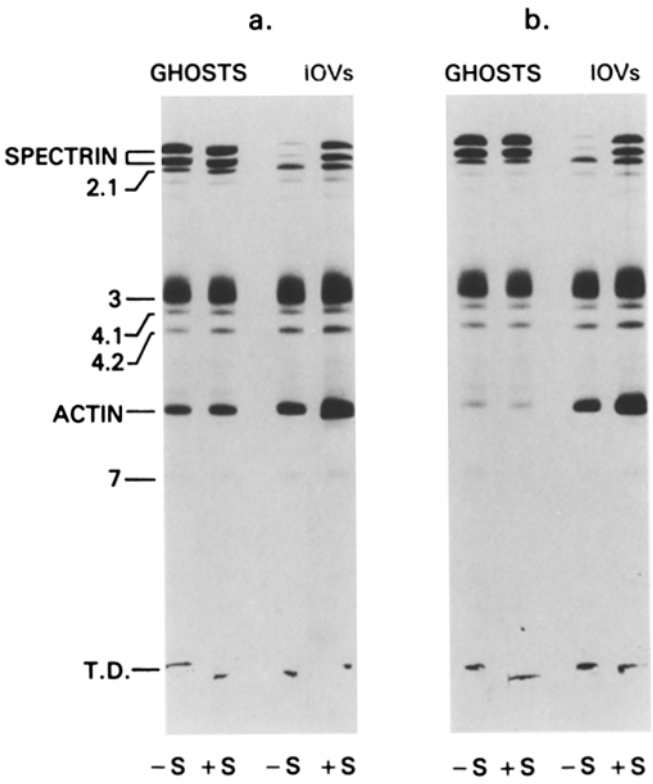

FIGURE 7 Effect of purified spectrin dimer on actin pelleting with erythrocyte membranes (ghosts) or spectrin-actin depleted inside-out vesicles (*IOVs*). Rabbit muscle (*a*) G-actin or (*b*) F-actin (polymerized as described in Fig. 4) was mixed with equivalent volumes of ghosts or inside-out vesicles in the presence (+S) or absence (−S) of spectrin, under polymerizing conditions as described in Materials and Methods. Final concentrations of actin, ghosts, inside-out vesicles, and spectrin were 200 μg/ml, 230 μg/ml, 200 μg/ml, and 30 μg/ml, respectively. Immediately after mixing, samples were incubated and centrifuged as described in the accompanying paper (26). The supernates were discarded and equivalent volumes of the resuspended pellets were electrophoresed on 5% SDS polyacrylamide tube gels (15).

[5] Under approximately physiological conditions (50 mM KCl, pH 7.0, $[Ca^{++}]_{free} = 10^{-8}$ M), low concentrations (<0.2 mg/ml) of purified human spectrin tetramer are not active in cross-linking actin unless band 4.1 is also present (18, 19). However, at pH 8.0 in the presence of 0.2 mM $CaCl_2$ and 2 mM $MgCl_2$, purified sheep spectrin tetramer has been reported to be active in cross-linking actin in the absence of band 4.1 (6).

