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THE ROLE OF BAND 4.1 IN THE ASSOCIATION OF ACTIN WITH ERYTHROCYTE MEMBRANES

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Spectrin stimulates the association of F-actin with erythrocyte inside-out vesicles. Although inside-out vesicles are nearly devoid of two of the three major cytoskeletal proteins, spectrin and actin, they retain nearly all of the cytoskeletal protein designated band 4.1. Inside-out vesicles which have been substantially depleted of band 4.1 by extraction in 1 M KCl, 0.4 M urea and then reconstituted with spectrin show a markedly diminished ability to bind actin by comparison with vesicles containing normal amounts of band 4.1. This diminution is not due to an impaired ability of the vesicles to bind spectrin. Addition of purified band 4.1 to vesicles either before or after they have been reconstituted with spectrin restores their actin binding capacity to near normal levels as does addition of a spectrin-band 4.1 complex prepared by sucrose gradient centrifugation. Band 4.1 bound to vesicles in the absence of added spectrin has no effect on actin binding. Our results suggest that a spectrin band 4.1 complex is responsible for binding actin to erythrocyte membranes.

Introduction

The cytoskeletons of eukaryotic cells appear to make contact with the plasma membrane in a variety of ways. In some cells focal contacts of actin filaments or filament bundles with specialized membrane sites as well as lateral contact of filaments with membranes have been observed [1,2] but the molecular details of such contacts remain obscure. More recent observations suggest that in some cases the outer boundary of the cytoskeleton may be so extensively membrane associated that the distinction between cytoskeletal and peripheral membrane proteins becomes arbitrary [3,4]. The cytoskeleton of the human erythrocyte may be unique because it is entirely membrane associated, being confined to a shallow region just beneath the plasma membrane and anchored there by multiple high-affinity contacts between the major cytoskeletal protein spectrin and its membrane binding site, ankyrin (for reviews see Ref. 5 and 6).

Considerable evidence suggests that the remarkable stability of the shell-like cytoskeleton which remains when red cells are extracted with non-ionic detergents is due to the high density of interconnections between the major cytoskeletal proteins spectrin, actin and band 4.1 Some of these interconnections or associations have been measured biochemically and have also been visualized by electron microscopy of purified cytoskeletal proteins in solution (for review, see Ref.

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Abbreviations: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

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5). The association between spectrin and actin is of particular interest to us because actin is ubiquitous in eukaryotic cells and spectrin is similar to actin binding proteins found in other cell types. Thus, insights into the interaction of these proteins in the erythrocyte cytoskeleton may provide paradigms for the study of similar associations in more complex cells.

Previous work has shown that spectrin dimers contain a single actin binding site at their tail while spectrin tetramers, formed by head to head association of two dimers, possess two actin binding sites, one at each end [7]. Because spectrin tetramers are bivalent in actin binding sites they can crosslink actin filaments in solution to form a gel. Although long actin filaments are not apparent in the red cell cytoskeleton the crosslinking of short actin filaments by spectrin has been proposed to be an important structural feature of the cytoskeleton [5,8,9].

While spectrin can bind to and crosslink actin filaments by itself [10], the addition of the third major cytoskeletal protein band 4.1 enhances the interaction between spectrin and actin in solution [9,11,12]. Previously, we examined the interaction between actin and spectrin which was bound to the erythrocyte membrane and showed that spectrin could stimulate binding of actin to inside-out vesicles and that added band 4.1 could augment this binding [13]. Similar effects of membrane-bound spectrin have also been detected by other techniques [14]. Here we examine further the role of band 4.1 and provide evidence that it is necessary for spectrin-dependent binding of actin to erythrocyte inside-out vesicles.

Methods

Preparation of actin

Actin was extracted from rabbit skeletal muscle by the method of Spudich and Watt [15] with the exception that the F-actin was extracted with 0.8 M KCl rather than 0.6 M KCl prior to sedimentation. ^{3}H-labeled G-actin was prepared as described in Cohen et al. [16] or by the following time saving modification. Actin was extracted from acetone powder in 2 mM Tes, pH 8.0, 0.2 mM $CaCl_2$, 0.5 mM ATP as described [15] except that dithiothreitol was omitted. The mixture was centrifuged for 30 min in a Sorvall SS34 Rotor at 10000 rpm and the crude extract was saved. *N*-[^{3}H]Ethylmaleimide (New England Nuclear, 692–1100 mCi/mmol) was diluted to a specific activity of 100 mCi/mmol by addition of unlabeled *N*-ethylmaleimide in pentane and 63 nmol was added to a clean glass tube per mg protein to be labeled. After the pentane was evaporated the crude actin extract was added to the tube and incubated at room temperature with end-over-end tumbling for 30 min, followed by additon of 5 mM β-mercaptoethanol. The actin was then polymerized and extracted in 0.8 M KCl as described above. After centrifugation at $220000 \times g$ for 2 h the F-actin was resuspended by homogenization and dialyzed at least 18 h against 2 mM Tes, pH 8.0, 0.2 mM $CaCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol. The final specific activity of the [^{3}H]actin was 800–2100 cpm/μg. Prior to use ^{3}H-labeled G-actin was polymerized at a concentration of 5.0 mg/ml in the presence of 100 mM KCl, 2 mM $MgCl_2$ at 25°C for 45 min.

Preparation of spectrin and band 4.1

Spectrin was prepared by minor modifications of previously described methods [17,18]. Briefly, erythrocyte ghosts prepared as described by Steck and Kant [19] were washed once in 30 vol. of ice-cold 0.1 mM EDTA, pH 8.5. The pelleted ghosts were resuspended in 0.5 vol. of the same buffer and incubated at 37°C for 40 min to extract spectrin dimers and then sedimented at $225000 \times g$ for 30 min. The supernate was chromatographed on a 2.5×90 cm column of Sepharose 4B at 4°C using 0.1 M KCl, 2 mM Tes, pH 7.6, as the eluting buffer. The dimer fractions were concentrated by ultrafiltration to $A_{280} = 1.0$.

