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Actin—Membrane Interactions: Association of G-Actin With the Red Cell Membrane

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Chemically tritiated actin from rabbit skeletal muscle was used to investigate the association of G-actin with the red cell membrane. The tritiated actin was shown to be identical to unmodified actin in its ability to polymerize and to activate heavy meromyosin ATPase. Using sealed and unsealed red cell ghosts we have shown that G-actin binds to the cytoplasmic but not the extracellular membrane surface of ghosts. Inside-out vesicles which have been stripped of endogenous actin and spectrin by low-ionic-strength incubation bind little G-actin. However, when a crude spectrin extract containing primarily spectrin, actin, and band 4.1 is added back to stripped vesicles, subsequent binding of G-actin can be increased up to 40-fold. Further, this crude spectrin extract can compete for and abolish G-actin binding to unsealed ghosts. Actin binding to ghosts increases linearly with added G-actin and requires the presence of magnesium. In addition, actin binding is inhibited by cytochalasin B and DNAase I. Negative staining reveals an abundance of actin filaments formed when G-actin is added to reconstituted inside-out vesicles but none when it is added to unreconstituted vesicles. These observations indicate that added G-actin binds to the red cell membrane via filament formation nucleated by some membrane component at the cytoplasmic surface.

Key words: spectrin, actin, actin binding to red cell membrane

The filamentous proteins actin and myosin are thought to be responsible for most types of eukaryotic cell motility and frequently form extensive trans-cellular networks which seem to determine and stabilize cell shape by a direct or indirect association with the plasma membrane [1–3]. Actin has been found in association with plasma membranes from a variety of cells [2, 4–7] and has also been shown to associate with membranes of

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secretory vesicles [8, 9]. In addition, there is suggestive evidence for a transmembrane linkage of cell surface receptors and integral membrane proteins with submembrane actin filaments [10, 11]. While such morphological and biochemical studies provide strong evidence that some fraction of a cell's actin complement is associated with its plasma membrane, the molecular nature of this association is not understood.

The red cell is known to contain membrane-associated actin [12, 13], and although this cell is nonmotile, its membrane serves as an important model for those of more complex cells. Further, its mechanochemical properties are interesting in their own right, since it is now recognized that red cell shape and deformability both in normal and disease states are primarily determined by the cell membrane [14–16]. Even in diseases where the initial lesion is not of membrane origin, such as sickle cell anemia, the final result can be loss of intrinsic membrane flexibility [16]. Since these properties of the membrane may determine the in vivo fate of both abnormal red cells and normal aged cells, an understanding of molecular interactions in the membrane is of interest from both a medical and a biochemical viewpoint.

Although it has been repeatedly suggested that the mechanical properties of the red cell membrane are controlled by its interaction with red cell actin and the high-molecular-weight protein spectrin, only recently has a specific association of spectrin with the membrane been demonstrated [17]. While actin is present in nearly a 1:1 molar ratio with spectrin, much less is known about its association with the membrane. The fact that actin and spectrin appear to coelute from the membrane at low ionic strength [18] and form a self-associating filamentous network upon solubilization of membrane lipids by detergent [19] has led to the proposal that these molecules may interact with each other in vivo [13]. Although recent work [20, 13] has provided some evidence of a spectrin–actin interaction, its nature and physiological relevance is far from clear.

To study how actin associates with membranes, we have begun an investigation of actin binding to the red cell membrane using ^{3}H-G-actin. The use of radioactive actin in these experiments offers several advantages: 1) It allows the quantification of membrane-associated actin; 2) it permits the study of actin binding to membranes at very low concentrations of either G- or F-actin, minimizing possible nonspecific associations. We describe here some preliminary studies of the associations formed when G-actin is added to the red cell membrane; a report of similar studies with F-actin is in preparation [C.M. Cohen, P.L. Jackson, and D. Branton, in preparation].

