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).
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), or a spectrin-band 4.1 complex (18, 19, 48), 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 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), 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 (ankyryn-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 Na3, 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 Na3, 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 determination 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 CaCl2, 0.2 mM ATP, 0.5 mM DTT, and 3.0 mM NaN3. 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).

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 EDTA, 0.1 mM CaCl2, and 1.0 mM MgATP (Ca2+ free, -10-6 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 concentration from ~1 x 10-6 M (free calcium ion concentrations calculated as in reference 18) to ~2 x 10-6 M inhibited the increases in viscosity of inside-out vesicle-spectrin-actin mixtures in some exper-
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 concentra-

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 (C) 0 µg/ml spectrin, (A) 2 µg/ml spectrin, (B) 5 µg/ml spectrin, (C) 30 µg/ml spectrin, or (D) 5 µg/ml heat-treated spectrin (5 min, 60°C). (A) 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.
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) supernatant 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.

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) the preformed F-actin to place it in the micropipettes used to measure viscosity.

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, spectrin, 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. 3a, 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. 7a 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. 8a 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 (35, 47) because similar results are obtained for inside-out vesicles reconstituted with purified spectrin tetramer instead of spectrin dimer (Fig. 8c 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), 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 spectrin-actin-depleted inside-out vesicles in the presence of spectrin dimer (18, 19). In the absence of membranes, 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 CaCl₂ and 2 mM MgCl₂, purified sheep spectrin tetramer has been reported to be active in cross-linking actin in the absence of band 4.1 (6).

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, 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 MgCl₂, 30 min, 32°C was mixed with inside-out vesicles and spectrin and incubated under polymerizing conditions as described in Materials and Methods. Additional MgCl₂ was added to the samples containing G-actin so that the final [MgCl₂] 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.
assays. 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 spectrin tetramer plus band 4.1 (Fig. 9a). In contrast, at 32°C, ankyrin markedly enhances the increases in viscosity induced by spectrin tetramer plus band 4.1 (Fig. 9b).

**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) 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. 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).

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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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