For some experiments spectrin was labeled with *N*-[^{3}H]ethylmaleimide by a procedure nearly identical to that described for labeling of actin above. Unbound *N*-[^{3}H]ethylmaleimide was removed by dialysis of spectrin against 2000 vol. of 0.3 mM sodium phosphate, pH 7.6, 0.5 mM β-mercaptoethanol at 4°C. At the end of 24 h 97% of the ^{3}H in the dialysis bag was precipitable by 10% trichoroacetic acid.

Purified band 4.1 was prepared from ghosts as described previously [13,20] and was dialysed against 5 mM sodium phosphate, pH 7.0 or pH

7.6, 0.2 mM dithiothreitol, 0.02% NaN_3 for 18 h prior to use. In several experiments band 4.1 was labeled with ^{125}I using Bolton-Hunter reagent as described [20].

Preparation of spectrin-band 4.1 complex

Spectrin (0.5 mg/ml) was incubated with 0.1 mg/ml band 4.1 in 50 mM KCl, 5 mM sodium phosphate, pH 7.6, 0.5 mM dithiothreitol, 0.2 mM EGTA, 0.15 mM $CaCl_2$ at 4°C for 1 h. The proteins were then loaded onto a 5–20% sucrose gradient made in the same medium and centrifuged for $2.7 \cdot 10^8\ g \cdot$ min max. in either an SW50.1 or an SW41 rotor. Gradients were fractionated into 20 fractions and protein peaks were located by either A_{280} or ^{125}I.

Preparation of membranes

Erythrocyte ghosts were prepared as described by Steck and Kant [19] by hypotonic lysis in 5 mM sodium phosphate, pH 7.6. Inside-out vesicles were prepared by diluting 1 vol. of packed ghosts directly into 40 vol. of 0.1 mM EDTA, pH 8.5 (pre-warmed to 37°C) and incubating at 37°C for 40 min. The vesicles were washed once in ice-cold 0.1 mM EDTA and once in 5 mM sodium phosphate, pH 7.6, in which they were stored with 0.02% NaN_3.

Inside-out vesicles depleted (but not devoid) of band 4.1 were prepared as described by Hargreaves et al. [21]. Briefly, inside-out vesicles were diluted into 20 vol. of ice-cold 1 M KCl, 0.4 M urea, 25 mM EDTA, 50 mM glycine, pH 6.75 and incubated on ice for 35 min. The vesicles were pelleted and washed once in this same medium followed by three washes in 40 vol. of 0.3 mM sodium phosphate, pH 7.6 and one wash in 5 mM sodium phosphate, pH 7.6 at 4°C.

Inside-out vesicles were reconstituted with spectrin or band 4.1 by incubating 0.6 mg/ml vesicles with the protein (concentrations indicated in figures) in 20 mM KCl, 2 mM $MgCl_2$, 5 mM sodium phospate, pH 7.6 at 4°C for 1 h. The vesicles were washed once in 5 mM sodium phosphate, pH 7.6 prior to further use.

Measurement of actin binding

Binding of F-actin to inside-out vesicles was quantified by measuring disappearance of ^3H-labeled F-actin form supernates obtained after sedimentation of vesicles as described [13]. Briefly, ^3H-labeled F-actin (100 μg/ml unless otherwise specified) was incubated with membranes at a protein concentration of 0.125 mg/ml in 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM ATP, 0.75 mM β-mercaptoethanol, 5 mM sodium phosphate, pH 7.0 (binding buffer) at 25°C for 1 h. After the incubation triplicate 0.2 ml aliqutos of the mixture were centrifuged at 12500 rpm for exactly 10 min in a Sorvall SS34 rotor at 4°C. 0.05 ml samples of each supernate were counted for ^3H and the amount of ^3H-labeled F-actin in the vesicle pellet was computed from the difference between supernate and initial reaction mixture. The amount of actin sedimenting in the absence of vesicles was measured at each actin concentration used in all experiments and was subtracted from the appropriate sample values.

All values of actin binding shown in the figures represent the means of three independent determinations on separate samples. The standard deviations of these values were, unless specifically indicated otherwise, less than 15% of the values shown.

Other procedures

Gel electrophoresis in the presence of SDS was done in 5% acrylamide gels according to the method of Fairbanks et al. [22], or in 5–15% acrylamide gradient gels by the method of Laemmli [23]. The Laemmli gel shown in Fig. 7 was stained by the ultrasensitive silver staining procedure of Oakley et al. [24].

Results

Fig. 1 shows that actin binding to inside-out vesicles reconstituted with spectrin was reduced if the vesicles were preextracted with various concentrations of KCl and urea. SDS gels of the extracted vesicles show that the major proteins removed by these treatments are band 4.1 and band 6 (gel a in Fig. 1 is identical to a gel of untreated inside-out vesicles). Band 6 was eluted by 0.4 M urea plus 0.1 M KCl (compare gels a, b) well before any reduction in actin binding was seen and consequently plays no role in actin binding under our conditions. In the presence of 0.4 M