MATERIALS AND METHODS

Preparation of ^{3}H-Actin

Actin was extracted from rabbit skeletal muscle by the method of Spudich and Watt [21] and stored as a powder after colyophilization with 2 mg sucrose/mg actin. Prior to labeling, 2–6 mg of actin powder was suspended at a protein concentration of 2–3 mg/ml in 3 mM imidazole, pH 7.5, 0.1 mM $CaCl_2$, 0.5 mM ATP, homogenized gently in a Teflon–glass Dounce homogenizer, and dialyzed for 18 hours against this same buffer at 4°C. Sulfhydryl reducing agents were omitted from the buffer so as not to interfere with the labeling reaction. The G-actin was then centrifuged at 150,000g for 30 minutes at 4°C to remove aggregated material, after which the protein concentration was determined by the Lowry method.

An amount of ^{3}H-N-ethylmaleimide (in pentane, New England Nuclear Corp., Boston, Mass) calculated to be 3.5 × the amount of actin to be labeled, on a molar basis,

was added to the bottom of a 5-ml glass test tube. The tube was then rotated, to allow the N-ethylmaleimide (NEM)–pentane to coat the sides, until all visible pentane had evaporated. G-Actin (typically 2 ml of a 2 mg/ml solution) was then added to the tube and incubated with the ^{3}H-NEM at room temperature with gentle agitation for 0.5 hours, after which the reaction was terminated by the addition of 2 mM β-mercaptoethanol (final concentration). Additional experiments demonstrated that lengthening the time of labeling beyond 0.5 hour or increasing the amount of ^{3}H-NEM added did not significantly increase the amount of ^{3}H-NEM incorporated into the actin. The G-actin was clarified by centrifugation at 150,000g for 30 minutes to remove any aggregated material which may have formed, polymerized by the addition of 0.1 M KCl and 2 mM $MgCl_2$ (final concentration), and incubated at 25°C for 1.5 hours. The F-actin was collected by centrifugation at 150,000g for 2.5 hours and resuspended to about 2 ml in 3 mM imidazole, pH 7.5, 0.1 mM $CaCl_2$, 0.5 mM ATP, and 0.75 mM β-mercaptoethanol. The resuspended actin was gently dispersed in a Teflon–glass Dounce homogenizer and dialyzed for 2 days at 4°C against 1 liter of the above buffer. Repeating this polymerization and dialysis had no effect upon the final properties of the labeled actin. After dialysis, the G-actin was again clarified by centrifugation at 150,000g for 30 minutes, made 0.02% w/v in Na azide, and assayed for protein and radioactivity. The ^{3}H-actin was stored in solution either in the G or F form at 4°C for no more than 10 days before use.

Preparation of Red Cell Membranes and Crude Spectrin

Whole blood obtained from donors was stored in plasma at 4°C and used within 1 week. Red cells were washed four times in 140 mM NaCl, 5 mM $NaPO_4$, pH 7.6, and lysed by rapid dilution into 30 volumes of ice-cold 20 mOsm $NaPO_4$, pH 7.6. The ghosts were washed 3–4 times in this buffer until they were white, each time removing the pellet of contaminating white cells [18]. For some experiments, fragmented ghosts were prepared by forceful passage of concentrated white ghosts 4–6 times through a ½ inch 27-g hypodermic needle on a 1-ml syringe until the ghosts had broken down into small vesicles as seen in the phase contrast microscope. The vesicles are morphologically right side out [17] and are unsealed, as shown by 100% accessibility of their cytoplasmic surface by glyceraldehyde 3-phosphate dehydrogenase assay [22; data not shown].

Unsealed spectrin–actin depleted inside-out vesicles were prepared as described by Bennett and Branton [17]. Briefly, ghosts were resuspended in 30 volumes of 0.3 mM $NaPO_4$, pH 7.6, and incubated at 37°C for 30 minutes. The small vesicles resulting from this incubation were centrifuged at 34,000g for 30 minutes and washed once in the same way with ice cold 0.3 mM $NaPO_4$, pH 7.6. In some experiments these vesicles were used directly for actin-binding studies while in others the vesicles were centrifuged on a 10% w/v Dextran T 110 shelf as described [17], and the pellet was used for binding studies. No major differences were observed between the two methods of preparation.

