

# Spectrin-dependent and -independent Association of F-Actin with the Erythrocyte Membrane

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**ABSTRACT** Binding of F-actin to spectrin-actin-depleted erythrocyte membrane inside-out vesicles was measured using [<sup>3</sup>H]F-actin. F-actin binding to vesicles at 25°C was stimulated 5–10-fold by addition of spectrin dimers or tetramers to vesicles. Spectrin tetramer was twice as effective as dimer in stimulating actin binding, but neither tetramer nor dimer stimulated binding at 4°C. The addition of purified erythrocyte membrane protein band 4.1 to spectrin-reconstituted vesicles doubled their actin-binding capacity. Trypsinization of unreconstituted vesicles that contain <10% of the spectrin but nearly all of the band 4.1, relative to ghosts, decreased their F-actin-binding capacity by 70%. Whereas little or none of the residual spectrin was affected by trypsinization, band 4.1 was significantly degraded. Our results show that spectrin can anchor actin filaments to the cytoplasmic surface of erythrocyte membranes and suggest that band 4.1 may be importantly involved in the association.

The role of intracellular actin filaments in the generation of cell motility and the maintenance of cell shape is well recognized, and there is considerable morphological and some biochemical evidence suggesting that microfilaments may function, at least in part, through their association with cell membranes (1, 13, 14, 15). Human erythrocytes, like other cells, contain membrane-bound actin as a major component of their cytoskeleton (17, 24) and, although membrane-bound actin filaments have not been detected morphologically, indirect evidence suggests that at least some of the actin is in the form of short filaments (4, 8, 18). An important role for these actin filaments in the erythrocyte cytoskeleton was suggested by experiments in which tetramers of spectrin, the major erythrocyte cytoskeletal protein, with or without erythrocyte band 4.1, cross-linked actin filaments and formed three-dimensional viscous gels or oligomeric complexes (see footnote 1 and references 5, 9, 11 and 28). These experiments showed that F-actin can bind to spectrin and provided some insight into the assembly of the erythrocyte cytoskeleton. It remains unclear, however, whether erythrocyte actin is bound to the membrane only through its association with spectrin or whether independent associations can be formed with other membrane components. Here, we show that spectrin tetramers or dimers can stimulate binding of preformed F-actin to erythrocyte inside-out vesicles, and that F-actin can also bind to spectrin-depleted membrane vesicles via a trypsin-sensitive interaction.

<sup>1</sup> Cohen, C. M., and C. Korsgren. Manuscript submitted for publication.

## MATERIALS AND METHODS

### Preparation of Actin

Actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt (20) and was used within 5 d after extraction from acetone powder. [<sup>3</sup>H]actin was prepared by the method of Cohen et al. (7), except that G-actin was chromatographed on Bio-Gel P-4 (Bio-Rad Laboratories, Richmond, Calif.) using 2 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), pH 7.6, 0.5 mM ATP, 0.1 mM CaCl<sub>2</sub>, as the eluting buffer to remove traces of β-mercaptoethanol before labeling with [<sup>3</sup>H]*n*-ethylmaleimide. The specific activity of the actin ranged from 800 to 2,100 cpm/μg. Actin was polymerized at a concentration of 5.0 mg/ml before binding experiments by the addition of 100 mM KCl and 2 mM MgCl<sub>2</sub> (final concentrations) and incubation at 25°C for 30 min.

### Preparation of Spectrin

Spectrin dimers and tetramers were prepared by minor modifications of established methods (5, 16, 19, 27). Erythrocyte ghosts prepared as described by Steck and Kant (23) were washed once in 30 vol of ice-cold 0.1 mM NaPO<sub>4</sub>, pH 7.6. The pelleted ghosts were resuspended with 0.5 vol of the same buffer and incubated at 37°C for 40 min to extract spectrin dimers and then sedimented at 225,000 g for 30 min. The supernate was chromatographed at 4°C in a 2.5 × 100-cm column of Sepharose 4B using 0.1 M NaCl, 25 mM Tris-HCl, pH 7.6, and 0.1 mM EDTA as the eluting buffer. The dimer fractions (Fig. 2, Panel I) were pooled, concentrated by ultrafiltration to  $A_{280} = 1.0$ , and either used directly after 18-h dialysis against 20 mM KCl, 2 mM TES, pH 7.6 or incubated at 29.5°C for 2 h to promote conversion of dimer to tetramer (27). The incubated samples were then rechromatographed as described above, and the fractions containing spectrin tetramer (Fig. 2, Panel II) were pooled, concentrated, and dialyzed as described above. This two-step procedure for the preparation of tetramers was adopted to minimize potential contamination by void volume fractions which frequently spill over into tetramer fractions when tetramers are prepared by direct chromatography of 4°C membrane extracts (5, 27).