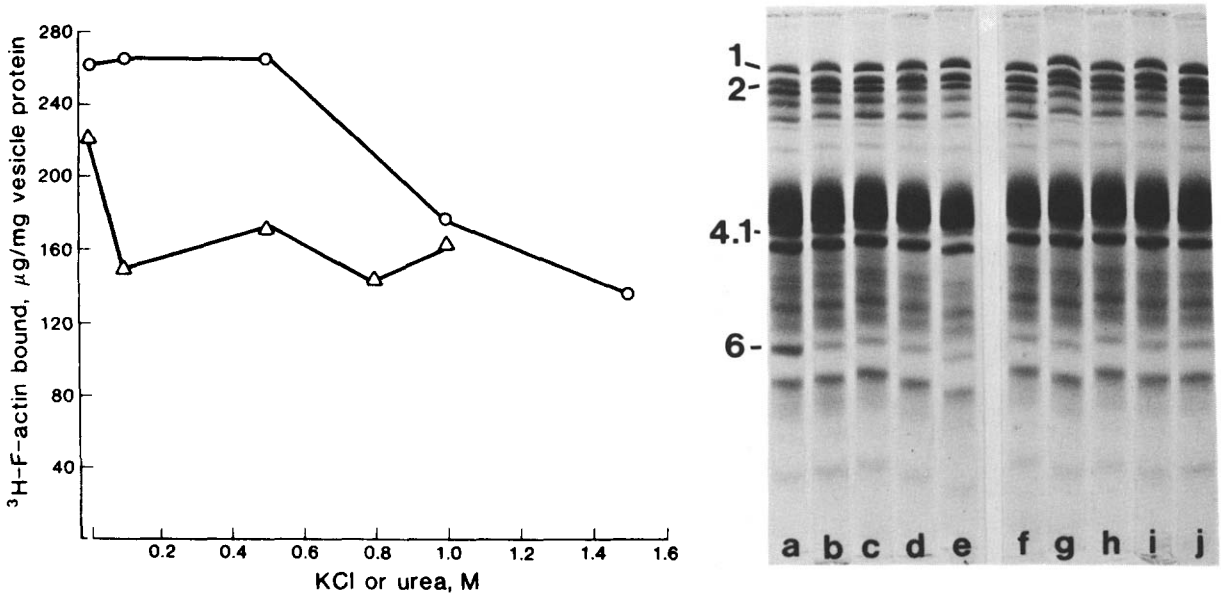

Fig. 1. Binding of F-actin to inside-out vesicles extracted in various media then reconstituted with spectrin. Samples of inside-out vesicles were extracted with 25 mM EDTA, 50 mM glycine, pH 6.75 and 0.4 M urea with increasing KCl (○——○) or 25 mM EDTA, 50 mM glycine, pH 6.75 and 1 M KCl with increasing urea (△——△) at 4°C for 35 min. The vesicles were washed once in the medium in which they were extracted, three times in 0.3 mM sodium phosphate, pH 7.6 and once in 5 mM sodium phosphate, pH 7.6. The vesicles were then reconstituted with 50 μg/ml spectrin (see Methods) and tested for their ability to bind 100 μg/ml added ^{3}H-labeled F-actin. Gels: vesicles extrated in the above buffer containing 0.4 M urea and 0 (a), 0.1 (b), 0.5 (c), 1.0 (d), or 1.5 (e), M KCl, or containing 1 M KCl and 0 (f), 0.1 (g), 0.5 (h), 0.8 (i) or 1.0 (j), M urea. Vesicles were reconstituted with spectrin after extraction. Gel (a) is identical to a gel of untreated vesicles reconstituted with spectrin (as above) which bound 260 μg F-actin/mg vesicle protein.

urea, KCl concentrations above 0.6 M caused a reduction in actin binding accompanied by a significant loss of band 4.1 (compare gels a, and d or e). In the presence of 1 M KCl even 0.1 M urea caused a reduction in actin binding and the gels show that in 1 M KCl band 4.1 was reduced at all urea concentrations.

While the extraction conditions used in Fig. 1 and those used to prepare band 4.1 depleted vesicles used in the following experiments eluted primarily bands 6 and 4.1 we frequently, but not always, observed some diminution in band 2.1 (ankyrin). Because ankyrin binds spectrin to the membrane and because F-actin binding to erythrocyte membranes is dependent upon membrane-bound spectrin in a dose-dependent manner [13] it is possible that the diminution in actin binding was due to diminished spectrin binding. While in some experiments vesicles depleted of band 4.1 bound less spectrin than control vesicles, this was not always the case. In several vesicle preparations, direct measurement of spectrin binding using [^{3}H]spectrin (Fig. 2) revealed no significant difference between control and 4.1 depleted vesicles, showing that depletion of band 4.1 does not in and of itself result in reduced spectrin binding. We did not investigate the effect on spectin binding of adding excess band 4.1 to normal inside-out vesicles, but Tyler et al. [25] reported that this can increase spectrin binding. Consequently, care was taken in all experiments to ensure that comparison of actin binding betweeen 4.1 depleted and control vesicles was done with vesicles containing as near as possible equivalent amounts of spectrin.

F-actin binding to normal inside-out vesicles increased nearly 4-fold upon prior addition of spectrin to the vesicles (Fig. 3 and gels a–e), but F-actin binding to inside-out vesicles depleted of band 4.1 hardly increased at all upon addition of