Resealed ghosts were prepared by incubating freshly prepared white ghosts in 20 mOsm $NaPO_4$, pH 7.6, plus 2 mM $MgCl_2$ at 37°C for 40 minutes followed by centrifugation on an equal volume of 1.03 g/ml dextran (160,000 mol wt, Sigma) in an SW 40 rotor at 40,000 rpm for 2 hours. Sealed ghosts were collected from the buffer–Dextran interface and washed twice in 20 mOsm $NaPO_4$, pH 7.6, 2 mM $MgCl_2$, prior to use. Sealed ghosts were generally 85–95% sealed as measured by the glyceraldehyde 3-phosphate dehydrogenase assay [22].

Crude spectrin, containing primarily spectrin (bands 1 and 2), actin (band 5), and band 4.1 (nonmenclature of Fairbanks et al [18]) was prepared from white ghosts made

from blood on the same day it was drawn. Ghosts were washed once in ice-cold 0.3 mM $NaPO_4$, pH 7.6, and then resuspended in a volume of this buffer approximately equal to that of the packed ghosts and incubated at 37°C for 25 minutes. The suspension was centrifuged at 150,000g for 30 minutes and the supernatant, containing the crude spectrin, was carefully extracted and the following components were added (final concentrations): 20 mM KCl, 0.7 mM $NaPO_4$, pH 7.6, 1 mM DTT, 0.1 mM $MgCl_2$, 0.05 mM ATP. After these additions, the pH was readjusted to 7.6.

Assay of ^{3}H-Actin Binding to Membranes

^{3}H-G-Actin at concentrations specified in the figure legends was incubated in a volume of 0.8 ml with membranes at a protein concentration of 0.25 mg/ml in 20 mM KCl, 5 mM $NaPO_4$, pH 6.5, 2 mM $MgCl_2$, 1 mM ATP, and 0.75 mM β-mercaptoethanol (actin-binding buffer). Incubations were done on ice for 1 hour, and three 0.2-ml samples were withdrawn and layered on 0.2 ml 20% w/v sucrose (made in actin-binding buffer) in 0.5-ml polyethylene microfuge tubes. The tubes were centrifuged at 18,000 rpm for 25 minutes in a Sorvall SS 34 rotor; and the tips, containing the membrane pellet, were frozen in liquid nitrogen, cut off, and placed into 10 ml of scintillation fluid [toluene: Protosol:Liquefluor (New England Nuclear Corp., Boston, Mass); 1 liter:50 ml:40ml]. The tubes were shaken vigorously and counted for ^{3}H. Control experiments demonstrated that the presence of sucrose from the clipped tube had no effect on counting efficiency. To further test the efficiency of ^{3}H counting by this method, samples containing ghost membranes and ^{3}H-G-actin were centrifuged as described, but the supernatant and sucrose were completely removed by aspiration and the ghost pellet resuspended in 0.4 ml of 1% SDS which was then transferred to scintillation fluid. The counts in these samples and in the clipped tube tips from identical incubations were equivalent to within 15%. No ^{3}H-G-actin sedimented through the sucrose in the absence of membranes under any of the conditions used. Recovery of ghost or vesicle membrane in the pellet was 70–100% (measured by membrane-bound ^{125}I-labeled wheat germ agglutinin) depending upon the conditions of incubation. Corrections for this were applied where appropriate.

Additional Methods

Heavy meromyosin (HMM) was prepared by the method of Lowey and Cohen [23]. Activation of HMM ATPase by F-actin was measured by incubating 50 μg of HMM in the presence or absence of 40 μg F-actin in a volume of 0.5 ml containing (final concentrations) 25 mM Tris, pH 7.0, 2.2 mM $MgCl_2$, 0.8 mM ATP. Incubations were carried out at 25°C for 5 minutes and terminated by the addition of 200 λ of 30% TCA. After centrifugation to remove the precipitate, the supernatant was assayed for inorganic PO_4.