## Preparation of Band 4.1

Band 4.1 was extracted from spectrin-depleted vesicles by minor modification of established methods (25, 28). Membranes remaining after extraction of spectrin by dialysis at 4°C were washed once in 30 vol of 150 mM NaCl, 5 mM NaPO<sub>4</sub>, pH 7.6, and resuspended to 1.5 times their packed volume in this buffer. The membranes were then extracted by the addition of 0.8 M KCl, 0.1 mM dithiothreitol (DTT), and 3 mM phenylmethyl sulfonyl fluoride (PMSF; final concentrations) and incubated at 4°C for 6–18 h with stirring. After extraction, the membranes were sedimented at 225,000 g for 30 min and the supernate was desalted on a 3 × 10-cm Bio-Gel P4 column equilibrated with 5 mM NaPO<sub>4</sub>, pH 7.6, 1 mM EDTA, 20 mM KCl, and 0.5 mM DTT. The void volume fractions were then loaded into a 1 × 5-cm column of DEAE cellulose that had been preequilibrated with the buffer described above, and the column was flushed with 10 ml of the same buffer. The column was then flushed with 10–15 ml of 5 mM NaPO<sub>4</sub>, pH 7.6, 1 mM EDTA, 65 mM KCl, and 0.5 mM DTT and finally with 5 mM NaPO<sub>4</sub>, pH 7.6, 1 mM EDTA, 120 mM KCl, and 0.5 mM DTT, which elutes band 4.1. Elution was monitored by the A<sub>280</sub> of the column effluent and the fractions containing band 4.1 were concentrated by ultrafiltration and dialyzed for 18 h against 20 mM KCl, 2 mM TES, pH 7.6, 0.1 mM EDTA, and 0.1 mM PMSF for use in binding experiments. Typically, ~2–6 ml of protein at a concentration of 0.1 mg/ml were obtained from 50 ml of ghosts (see Fig. 4 for gel of purified band 4.1).

## Preparation of Membranes

Erythrocyte inside-out vesicles were prepared from ghosts (23) by incubation in 30 vol of 0.3 mM NaPO<sub>4</sub>, pH 7.6, or 0.1 mM EDTA, pH 8.5, at 37°C for 30 min. This simple treatment results in the elution of >90% of spectrin and actin and leads to formation of small (<0.5-μm) membrane vesicles that morphologically are inside-out (23). These spectrin-actin-depleted inside-out vesicles are referred to in the text simply as vesicles. Sealed ghosts were prepared by incubating freshly made ghosts in 5 mM NaPO<sub>4</sub>, pH 7.6, and 2 mM MgCl<sub>2</sub> at 37°C for 1 h.

## Measurement of Actin Binding

Binding of F-actin was done by incubating membranes at protein concentrations ranging from 0.15 to 0.5 mg/ml in 50 mM KCl, 2 mM MgCl<sub>2</sub>, 0.5 mM ATP, 5.0 mM NaPO<sub>4</sub>, pH 6.5, and 0.75 mM β-mercaptoethanol (binding buffer) with the appropriate concentrations of actin at 25°C for 1 h. After the incubation, triplicate 0.2-ml aliquots of the mixture were centrifuged at 12,500 rpm for exactly 10 min in a Sorvall SS34 rotor at 4°C (DuPont Instruments-Sorvall, DuPont, Co., Newtown, Conn.). 0.05-ml aliquots of each supernate were counted for <sup>3</sup>H, and the amount of [<sup>3</sup>H]F-actin in the pellet was computed from the difference between supernate and initial incubation mixture. Standard deviations were generally 12–17% of the values shown in the figures. Control experiments with vesicles labeled by phosphorylation with [γ-<sup>32</sup>P]ATP by standard methods (2) showed that 97–100% of vesicles and ghosts sedimented under these conditions, whereas <10% of the actin found in the pellet with spectrin-reconstituted vesicles (see Results) sedimented in their absence. The amount of actin sedimenting in the absence of vesicles was measured at each actin concentration in all experiments and was subtracted from the appropriate sample values. Vesicles were reconstituted with spectrin by incubating 0.6 mg/ml vesicles at 4°C for 1 h in binding buffer (pH 7.6) containing the final concentrations of spectrin indicated in the figures, sedimented, and, after all supernate had been carefully removed, resuspended to their original volume for actin binding measurements.