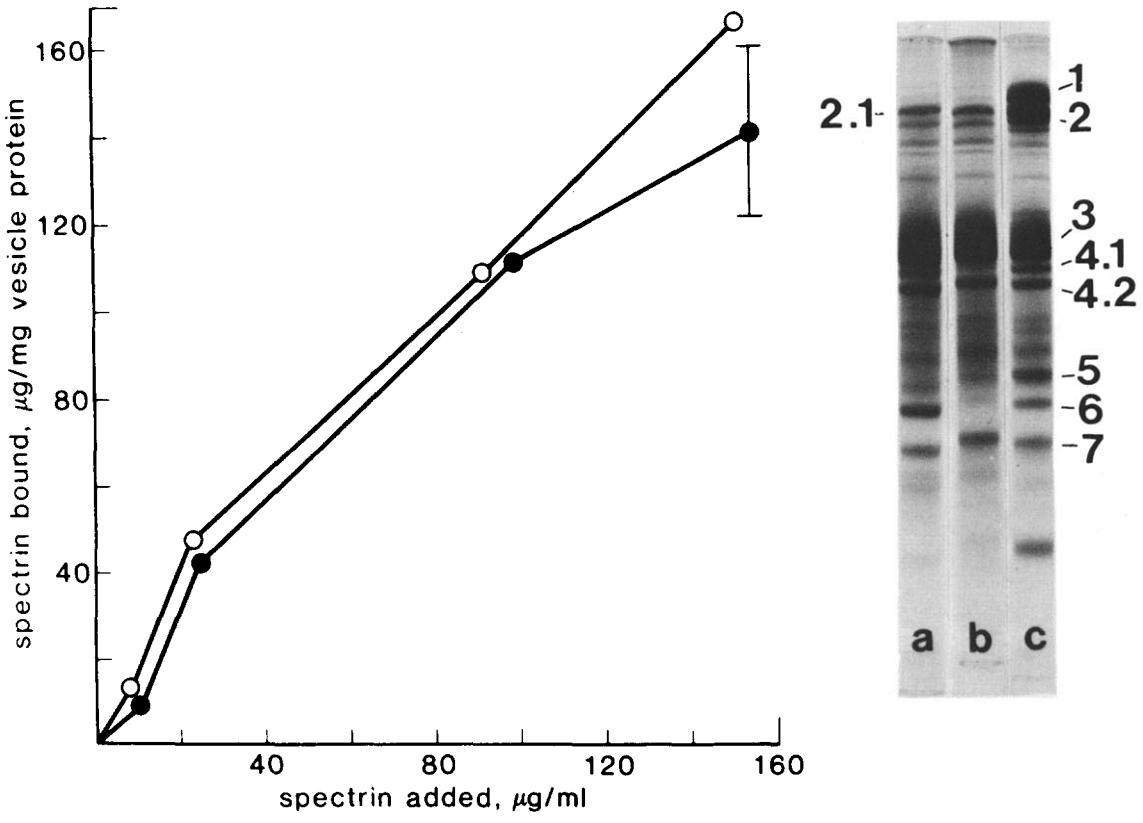

Fig. 2. [^{3}H]spectrin binding to inside-out vesicles. Control (●———●) and band 4.1 depleted (○———○) inside-out vesicles at a concentration of 0.25 mg/ml were incubated with the indicated concentrations of [^{3}H]spectrin in 50 mM KCl, 2 mM $MgCl_2$, 5 mM sodium phosphate, pH 7.6 at 4°C. At the end of 1 h, triplicate 0.2 ml aliquots of each mixture were centrifuged in a Sorvall SS34 rotor at 18000 rpm for 15 min to sediment vesicles and bound spectrin. 0.025 ml of each supernate was counted for ^{3}H, and the amount of spectrin bound was determined by measuring disappearance of spectrin from the supernate relative to the initial reaction mixture. Gels: (a) inside-out vesicles, (b) 4.1 depleted inside-out vesicles, (c) ghosts.

the same concentrations of spectrin. Comparison of the gels of the 4.1 depleted vesicles (Fig. 3, gels f–j) and the normal vesicles (Fig. 3, gels a–e) shows that the two types of vesicle have about the same amount of spectrin on them at each spectrin concentration. Fig. 4 shows that when actin binding was plotted versus the amount of spectrin bound to the vesicles the 4.1 depleted vesicles had a smaller increase in actin binding per unit bound spectrin than did control vesicles. Thus, spectrin bound to 4.1 depleted vesicles cannot bind actin to the same degree as spectrin bound to normal vesicles can.

If the reduction of actin binding to vesicles was the result of 4.1 depletion and not, for example, denaturation of some vesicle component by the KCl-urea extraction, then re-addition of purified band 4.1 to 4.1 depleted vesicles should restore their actin binding capacity. Fig. 5 shows that addition of purified band 4.1 to 4.1 depleted vesicles which were reconstituted with spectrin increased their actin binding capacity to a nearly normal level. Also, a given amount of band 4.1 enhanced actin binding to the same extent whether it was added to the vesicles before or after spectrin. Fig. 5 also shows the results of a separate experiment which demonstrates that band 4.1 by itself had no effect on actin binding when spectrin was not added to vesicles.

Because it is known that spectrin plus band 4.1 can, under certain circumstances, cosediment with F-actin we considered the possibility that some of