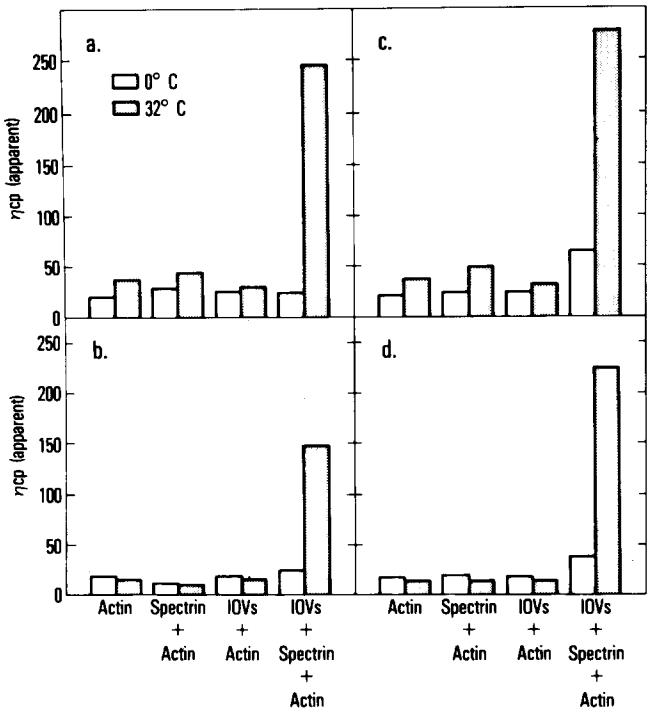

FIGURE 8 Inhibition at 0°C of increases in viscosity of actin induced by spectrin-actin-depleted inside-out vesicles (*IOVs*) reconstituted with *a* and *b*) purified spectrin dimer or (*c* and *d*) purified spectrin tetramer. Rabbit muscle (*a* and *c*) G-actin or (*b* and *d*) F-actin (polymerized as in Fig. 4) was mixed with inside-out vesicles in the presence of spectrin dimer or tetramer as described in Materials and Methods, at final concentrations of 0.8 mg/ml actin, 75 μg/ml inside-out vesicles, and 30 μg/ml spectrin dimer or tetramer. Samples were incubated under ionic conditions favoring polymerization (blank bars) for 4 h at 0°C or (dotted bars) for 4 h at 32°C before the viscosities were measured.

spectrin tetramer plus band 4.1 (Fig. 9*a*). In contrast, at 32°C, ankyrin markedly enhances the increases in viscosity induced by spectrin tetramer plus band 4.1 (Fig. 9*b*).

DISCUSSION

Our results show that the ability of inside-out vesicles to cross-link and increase the viscosity of actin preparations is dependent on the specific reassociation of spectrin with the membrane. We conclude that F-actin can attach to the erythrocyte membrane via an interaction with spectrin. This conclusion extends previous observations of spectrin-actin interactions in solution (6, 18, 19, 48)[2] and agrees with recent direct measurements of actin binding to membranes using radiolabeled F-actin (12).

Actin Associates with Membrane-bound Spectrin

In all of our experiments the increases in viscosity of actin-containing solutions are attributable to spectrin (either spectrin heterodimers or tetramers) bound to the inside-out vesicles. Conditions known to reduce spectrin reassociation with the membrane inhibit the increases in viscosity; conditions that maximize specific spectrin binding maximize the increases in viscosity. Although it is known that, in the absence of membranes, spectrin alone or in the presence of band 4.1 can cross-link actin, such cross-linking requires spectrin tetramer and requires spectrin concentrations higher than those used in our assays.[5] It is unlikely that in our experiments membranes simply provide a source of soluble band 4.1. Band 4.1 does not elute from the membrane under our conditions of ionic strength and pH (40, 41, 45). Furthermore, the maximal amount of band 4.1 that could conceivably be eluted from the concentrations of inside-out vesicles used in our assays is <5 μg/ml. This amount of band 4.1 has no effect on the actin cross-linking activity of low concentrations of spectrin in the absence of membranes (Fig. 9 and see references 18 and 19).

Further evidence that the role of the inside-out vesicles is not simply to furnish band 4.1 for spectrin-band 4.1-actin cross-linking in solution is the fact that the 72,000-dalton polypeptide