## Other procedures

Gel electrophoresis in 5% SDS polyacrylamide was performed according to the method of Fairbanks et al. (10), and protein bands were labeled in accordance with standard nomenclature (10, 22).

## RESULTS

Fig. 1 shows that inside-out vesicles (Fig. 1, gel a), which have 5–10% of the spectrin content of ghosts (Fig. 1, gel c), bind added F-actin, and that reconstituting these vesicles with spectrin dimers (Fig. 1, gel b) greatly increases their capacity to bind F-actin. No binding of F-actin to sealed ghosts was detected, showing that, whether or not vesicles were reconstituted with spectrin, the binding was specific to the cytoplasmic membrane surface.

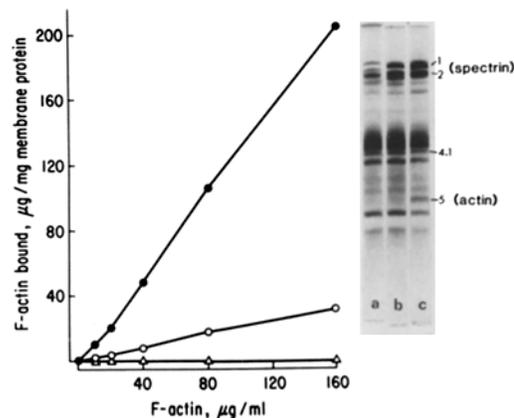


FIGURE 1 Binding of [<sup>3</sup>H]F-actin to sealed ghosts (Δ), inside-out vesicles (○), and inside-out vesicles reconstituted with purified spectrin dimer (●). Actin binding at 25°C and reconstitution of vesicles were performed as described in Methods. Gels: (a) inside-out vesicles; (b) inside-out vesicles reconstituted with 100 μg/ml spectrin dimer; and (c) sealed ghosts.

TABLE I  
pH Dependence of F-Actin Binding to Spectrin-Reconstituted Inside-Out Vesicles

| pH  | Actin binding<br>μg/mg vesicle protein |
|-----|--|
| 6.0 | 240                                    |
| 6.5 | 188                                    |
| 7.0 | 175                                    |
| 7.5 | 120                                    |
| 8.0 | 105                                    |

Inside-out vesicles were reconstituted with 25 μg/ml spectrin dimer as described in Methods. Actin binding was measured at the indicated pH values at an actin concentration of 100 μg/ml. Values shown are the means of three independent measurements and have standard deviations of 10–17%.

Actin binding to reconstituted vesicles depended upon pH, there being, on the average, a 28% decrease in binding for each pH unit above pH 6.0 (Table I). Our binding studies were routinely done at pH 6.5, at which pH the binding was ~36% greater than at the physiological pH of 7.5.

The spectrin dimer, which consists of two polypeptide chains, band 1 (240,000 daltons), and band 2 (220,000 daltons) can self-associate to form a tetramer, (1 + 2)<sub>2</sub>, which can be separated from the dimer by column chromatography (Fig. 2). Fig. 3 shows that actin binding to vesicles increased with added spectrin but was independent of whether the spectrin was in the dimeric or tetrameric form. Fig. 3 also shows that spectrin-stimulated actin binding was observed at 25°C but not at 4°C, a finding that correlates well with our observation<sup>1</sup> that spectrin cross-linking of F-actin in solution is much greater at 25°C than 4°C.