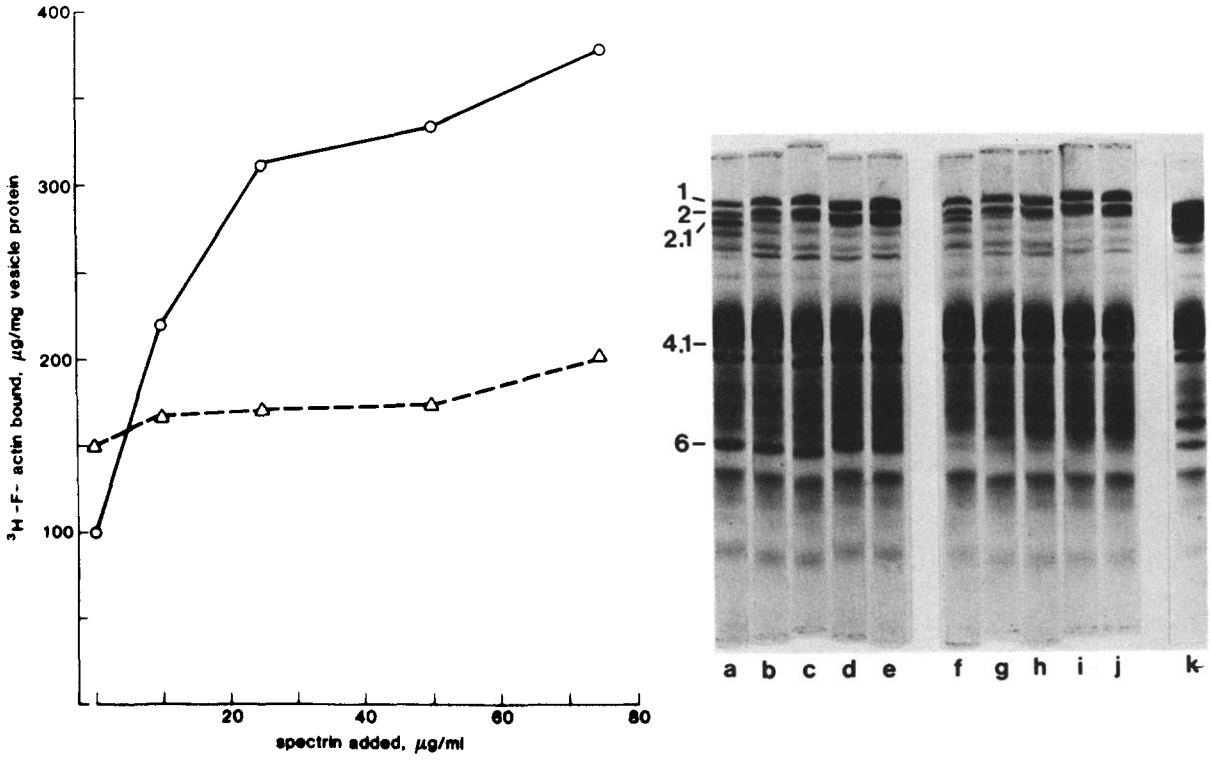

Fig. 3. Binding of F-actin to inside-out vesicles and 4.1 depleted inside-out vesicles. Inside-out vesicles (○——○) and 4.1 depleted inside-out vesicles (△------△), (prepared as described in Methods) were reconstituted with the concentrations of spectrin shown. Actin binding was measured as described in Methods. Gels: normal vesicles reconstituted with 0 (a), 10 (b), 25 (c), 50 (d), 75 (e) μg/ml spectrin. 4.1 depleted vesicles reconstituted with 0 (f), 10 (g), 25 (h), 50 (i), 75 (j) μg/ml spectrin. Each gel contains 40 μg of protein.

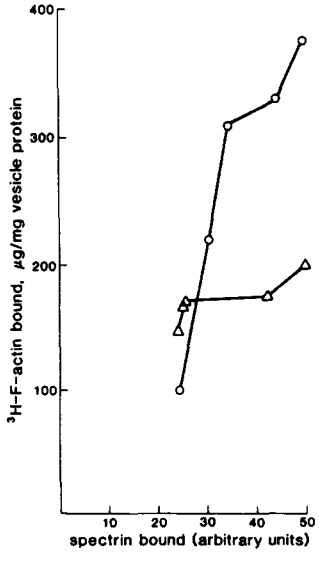

Fig. 4. The gels shown in Fig. 3 were scanned on a densitometer and the peaks corresponding to spectrin were cut out and weighed. The values for bound actin were then plotted versus the amount (arbitrary units) of spectrin bound to each sample of inside-out vesicles. ○——○, inside-out vesicles; △——△, 4.1 depleted inside-out vesicles.

the binding we were measuring resulted from artifactual cosedimentation of these proteins independent of the vesicles. To test this hypothesis we incubated 25 μg/ml band 4.1 with concentrations of spectrin dimer ranging from 0 to 50 μg/ml in the presence of 100 μg/ml ^{3}H-labeled F-actin, under conditions identical to those used for the vesicle actin binding assay, but in the absence of vesicles. Samples were centrifuged and sampled for actin sedimentation as though there were vesicles present. We found at most a 3 to 4 μg/ml disappearance of actin from the supernatant. Because a typical assay contained 0.125 mg/ml vesicle protein, this corresponds to maximal contribu-