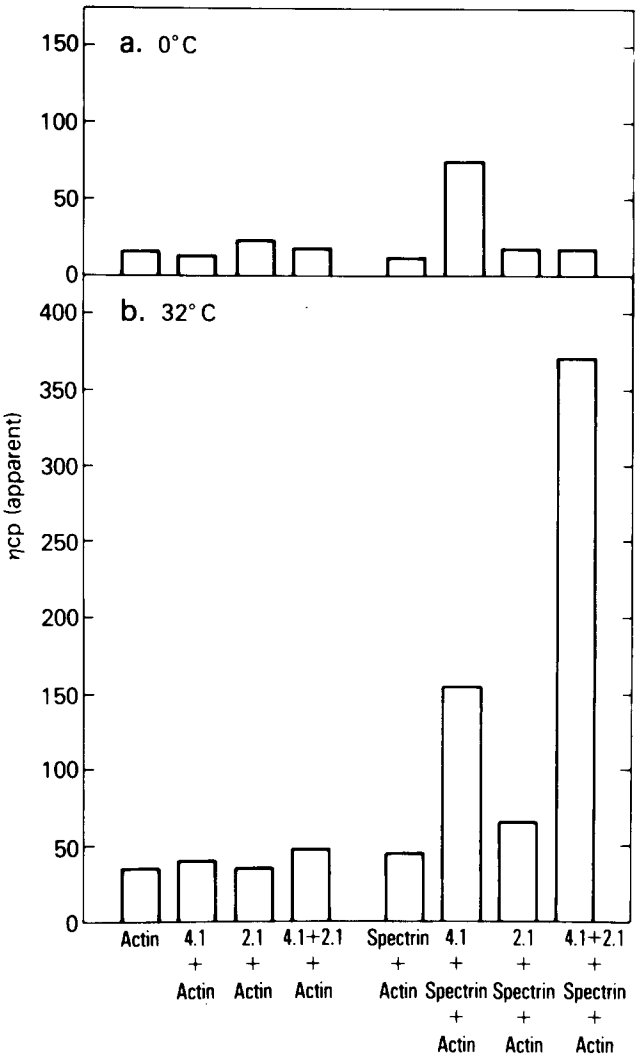

FIGURE 9 Effect of purified ankyrin (2.1) on the increases in viscosity of actin at (*a*) 0°C or (*b*) 32°C induced by purified spectrin tetramer plus purified band 4.1. Ankyrin and band 4.1 were purified as previously described (21, 45). Rabbit muscle G-actin was used at a final concentration of 0.8 mg/ml, spectrin tetramer at (*a*) 30 μg/ml or (*b*) 50 μg/ml, band 4.1 at 8 μg/ml, and ankyrin at 20 μg/ml. Components were added to the assay mixture in the following order: spectrin, band 4.1, ankyrin, actin. Samples were drawn up into the micropipettes immediately after all components were added to the assay mixture, and incubated under ionic conditions favoring polymerization at (*a*) 0°C or (*b*) 32°C for 4 h before the viscosities were measured. Ankyrin also enhances actin cross-linking by spectrin dimer plus band 4.1 at 32°C (not shown), a temperature which favors dimer to tetramer conversion (47).

does not inhibit the increases in viscosity observed at 32°C when spectrin, band 4.1, and actin are mixed in appropriate concentrations in the absence of membranes (not shown). However, it is possible that interactions of spectrin with band 4.1 *in situ* on the membrane may enhance the spectrin-mediated interaction of actin with the cytoplasmic surface of the membrane. Our data do not directly address this question because we were not able to selectively extract all of the band 4.1 from the membrane without also removing some of the ankyrin (see Fig. 1, lane *c*).

Does Actin Interact with Components Other Than Spectrin on the Cytoplasmic Surface of the Membrane?

In contrast to the increases in viscosity of actin induced by low concentrations of inside-out vesicles reconstituted with purified spectrin, comparable concentrations of inside-out vesicles alone have relatively little effect on the viscosity of actin. The increases in viscosity of actin observed at very high concentrations of inside-out vesicles in the absence of added spectrin (Fig. 2, *inset*) may reflect incomplete extraction of the endogenous spectrin from the membrane (see Fig. 1, lane *b*), or perhaps a lower affinity interaction of actin with a nonspectrin component (e.g., band 4.1) (12). Under conditions where inside-out vesicles alone have no effect on the viscosity of the actin, some actin does pellet with the spectrin-actin-depleted inside-out vesicles, even in the absence of added spectrin (Fig. 7, and reference 12). However, it is difficult to evaluate F-actin binding in the absence of independent measures of polymerization and filament length. For example, the number of actin filaments associated with the membrane could change (as reflected in differences in viscosity), with no corresponding change in the absolute amount of actin pelleting with the vesicles.