SDS polyacrylamide gel electrophoresis was done by the method of Fairbanks et al [18]. For counting ^{3}H, the gels were frozen and sliced into 1.2-mm sections, each slice was then soaked in 5 ml scintillation fluid (see above) overnight, and counted for ^{3}H. By this procedure, 97–100% of the ^{3}H counts electrophoresed were recovered.

RESULTS

Several properties of the ^{3}H-actin are listed in Table I. The molar ratio of incorporated ^{3}H-NEM to actin varied between 0.7 and 0.95 in 20 separate preparations. This ratio is consistent with observations by others that nondenaturing sulfhydryl reagents such as NEM react rapidly with only one, or possibly two, of actin's six sulfhydryl groups [24–27] and

that stoichiometric labeling (1 mole/mole) of actin with either ^{14}C-NEM or spin-labeled NEM can be easily achieved [25, 28]. Table I also shows that the labeled actin is functionally identical to native actin since its ability to activate HMM-ATPase, and to form filaments (as assayed by sedimentation) is not affected by labeling. When ^{3}H-actin was electrophoresed in an SDS polyacrylamide gel (Fig. 1), approximately 97% of the ^{3}H added to the gel comigrated with actin as a single band.

Table II shows the results obtained when various red cell membrane preparations were tested for their ability to bind ^{3}H-G-actin. Resealed ghosts bound little or no ^{3}H-G-actin at the low actin concentration used in this experiment (35 μg/ml). G-Actin binding was generally done at low concentrations and at 0°C to reduce the possibility of spontaneous polymerization. Unsealed ghosts, however, under the same conditions, bound 25.8 μg of the added ^{3}H-G-actin/mg membrane protein. Since these unsealed ghosts already contain ~ 45 μg endogenous actin/mg protein (based on integrated staining intensity of band 5 [29]), the amount of ^{3}H-G-actin bound represents a 50% increase over the endogenous membrane actin. Table II also shows that actin binding to ghosts requires the presence of MgATP in the binding medium. While Mg can cause ghosts to reseal, this generally occurs only at elevated temperatures (these experiments were done at 0°C) so that the MgATP dependence of actin binding is unlikely to result from trapping of actin inside ghosts. In addition, estimation of the internal volume of the ghosts showed that

TABLE I. Properties of (^{3}H)-N-Ethyl Maleimide Actin

Moles N-ethyl maleimide/mole actin	0.7–0.95
Specific activity of ^{3}H-actin	1300–1800 CPM/μg
Activation of HMM-ATPase by ^{3}H-F-actin (% relative to unmodified F-actin)	90–100%
Recovery of ^{3}H-F-actin in 2.3 X 10^{7}g X min pellet (% relative to unmodified F-actin)	100–109%

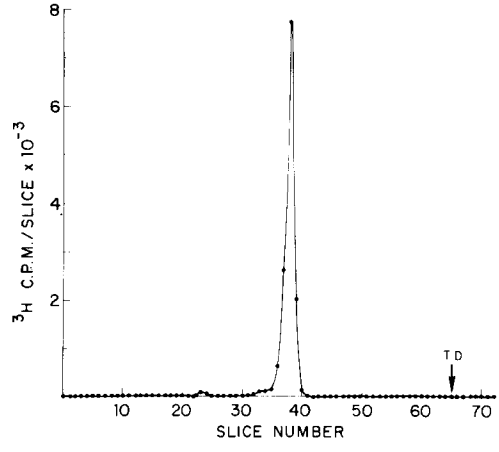

Fig. 1. SDS-polyacrylamide gel electrophoresis of 10 μg of ^{3}H-G-actin. The gel was sliced and counted for ^{3}H as described in Methods. The peak of radioactivity comigrated with the coomassie blue stained actin band (not shown). T D: Tracker Dye.