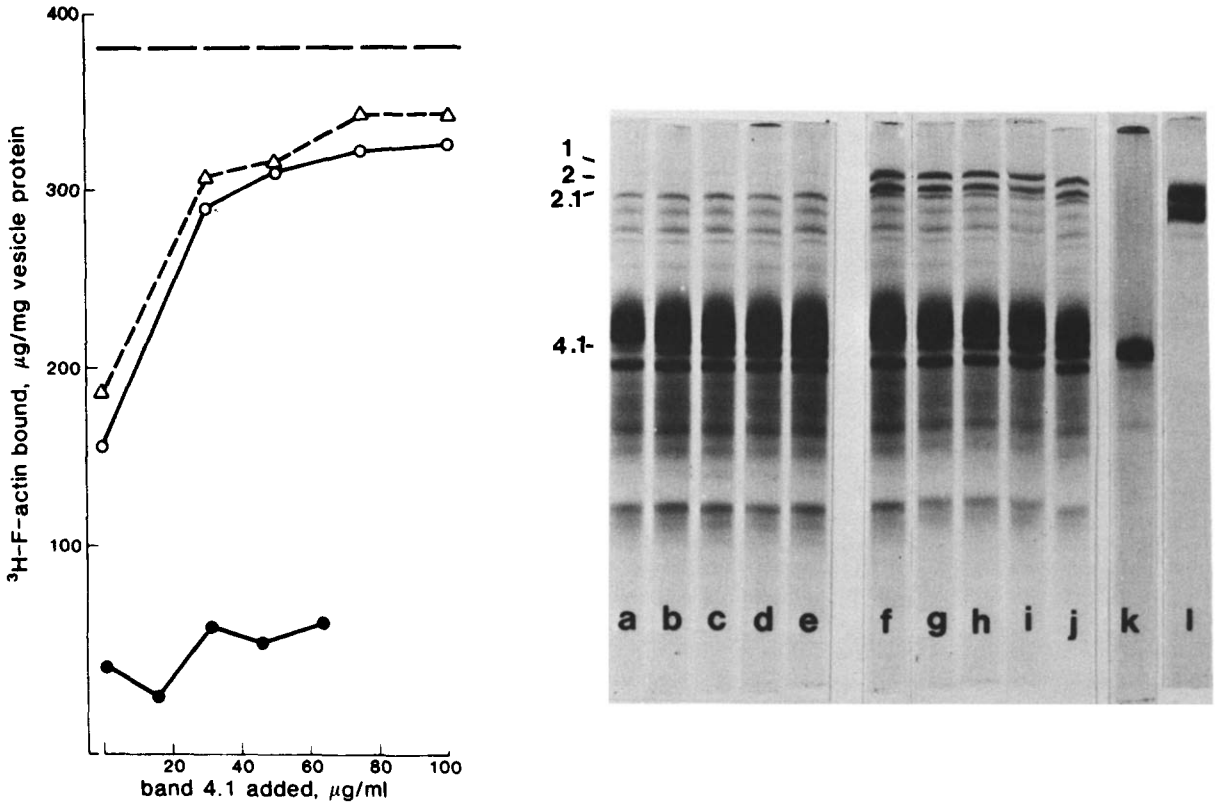

Fig. 5. F-actin binding to band 4.1 depleted vesicles after reconstitution with purified band 4.1 and spectrin. Band 4.1 depleted inside-out vesicles were incubated with the indicated concentrations of purified band 4.1 as described in Methods, washed once and then reconstituted with 150 μg/ml spectrin (O——O) or, reconstituted with 150 μg/ml spectrin first, then with band 4.1 (△------△). Actin binding was measured as described in Methods. (– –) actin binding to unextracted (normal 4.1 content) inside-out vesicles reconstituted with 150 μg/ml spectrin and no added band 4.1. (●——●) actin binding to 4.1 depleted inside-out vesicles reconstituted with the indicated concentrations of band 4.1 and no added spectrin (these latter vesicles were from a different preparation from those used in the other experiments in this figure). Gels: band 4.1 depleted vesicles reconstructed with 0 (a), 20 (b), 40 (c), 60 (d), 80 (e) μg/ml band 4.1. Band 4.1 depleted vesicles reconstituted with 150 μg/ml spectrin followed by 0 (f), 20 (g), 40 (h), 60 (i), 80 (j) μg/ml band 4.1. Vesicles reconstituted with spectrin and band 4.1 in the reverse order were nearly identical to those in appearance. (k) purified band 4.1; (l) purified spectrin.

tion of 32 μg ^{3}H-labeled F-actin/mg vesicle protein to vesicle binding, which is a small fraction of the total amount of actin bound in a typical experiment. In practice it is unlikely that such high concentrations of free spectrin and band 4.1 could be present during the binding assay since the vesicles were always washed once after being reconstituted with these proteins.

The gels in Fig. 5 show that the band 4.1 content of the vesicles increased with added band 4.1. However, Fig. 5 shows that while 30 μg/ml added band 4.1 doubled actin binding to vesicles, higher concentrations failed to increase actin binding proportionately. To determine whether this was the result of a limitation in the amount of band 4.1 which could be bound to the vesicles we measured binding of ^{125}I-labeled band 4.1 to inside-out vesicles. Fig. 6 shows a representative study from a number of experiments from which we have concluded the following: (a) band 4.1 binding to normal or 4.1 depleted vesicles increases with added 4.1 in an apparently non-saturable manner. It is unlikely that this apparent non-saturability is due to the presence of a high proportion of denatured band 4.1 because in concurrent experiments we found that typically 60–75% of band 4.1 bound

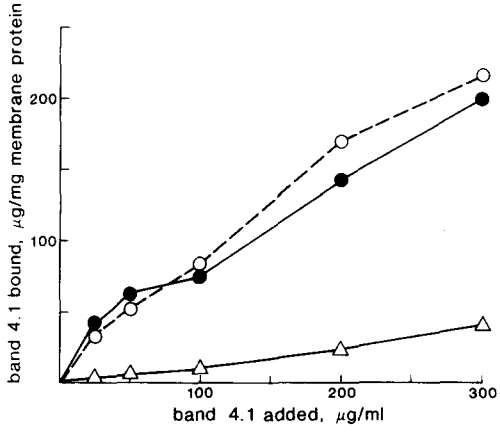

Fig. 6. Binding of ^{125}I-band 4.1 to inside-out vesicles and sealed ghosts. Inside-out vesicles (O------O), inside-out vesicles reconstituted with 100 μg/ml spectrin (●———●) or sealed ghosts (△———△) were incubated at a protein concentration of 0.25 mg/ml with the indicated concentrations of ^{125}I-band 4.1 (2384 cpm/μg) in 50 mM KCl, 2 mM $MgCl_2$, 5 mM sodiumphosphate, pH 7.6, 0.5 mM ATP, 0.75 mM β-mercaptoethanol at 25°C for 1 h. Bound ^{125}I-band 4.1 was measured in the same way as actin binding (see Methods). Each data point represents the mean of triplicate determinations, with a standard deviation of less than 15% of the values shown. The amount of band 4.1 contained in ghosts is about 40 μg/mg membrane protein [28].