Mode of Attachment of Actin to the Inside-Out Vesicles: Lateral vs. End-on Association

An actin oligomer or filament could form either end-on or lateral attachments to components on the surface of an inside-out vesicle. Assuming that spectrin does not bind equally to both the sides and ends of actin filaments, the following considerations lead us to favor lateral attachments for actin-spectrin-inside-out vesicle interactions. First, because the two ends of a single actin filament are not identical (31), both ends cannot bind with the same specificity. On the other hand, actin filaments could associate laterally with identical components on two or more vesicles, thus linking them to one another. Second, if end-on attachment of actin to the inside-out vesicles occurred, then as the number of sites available for end-on attachment increased with increasing inside-out vesicle and spectrin concentration, the length of the actin filaments attached to the inside-out vesicles would become shorter and shorter. Shortening of filaments would probably tend to decrease, not increase, the viscosity of actin as the inside-out vesicle concentration was increased. Third, electron microscope images of actin-spectrin-band 4.1 interactions show that spectrin associates laterally rather than at the ends of actin filaments (13, 48): actin filaments are bridged by spectrin to neighboring filaments along the entire length of the actin filaments.

A combination of end-on attachment of actin filaments to a component on one inside-out vesicle and lateral associations with a different component on another inside-out vesicle could also lead to the increases in viscosity and gelation observed. This may account for actin binding to low concentrations of inside-out vesicles in the absence of spectrin as well as spectrin-stimulated increases in actin binding (this paper and see reference 12).

Interaction of Actin with the Cytoplasmic Surface of the Erythrocyte Membrane In Vivo

It is important to note that we have no independent criteria for assessing whether the actin-spectrin-membrane associations we observe reconstitute the native cytoskeletal spectrin-actin network. Some indication of the complex interrelations that may exist among cytoskeletal components in the erythrocyte is provided by our observations of temperature effects on actin-membrane interactions. Inside-out vesicles reconstituted with purified spectrin do not cross-link actin at 0°C and, in the absence of membranes, purified ankyrin inhibits spectrin tetramer-band 4.1-actin cross-linking at 0°C and enhances it at 32°C (Figs. 8 and 9). This may be because of an ankyrin-induced temperature-dependent conformational change in the spectrin (or in a spectrin-band 4.1 complex) or, alternatively, it may be because of a temperature-dependent formation of additional cross-links between ankyrin, actin, spectrin, and/or band 4.1.

Application of Falling Ball Viscometry to the Study of Cytoskeleton-Membrane Interactions

The excellent agreement between our results using viscometric assays and a recent study using direct binding measurements (12) is evidence that the falling ball viscometer can provide a valid indication of how membranes and actin may interact. But, while actin cross-linking by membranes requires actin to be bound to the membrane, actin binding to the membrane may not necessarily result in cross-linking of actin by membranes. The technique of low-shear viscometry may be particularly sensitive to associations of actin with membranes which lead to extensive cross-linking and gelation of actin filaments but may be insensitive to other modes of association.

Because low-shear viscometry is rapidly and easily performed, this assay in combination with selective stripping techniques could be useful in the preliminary identification of actin-binding components of intracellular organelles such as endocytic vesicles and secretory vesicles, as well as inverted vesicles from the plasma membrane itself. We have recently applied this technique to study actin-binding components in membranes from the ameboid stage of *Dictyostelium discoideum* (26).