TABLE II. G-Actin Binding to Red Cell Membranes

	Actin bound μg/mg membrane protein
Resealed ghosts	0.05
Unsealed ghosts	
Control	25.8
Minus MgATP	0.4
Plus crude spectrin[a]	3.2
Plus heat-denatured crude spectrin[b]	24.2
Inside-out vesicles	
Control	0.5
Minus MgATP	0.4
Reconstituted with crude spectrin[c]	20.8
Reconstituted:minus MgATP[d]	0.3

Red cell membranes at a protein concentration of 0.25 mg/ml were incubated with ^{3}H-G-actin at a concentration of 35 μg/ml in actin-binding buffer (see Methods) for 1 hour at 0°C. ^{3}H-G-Actin binding was measured as described in Methods. All values are the mean of three determinations; the standard deviation was generally less than 15% of the values shown.

[a]Unsealed ghosts plus ^{3}H-G-actin were incubated as described above with the addition of 50 μg/ml crude spectrin.

[b]Crude spectrin was denatured by heating to 70° C for 5 minutes before inclusion in the assay as described in (a).

[c]Inside-out vesicles (0.3 mg/ml) were incubated with 12.5 μg/ml crude spectrin in actin-binding buffer (see Methods) for 1 hour at 4°C, washed once, resuspended to 0.25 mg/ml, and assayed for actin binding.

[d]Same as (c) except actin binding was done in the absence of MgATP.

actin entrapment could account for less than 20% of the actin bound. The fact that MgATP also greatly stimulates binding to reconstituted inside-out vesicles (see below and Table II) but has little effect on unreconstituted vesicles further demonstrates that factors other than membrane sealing are responsible for actin binding.

Since spectrin is the major protein at the cytoplasmic membrane surface and has been reported to interact with actin [20, 13], we tested the ability of a crude spectrin extract [Fig. 2, gel C), obtained from a separate batch of fresh ghosts, to compete for or inhibit actin binding to unsealed ghosts. Table II shows that when unsealed ghosts were incubated with ^{3}H-G-actin plus a small amount of crude spectrin, actin binding to the ghosts was reduced by nearly 90%. If, however, the crude spectrin was first denatured by heating to 70°C for 5 minutes, it did not inhibit binding to ghosts. These results suggest either that actin bound to some component of free crude spectrin and was prevented from binding to the ghost or that the crude spectrin associated with the ghost in such a way as to prevent actin binding.

To further examine these possibilities, we studied actin binding directly to the cytoplasmic membrane surface by using inside-out vesicles. These vesicles are depleted of most of their endogenous spectrin and actin (Fig. 2, gel B) compared to ghosts (Fig. 2, gel A) and are morphologically inside-out as judged by intramembrane partical distribution seen by freeze fracture [17]. Table II shows that these vesicles bind only a small amount of G-actin. However, if they are first reconstituted with crude spectrin (Fig. 2, gel D) their ability to bind G-actin is increased more than 40-fold, and is close to that of unsealed ghosts. ^{3}H-G-Actin binding to these reconstituted vesicles is also dependent upon the

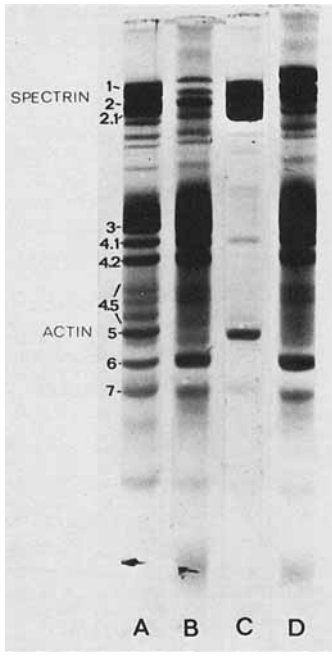

Fig. 2. SDS-polyacrylamide gels of red cell membranes and crude spectrin: a) Unsealed ghosts; b) inside-out vesicles; c) crude spectrin; d) inside-out vesicles reconstituted with crude spectrin (see legend, Table II).

presence of MgATP and is abolished when this is omitted. These experiments show that the inhibition of actin binding to ghosts by crude spectrin described above must be due to actin binding to some component of the free crude spectrin, since if the crude spectrin had associated with the ghost actin binding would have been stimulated rather than inhibited.