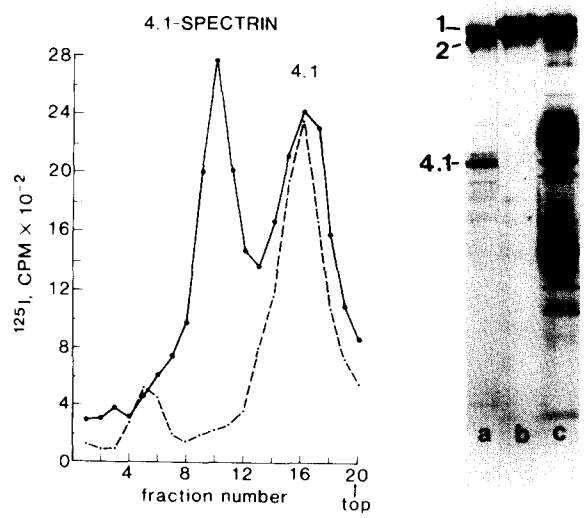

Fig. 7. Preparation of spectrin-4.1 complex. ^{125}I-labeled band 4.1 was incubated with spectrin (●———●) or by itself (– · –) (sample without spectrin contained about 1/3 as much ^{125}I-labeled band 4.1 as that with spectrin) and centrifuged on a 5–20% sucrose gradient as described in Methods. The gradient shown here was spun in a Bechman SW 50.1 rotor, and the ^{125}I counts refer to the whole of each fraction. Gels were taken from a gradient prepared in a similar fashion but in an SW 41 rotor using nonradioactive band 4.1. Gels: (a) spectrin-4.1 complex, fraction 10, (b) spectrin without band 4.1 prepared on a separate gradient, (c) ghost standard. Note that in the Laemmli gels shown here band 4.1 migrates as a dimer, often referred to as 4.1 a and b.

to spectrin in solution (data not shown). (b) The amount of band 4.1 bound to vesicles is independent of their spectrin content. (c) Band 4.1 binding after 60 min is the same at 4°C as 25°C. (d) There is significantly less binding of band 4.1 to sealed ghosts than to inside-out vesicles. Conclusion (a) above, implies that the inability of high concentrations of band 4.1 to increase actin binding to spectrin-containing vesicles in proportion to added 4.1 was not due to a limitation in band 4.1 binding to vesicles.

Because both spectrin and band 4.1 were required for actin binding, and because spectrin binds to band 4.1 in solution [20] we hypothesized that band 4.1 enhanced actin binding to vesicles by forming a complex with spectrin on the membrane. To test this hypothesis we prepared a complex of spectrin and band 4.1 by rate zonal sedimentation through sucrose using methods similar to those described by Tyler et al. [20]. Fig. 7 shows that while band 4.1 alone migrated near the top of a 5–20% sucrose gradient, in the presence of spectrin about half of the band 4.1 comigrated with spectrin further into the gradient. Gel electrophoresis of fraction 10 of the gradient shown (gel a Fig. 7) reveals the presence of both spectrin and band 4.1. Fig. 8 shows that when band 4.1 depleted vesicles were reconstituted with this complex they bound about twice as much actin as those which were reconstituted with spectrin alone (prepared by sucrose gradient centrifugation in the same way as the spectrin-4.1 complex; Fig. 7, gel b). Further, 4.1 depleted vesicles reconstituted with the spectrin-4.1 complex bound as much actin as 4.1 depleted vesicles reconstituted with spectrin plus band 4.1 added separately or as control (no 4.1 extracted) vesicles reconstituted with spectrin. Vesicles reconstituted with the spectrin-4.1 complex contained as much spectrin as those reconstituted with spectrin alone (Fig. 8, gels b, c).

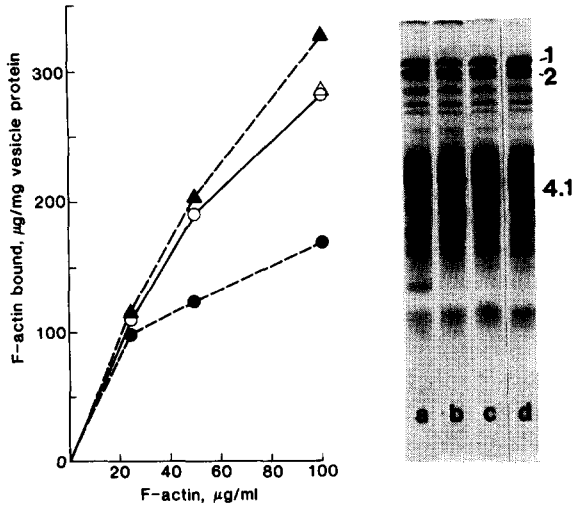

Fig. 8. Actin binding to 4.1 depleted vesicles reconstituted with spectrin-4.1 complex. The spectrin-4.1 complex was prepared as described in Methods and Fig. 7 and is shown in Fig. 7 gel a. Spectrin for this experiment was treated in the same way as the spectrin-4.1 complex and was also spun in a 5–20% sucrose gradient (Fig. 7, gel b). Band 4.1 depleted inside-out vesicles were reconstituted with 50 μg/ml spectrin (●------●) or 60 μg/ml spectrin-4.1 complex (○——○) (more protein was added to account for the fact that 15–20% of the protein in the spectrin-4.1 complex was band 4.1), or with 50μg/ml spectrin plus 8 μg/ml band 4.1 added separately (▲------▲). Also shown is one point corresponding to unextracted inside out vesicles reconstituted with 50 μg/ml spectrin (△), all of the other data points for this sample fell exactly on top of those from the sample reconstituted with the spectrin-4.1 complex (○——○). Actin binding to the various vesicles was measured at three actin concentrations as described in Methods. Gels: (a) control vesicles (no 4.1 extracted) reconstituted with 50 μg/ml spectrin from sucrose gradient, (b) 4.1 depleted vesicles reconstituted with 50 μg/ml spectrin from sucrose gradient, (c) 4.1 depleted vesicles reconstituted with 60 μg/ml spectrin-4.1 complex, (d) 4.1 depleted vesicles reconstituted with 50 μg/ml spectrin plus 8 μg/ml band 4.1, added separately.