Because other actin-binding proteins are known to cause F-actin to sediment under conditions similar to the ones used here but in the absence of vesicles (6), we considered the possibility that residual spectrin not bound to vesicles during reconstitution was responsible for the increase in sedimentable F-actin. However, spectrin dimer or tetramer alone, at concentrations up to 10 μg/ml, in the absence of vesicles, failed to cause F-actin to sediment under the conditions used. (The concentration 10 μg/ml was chosen because we estimated that after reconstitution with spectrin no more than 5–10% of the supernate could have remained behind, and the highest con-

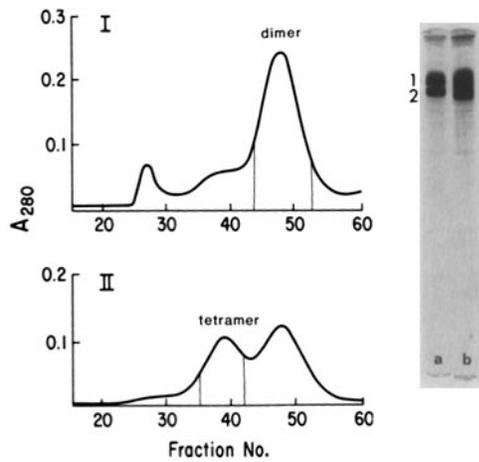


FIGURE 2 Chromatographic separation of spectrin dimers and tetramers, performed as described in Methods. Gels: (a) spectrin dimer, (b) spectrin tetramer. The minor bands seen in the gels are proteolytic fragments of spectrin of >120,000 mol wt.

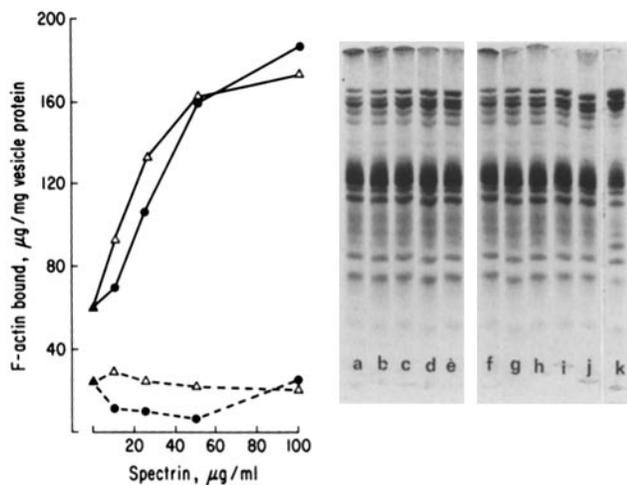


FIGURE 3 F-actin binding to spectrin-reconstituted vesicles. Vesicles were reconstituted as described in Methods with increasing concentrations of spectrin dimer (●) or tetramer (Δ). Following reconstitution, binding of 80 µg/ml [<sup>3</sup>H]F-actin was measured at a vesicle concentration of 0.25 mg/ml at either 4°C (dashed lines) or 25°C (solid lines). Gels: vesicles reconstituted with 0 (a), 10 (b), 25 (c), 50 (d), and 100 (e) µg/ml spectrin tetramer, and with 0 (f), 10 (g), 25 (h), 50 (i), 100 (j) µg/ml spectrin dimer. (k) Erythrocyte ghosts. At the highest spectrin concentration, 75% of the added F-actin was bound to vesicles.

centration of spectrin used was 100 µg/ml.) In addition, supernates obtained by sedimentation of spectrin-reconstituted vesicles that had been incubated under actin-binding conditions (without added actin) failed to stimulate sedimentation of subsequently added F-actin.

The data of Fig. 3 show that the tetramer and the dimer stimulated actin binding to nearly the same extent, and the gels show that there was an equivalent amount of total spectrin bound in both cases. That per unit weight there are half as many tetramers as dimers implies that tetramers are twice as efficient as dimers at binding actin to the membranes.

To determine how much actin could be bound to reconstituted vesicles, we measured actin binding at actin concentrations ranging from 0.05 to 1.0 mg/ml. Binding increased to a maximum of 315 µg/mg vesicle protein at 0.2 mg/ml actin but

decreased thereafter because of a failure of the vesicles to sediment at high actin concentrations under the centrifugation conditions used. By determining the amount of spectrin on reconstituted vesicles by densitometry of gels, and assuming an average of 1,000 actin monomers per filament, we calculate that at 0.2 mg/ml actin there was one actin filament bound for every 5–10 spectrin molecules on the vesicle. It is, of course, possible that single filaments could be anchored by more than one spectrin molecule.