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REFERENCES

1. Bennett, V., and D. Branton. 1977. Selective association of spectrin with the cytoplasmic surface of human erythrocyte plasma membranes. *J. Biol. Chem.* 252:2753–2763.
2. Bennett, V. 1978. Purification of an active proteolytic fragment of the membrane attachment site for human erythrocyte spectrin. *J. Biol. Chem.* 253:2292–2299.
3. Bennett, V., and P. Stenbuck. 1979. Identification and partial purification of ankyrin, the high affinity membrane attachment site for human erythrocyte spectrin. *J. Biol. Chem.* 254:2533–2541.
4. Bennett, V., and P. J. Stenbuck. 1979. The membrane attachment protein for spectrin is associated with band 3 in human erythrocyte membranes. *Nature (Lond.).* 280:468–473.
5. Bennett, V., and P. J. Stenbuck. 1980. Human erythrocyte ankyrin. Purification and properties. *J. Biol. Chem.* 255:2540–2548.
6. Brenner, S. L., and E. D. Korn. 1979. Spectrin-actin interaction. Phosphorylated and dephosphorylated spectrin tetramer cross-link F-actin. *J. Biol. Chem.* 254:8620–8627.
7. Brenner, S. L., and E. D. Korn. 1980. Spectrin/actin complex isolated from sheep erythrocytes accelerates actin polymerization by simple nucleation. Evidence for oligomeric actin in the erythrocyte cytoskeleton. *J. Biol. Chem.* 255:1670–1676.
8. Brotschi, E. A., J. H. Hartwig, and T. P. Stossel. 1978. The gelation of actin by actin-binding protein. *J. Biol. Chem.* 253:8988–8993.
9. Clarke, M., and J. A. Spudich. 1977. Non-muscle contractile proteins. The role of actin and myosin in cell motility and shape determination. *Annu. Rev. Biochem.* 46:797–822.
10. Cohen, C. M., P. L. Jackson, and D. Branton. 1978. Actin-membrane interactions: association of G-actin with the red cell membrane. *J. Supramol. Struct.* 9:113–124.
11. Cohen, C. M., and D. Branton. 1979. Erythrocyte membrane stimulated actin polymerization: the role of spectrin. *Nature (Lond.).* 279:163–165.
12. Cohen, C. M., and S. F. Foley. 1980. Spectrin dependent and independent association of F-actin with red cell membranes. *J. Cell Biol.* 86:694–698.
13. Cohen, C. M., J. M. Tyler, and D. Branton. 1980. Spectrin-actin associations studied by electron microscopy of shadowed preparations. *Cell.* 21:875–883.
14. Elgsaeter, A., and D. Branton. 1974. Intramembrane particle aggregation in erythrocyte ghosts. I. The effects of protein removal. *J. Cell Biol.* 63:1018–1030.
15. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606–2617.
16. Flanagan, M. D., and S. Lin. 1980. Cytochalasins block actin filament elongation by binding to high affinity sites associated with F-actin. *J. Biol. Chem.* 255:835–838.
17. Fowler, V., and V. Bennett. 1978. Association of spectrin with its membrane attachment site restricts lateral mobility of human erythrocyte membrane proteins. *J. Supramol. Struct.* 8:215–221.
18. Fowler, V., and D. L. Taylor. 1980. Spectrin plus band 4.1 cross-link actin. Regulation by micromolar calcium. *J. Cell Biol.* 85:361–376.
19. Fowler, V. 1980. Cytoskeletal structures and membrane protein mobility in the human erythrocyte. Ph.D. Thesis. The Biology Department. Harvard University, Cambridge, Mass.
20. Griffith, L. M., and T. D. Pollard. 1978. Evidence for actin filament-microtubule interaction mediated by microtubule-associated proteins. *J. Cell Biol.* 78:958–965.
21. Hargreaves, W. R., K. N. Giedd, A. Verkleij, and D. Branton. 1980. Reassociation of ankyrin with band 3 in erythrocyte membranes and in lipid vesicles. *J. Biol. Chem.* 255. In press.
22. Korn, E. D. 1978. Biochemistry of actomyosin-dependent cell motility. *Proc. Natl. Acad. Sci. U. S. A.* 75:588–599.
23. Lin, D. C., and S. Lin. 1979. Actin polymerization induced by a motility-related high-affinity cytochalasin binding complex from human erythrocyte membrane. *Proc. Natl. Acad. Sci. U. S. A.* 76:2345–2349.
24. Lowry, O. H., N. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
25. Luna, E. J., G. H. Kidd, and D. Branton. 1979. Identification by peptide analysis of the spectrin-binding protein in human erythrocytes. *J. Biol. Chem.* 254:2526–2532.