Discussion

Our results show that band 4.1 can modulate spectrin-dependent attachment of actin to red cell membranes. The most likely explanation for this phenomenon is that band 4.1 forms a complex with membrane bound spectrin and it is this complex which is responsible for the association of actin with the membrane. This interpretation explains why increasing concentrations of band 4.1 added to 4.1 depleted vesicles fail to increase actin binding above a certain level (Fig. 6). Once spectrin's binding sites for band 4.1 have been saturated, excess band 4.1, although bound to the membrane, has no effect. Band 4.1 binds to spectrin in solution with a K_d of approx. 10^{-7} M at pH 7.6 and saturates at 2 mol of band 4.1 per spectrin heterodimer [25]. Electron microscopy shows that both band 4.1 and actin bind to the tail of the spectrin dimer [7,25] but the precise arrangement of these molecules when both are bound simultaneously is not known. It is possible that band 4.1 acts as a link between spectrin and actin or that band 4.1 binds to spectrin's tail at a site somewhat removed form the actin binding site and affects spectrin-actin binding allosterically.

Studies of the interaction of spectrin and actin in solution have shown that while spectrin can bind to actin in the absence of added band 4.1 [10,12] band 4.1 can dramatically strengthen or enhance this interaction in a way which is still not understood [9,11,12]. While our present results show that spectrin-stimulated actin binding is strongly dependent upon band 4.1 we cannot rule out that some of the binding resulted from spectrin-actin contacts not involving band 4.1. Thus the small stimulation by spectrin of actin binding to 4.1 depleted vesicles without added 4.1 could have been the result of such binding. However, it was never possible for us to elute all of band 4.1, and the small amount left on the membrane could have been responsible for the observed spectrin-stimulated actin binding in the absence of added band 4.1.

If band 4.1 acts as a bridge to connect spectrin with actin then an association between actin and band 4.1 may be detectable in the absence of spectrin. We attempted to detect such an association by sucrose gradient sedimentation of G-actin plus band 4.1 under a variety of conditions, as well as by co-sedimentation of band 4.1 and F-actin. Although we found no association between G-actin and band 4.1, we did find a significant association of band 4.1 with F-actin, but only at pH 6.5 or below (Cohen, C.M. and Foley, S.F., unpublished data). These experiments were done by measuring F-actin-induced sedimentation of band 4.1 in 50 mM KCl, 5 mM sodium phosphate, 2 mM $MgCl_2$, 0.5 mM ATP, 0.1 mg/ml bovine serum albumin

after incubation at 25°C. Interestingly, this association was sensitive to Ca^{2+}, being maximal at somewhat less than 10^{-5} M Ca^{2+} and undetectable at less than 10^{-6} M and above 10^{-4} M Ca^{2+}. However, our inability to measure this association at pH 7.0–7.6 (at which all of the present experiments were done) makes us skeptical of its relevance to the present observations. In the absence of additional data we cannot conclude whether spectrin associated band 4.1 is actually in contact with F-actin on the membrane. We previously proposed that the relatively small amount of F-actin binding to inside-out vesicles containing no added spectrin was due to F-actin binding to band 4.1 [13], possibly via multi-point binding of F-actin to membrane bound 4.1, which would strengthen an otherwise weak association. This conclusion was based primarily on a correlation between reduction in actin binding to trypsinized vesicles and the loss of band 4.1 seen on SDS gels of the vesicles. Our present results clearly show that only when spectrin is present changes in band 4.1 content are reflected in changes in actin binding. Thus, the reduction in actin binding to trypsin-treated vesicles was likely the result of reduction in non-specific actin binding due to loss of protein, or to loss of some other unidentified membrane actin binding protein.

The association between band 4.1 and the erythrocyte membrane deserves further study. We found that band 4.1 could enhance actin binding to about the same extent whether it was added to membranes before spectrin, after spectrin, or as a spectrin-4.1 complex. This implies that band 4.1 can redistribute from whatever it is bound to when it is first added to membranes, to the tail of the spectrin dimer. The number of spectrin-associated band 4.1 binding sites is limited to two per dimer, and a simple calculation shows that binding to these sites on spectrin-containing vesicles (as in Fig. 6) should saturate at about 60–80 μg band 4.1 per mg vesicle protein. The nature of the membrane binding sites in excess of this value is not known nor is the significance of the apparent selectivity in 4.1 binding to the cytoplasmic membrane surface.

Our results, along with previous work [9,11,12] demonstrate the importance of band 4.1 in promoting or enhancing the association between spectrin and actin. Moreover, the ability of band 4.1 to cause spectrin-actin gels to become thixotropic [12] suggests that this protein may be involved in maintaining the flexibility of the red cell membrane. That band 4.1 contributes importantly to the stability of the red cell cytoskeleton is further shown by studies of red cells deficient in or lacking band 4.1 whose cytoskeletons are far less stable than normal cytoskeletons [26,27].

Finally, it is interesting to note that while red cell spectrin and actin both have structural analogues in other eukaryotic cells, no protein similar to band 4.1 has been reported in any other cell. While proteins such as gelsolin can confer Ca^{2+} sensitivity on actin gels (as band 4.1 can [11,12]) gelsolin destroys the gel rather than strengthening it and seems to act on actin rather than in concert with actin binding protein. It will be interesting to see if proteins similar to band 4.1 can be found in more complex cells by immunochemical or other methods.

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