Whereas spectrin alone can cross-link and bind to actin filaments (see footnote 1 and reference 5), recent studies have shown that erythrocyte membrane protein band 4.1 can stimulate or enhance this interaction (see footnote 1 and references 11 and 28). Consistent with this idea, Fig. 4 shows that, in the presence of spectrin, addition of band 4.1 doubled the actin-binding capacity of vesicles, whereas in the absence of added spectrin, addition of band 4.1 had little effect on actin binding. It should be noted, however, that the spectrin-depleted vesicles still contain nearly all of their original band 4.1.

Inside-out vesicles that are not reconstituted with spectrin retain 5–10% of the spectrin content and, as indicated above, 90% or more of the band 4.1 content of ghost membranes (measured by densitometry of gels). To determine whether these residual proteins were responsible for the binding of F-actin to unreconstituted vesicles, we trypsinized the vesicles and measured their subsequent ability to bind actin. Fig. 5 shows that trypsinization reduced the actin-binding capacity of vesicles by 70%, whereas SDS gels of these same vesicles (before addition of actin) show that no detectable loss or degradation of spectrin had occurred. Since membrane-bound spectrin is known to be trypsin sensitive (21), this lack of degradation could be explained if the spectrin were trapped within the small percentage of sealed right-side-out vesicles that invariably contaminate inside-out vesicles.

The gels do show that several protein bands were degraded by the trypsin, most notably bands 4.1, 4.2, 3, and 6. Separate

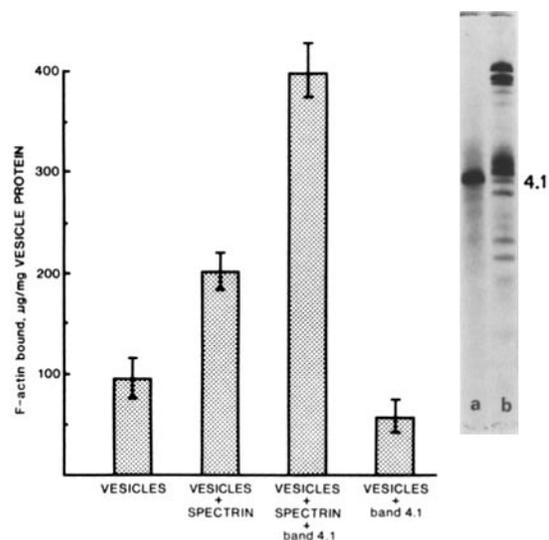


FIGURE 4 F-actin binding to vesicles with or without added spectrin dimer or band 4.1. Vesicles were reconstituted with 100 µg/ml spectrin dimer as described in Methods. The reconstituted or unreconstituted vesicles at a protein concentration of 0.125 mg/ml were tested for binding of 100 µg/ml F-actin at 25°C as described in Methods with or without the inclusion of 25 µg/ml band 4.1. Gels: (a) purified band 4.1, (b) ghosts.