Figure 3 shows the concentration dependence of ^{3}H-G-actin binding to the various membrane preparations described above. For these experiments, fragmented ghosts were used (see Methods) since it was found that they bound more ^{3}H-G-actin under all conditions tested than intact ghosts. Fragmented ghosts have a polypeptide composition identical to intact ghosts but are presumably more permeable to G-actin. Figure 3 shows a linear dependence of bound actin on added ^{3}H-G-actin for fragmented ghosts, while the curve for inside-out vesicles reconstituted with crude spectrin is slightly concave. The crude spectrin varied in its ability to stimulate binding of actin to inside-out vesicles (eg compare the stimulation of G-actin binding to inside-out vesicles by crude spectrin in Table II and Fig. 3). The reason for this variability is unclear, but it may be related to the state of phosphorylation of spectrin upon extraction [20] or to the variable presence of F-actin in the crude spectrin (see Discussion). At very low actin concentration (<20 μg/ml), the fragmented ghosts bind a small but nearly constant amount of actin, which then increases sharply above 20 μg/ml actin. This transition, along with the concave binding curve for reconstituted inside out vesicles, suggests that actin binding may result from induced polymerization of actin on the membrane above a certain critical concentration.

To explore further the possibility that filament formation is involved in actin binding, we tested the effects of DNAase I which depolymerizes F-actin and forms a highly

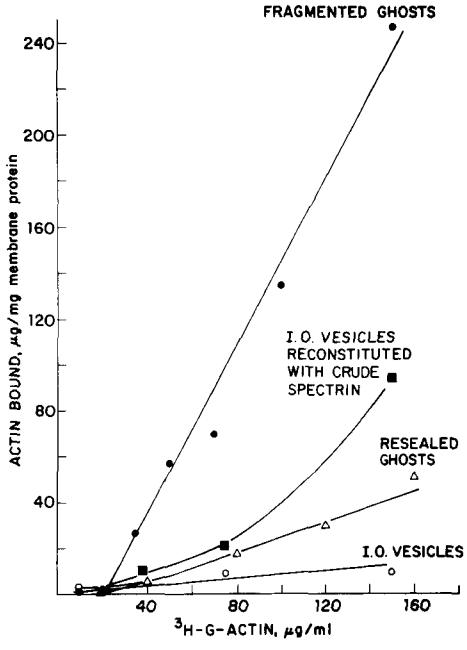

Fig. 3. Concentration dependence of ^{3}H-G-actin binding to red cell membranes. Vesicles or ghosts at a protein concentration of 0.25 mg/ml were incubated in actin binding buffer for 1 hour at 0°C with ^{3}H-G-actin at the concentrations indicated. Inside-out vesicles were reconstituted with crude spectrin as described (see legend, Table II). In this experiment the sealed ghosts bound more ^{3}H-G-actin than the unreconstituted inside-out vesicles (compare with Table II), probably due to contamination of sealed ghosts with unsealed ghosts. All points are the mean of three determinations; the standard deviation was less than 15% of the values shown.

stable 1:1 complex with G-actin [30] and cytochalasin B which has a wide variety of disruptive effects on actin filament-related cellular phenomena [2] and is thought to affect actin filament–actin binding protein interactions [31, 32]. Figure 4a shows that 5 μM cytochalasin B virtually eliminates actin binding to reconstituted inside-out vesicles (dimethyl sulfoxide alone, in which the cytochalasin was added, had no effect) and Figure 4b shows that 50 μg/ml DNAase I (which is 1.6 × molar excess over the G-actin present) completely prevents actin binding. These results suggest that filament formation and filament membrane association are required for binding. This observation was confirmed by negative staining of inside-out vesicles which were reconstituted with crude spectrin and then incubated with G-actin. Figure 5b shows that these vesicles clearly have a large number of actin filaments associated with them, whereas unreconstituted inside-out vesicles treated in the same way had no actin filaments. (Fig. 5a).