26. Luna, E. J., V. M. Fowler, J. Swanson, D. Branton, and D. L. Taylor. 1980. A membrane cytoskeleton from *Dictyostelium discoideum*. I. Identification and partial characterization of an actin-binding activity. *J. Cell Biol.* 88:396–409.
27. Lux, S. E. 1979. Spectrin-actin membrane skeleton of normal and abnormal red blood cells. *Semin. Hematol.* 16:21–51.
28. Lux, S. E. 1979. Dissecting the red cell membrane skeleton. *Nature (Lond.).* 281:426–429.
29. MacLean-Fletcher, S. D., and T. D. Pollard. 1980. Viscometric analysis of the gelation of *Acanthamoeba* extracts and purification of two gelation factors. *J. Cell Biol.* 85:414–428.
30. Nicolson, G. L., and R. G. Painter. 1973. Anionic sites of human erythrocyte membranes. II. Antispectrin induced transmembrane aggregation of the binding sites for positively charged collodial particles. *J. Cell Biol.* 59:395–406.
31. Oosawa, F., and M. Kasai. 1979. Actin. *In* Subunits in Biological Systems. S. N. Timasheff and G. D. Fasman, editors. Marcel Dekker, New York. 261–322.
32. Palek, J., and S-.C. Liu. 1979. Dependence of spectrin organization in red blood cell membranes on cell metabolism: implication for control of red cell shape, deformability, and surface area. *Semin. Hematol.* 16:75–93.
33. Pinder, J. C., E. Ungewickell, R. Calvert, E. Morris, and W. B. Gratzer. 1979. Polymerization of G-actin by spectrin preparations: identification of the active constituent. *FEBS* (Fed. Eur. Biochem. Soc.) *Lett.* 104:396–400.
34. Pinder, J. C., D. Bray, and W. B. Gratzer. 1977. Control of interaction of spectrin and actin by phosphorylation. *Nature (Lond.).* 270:752–754.
35. Ralston, G. B., J. Dunbar, and M. White. 1977. The temperature-dependent dissociation of spectrin. *Biochim. Biophys. Acta.* 491:345–348.
36. Sheetz, M. P., and D. Sawyer. 1978. Triton shells of intact erythrocytes. *J. Supramol. Struct.* 8:399–412.
37. Sheetz, M. P. 1979. Integral membrane protein interaction with Triton cytoskeletons of erythrocytes. *Biochim. Biophys. Acta.* 577:122–134.
38. Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the Tm-Tn complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.
39. Steck, T. L. 1972. Cross-linking the major proteins of the isolated erythrocyte membrane. *J. Mol. Biol.* 66:295–305.
40. Steck, T. L., and J. Yu. 1973. Selective solubilization of proteins from red blood cell membranes by protein perturbants. *J. Supramol. Struct.* 1:220–232.
41. Steck, T. L. 1974. The organization of proteins in the human red blood cell membrane. *J. Cell Biol.* 62:1–19.
42. Stossel, T. P. 1978. Contractile proteins in cell structure and function. *Annu. Rev. Med.* 29: 427–457.
43. Taylor, D. L., and J. S. Condeelis. 1979. Cytoplasmic structure and contractility in amoeboid cells. *Int. Rev. Cytol.* 56:57–144.
44. Tilney, L. G., and P. Detmers. 1975. Actin in erythrocyte ghosts and its association with spectrin. Evidence for a non-filamentous form of these two molecules *in situ*. *J. Cell Biol.* 66:508–520.
45. Tyler, J. M., W. R. Hargreaves, and D. Branton. 1979. Purification of two spectrin-binding proteins: biochemical and electron microscopical evidence for site-specific reassociation between spectrin and bands 2.1 and 4.1. *Proc. Natl. Acad. Sci. U. S. A.* 76:5192–5196.
46. Tyler, J. M., B. N. Reinhardt, and D. Branton. 1980. Associations of erythrocyte cytoskeletal proteins: binding of purified bands 2.1 and 4.1 to spectrin. *J. Biol. Chem.* 255:7034–7039.
47. Ungewickell, E., and W. Gratzer. 1978. Self-association of human spectrin. A thermodynamic and kinetic study. *Eur. J. Biochem.* 88:379–385.
48. Ungewickell, E., P. M. Bennett, R. Calvert, V. Ohanian, and W. B. Gratzer. 1979. *In vitro* formation of a complex between cytoskeletal proteins of the human erythrocyte. *Nature (Lond.)* 280:811–814.
49. Yu, J., and S. R. Goodman. 1979. Syndeins: the spectrin-binding protein(s) of the human erythrocyte membrane. *Proc. Natl. Acad. Sci. U. S. A.* 76:2340–2344.