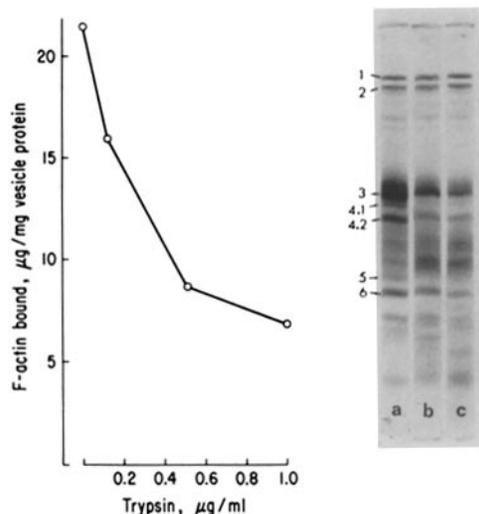


FIGURE 5 Reduction of F-actin binding to unreconstituted vesicles by trypsinization. Vesicles at a concentration of 0.5 mg/ml were incubated with the indicated concentrations of trypsin in binding buffer (pH 7.6) at 25°C for 0.5 h, at which time a 15-fold weight excess (relative to trypsin) of PMSF was added to all samples (including control). Vesicles were washed once in binding buffer containing 15 µg/ml PMSF and resuspended for measurement of F-actin binding at 25°C at an actin concentration of 80 µg/ml. Gel: (a) control vesicles, 0 µg/ml trypsin; (b) 0.5 µg/ml trypsin; and (c) 1.0 µg/ml trypsin. Sedimentation of vesicles for the binding assay resulted in 90–100% vesicle recovery at all trypsin concentrations, measured as described in Methods.

experiments (not shown) demonstrate that vesicles devoid of band 6 bind as much actin as those with band 6. Further work is in progress to determine which of the remaining proteins may bind actin to the membrane. In light of the results described above, however, we suggest that the band 4.1 protein on the membrane may be responsible for at least part of the observed F-actin binding to unreconstituted vesicles.

## DISCUSSION

The results of our experiments show that spectrin can anchor actin filaments to erythrocyte membranes and that band 4.1 may be importantly involved in this association. Because it has been reported that spectrin can bind to band 4.1 (25), our findings imply that spectrin and actin may both associate with band 4.1 on the membrane and share it as a common membrane attachment site. Thus, band 4.1 may play the dual role of helping to anchor the erythrocyte cytoskeleton to the membrane and promoting the association of the two major cytoskeletal components, spectrin and actin.

Our findings are consistent with the known geometry of the actin- and membrane-binding sites on the spectrin molecule. Spectrin binds to inside-out vesicles through a high-affinity association with the membrane protein band 2.1 (3, 12, 29). The work of Tyler et al. (25) shows that the 2.1 binding site is near the head of the dimer or near the middle of the tetramer, which is formed by head-to-head association of two dimers (19, 27). More recently work, using the technique of low-angle shadowing,<sup>2</sup> demonstrates that spectrin's actin-binding sites lie at the tails of the tetramer molecule, making each tetramer

bivalent in actin-binding sites and consequently able to cross-link adjacent actin filaments. Thus, dimers can bind to membranes at a site near their head and would have one actin-binding site available at their free tail, whereas tetramers could bind to membranes near their middle and would have two actin-binding sites available.

Whereas band 4.1 may be required for spectrin-stimulated actin binding, we cannot conclude from our findings whether band 4.1 is specifically involved in actin binding to unreconstituted vesicles. The reduction in actin binding to vesicles by proteolysis shown in Fig. 5 could simply reflect reduction of nonspecific (possibly electrostatic) binding caused by loss of vesicle protein rather than loss of a specific protein.

Our current findings are in contrast to but consistent with our previous report, which showed that purified spectrin dimers would not stimulate binding of added G-actin to vesicles, whereas an oligomeric complex of spectrin, actin, and band 4.1 would (8). In that report we put forth a proposal, subsequently partially confirmed by Brenner and Korn (4), that the oligomeric complex stimulated binding because it itself bound to the membrane and contained actin filament seeds that stimulated polymerization and growth of F-actin from the membrane. Because those experiments (8) were performed at 4°C and low G-actin concentration (40 µg/ml), filament formation without added nucleation sites would have been slow. Consequently, in the absence of the complex, there would have been few filaments to bind to the reconstituted vesicles. Furthermore, our current findings show that there is no stimulation of F-actin binding by spectrin dimers at 4°C, the temperature at which binding was measured previously.

Spectrin's similarity to filamin and actin-binding protein from macrophages has recently been demonstrated structurally by low-angle shadowing (26) and biochemically by its ability to cross-link and gel actin filaments (see footnote 1 and references 5, 9, 11, 28). It seems likely that these similarities would arise from a common intracellular function. Although the binding of filamin or actin-binding protein or plasma membranes has yet to be demonstrated, the results of our experiments suggest that an important role of such proteins, with or without such accessory proteins as band 4.1, may be to anchor actin filaments to plasma membranes.

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*Note Added in Proof:* After this work was completed, similar conclusions were reached by V. Fowler, E. J. Luna, W. R. Hargreaves, D. L. Taylor, and D. Branton (manuscript submitted for publication) using different techniques.

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