DISCUSSION

We have demonstrated that G-actin from rabbit skeletal muscle is capable of forming a specific association with the cytoplasmic surface of the red cell membrane. A similar result has also been reported recently by Birchmeier and Singer [33]. Our data suggest that G-actin binding results from an association of actin with some membrane component present in crude spectrin, which consists primarily of spectrin, actin, and band 4.1. This

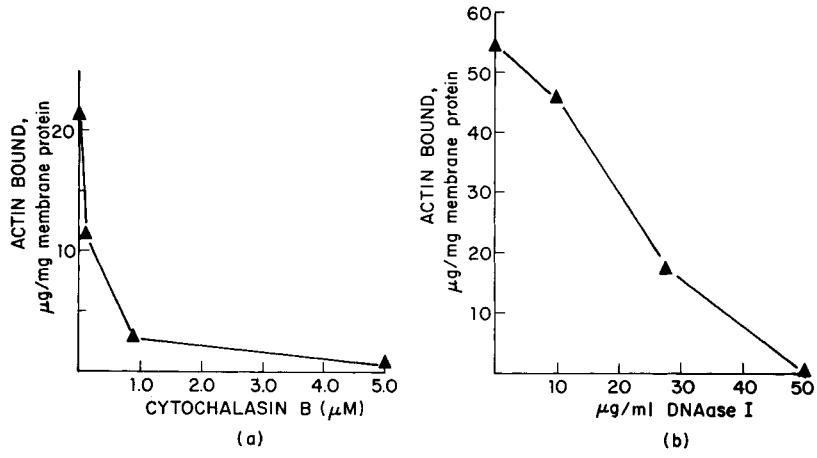

Fig. 4. a) Effect of cytochalasin B on G-actin binding to reconstituted inside-out vesicles. Inside-out vesicles reconstituted with crude spectrin, as described in Table II, were incubated with 48 μg/ml ^{3}H-G-actin in the presence of the indicated concentrations of cytochalasin B obtained by adding 5 μl of 20 μM, 130 μM, 1 mM cytochalasin B in DMSO to a 1 ml incubation. b) Effect of DNAase I on G-actin binding to reconstituted inside-out vesicles. DNAase I in binding buffer was added as a concentrate to vesicles plus 48 μg/ml G-actin. In a) and b) actin binding was measured as described in Methods except that the incubations were only 40 minutes. All points are the mean of three determinations; the standard deviation was less than 15% of the values shown.

conclusion is supported by several observations: 1) Red cell ghost membranes lose their capacity to bind G-actin when converted into inside-out vesicles, a process which results in the specific release from the membrane of spectrin, actin, and some of band 4.1; 2) when inside-out vesicles are reconstituted with crude spectrin, they regain their ability to bind G-actin; 3) crude spectrin in solution will inhibit the binding of G-actin to unsealed ghosts presumably by binding G-actin in solution and preventing it from binding to the ghost membrane. Since this inhibition does not occur after crude spectrin is denatured by heating, it probably represents a specific interaction of actin with some component of the crude spectrin.

The nature of the association between actin and the red cell membrane remains unclear. Although we have shown that G-actin binding is greatly enhanced by the presence of MgATP, ATP itself does not appear to play a role since Mg alone can stimulate binding to the same extent (data not shown). The linear dependence of bound actin on added actin suggests a nonsaturable type of association. While such a feature is generally thought to indicate nonspecific association, this would be hard to reconcile with the high degree of specificity of binding to the cytoplasmic surface and the marked dependence upon Mg. While we cannot rule out the possibility that some of the binding we observe is due to G-actin which binds to the membrane and remains there as monomeric actin, the lack of saturability and dependence on the presence of a divalent cation is consistent with the interpretation that actin binding proceeds by polymerization of G-actin on or from the cytoplasmic membrane surface. This conclusion is supported by our observation that reconstituted inside-out vesicles which have been incubated with G-actin can clearly be seen to have F-actin filaments associated with them (Fig. 5b). Since actin binding is prevented by DNAase I which disrupts or prevents filament formation and cytochalasin B, which may act by inhibiting actin binding protein–actin filament interactions, it is clear

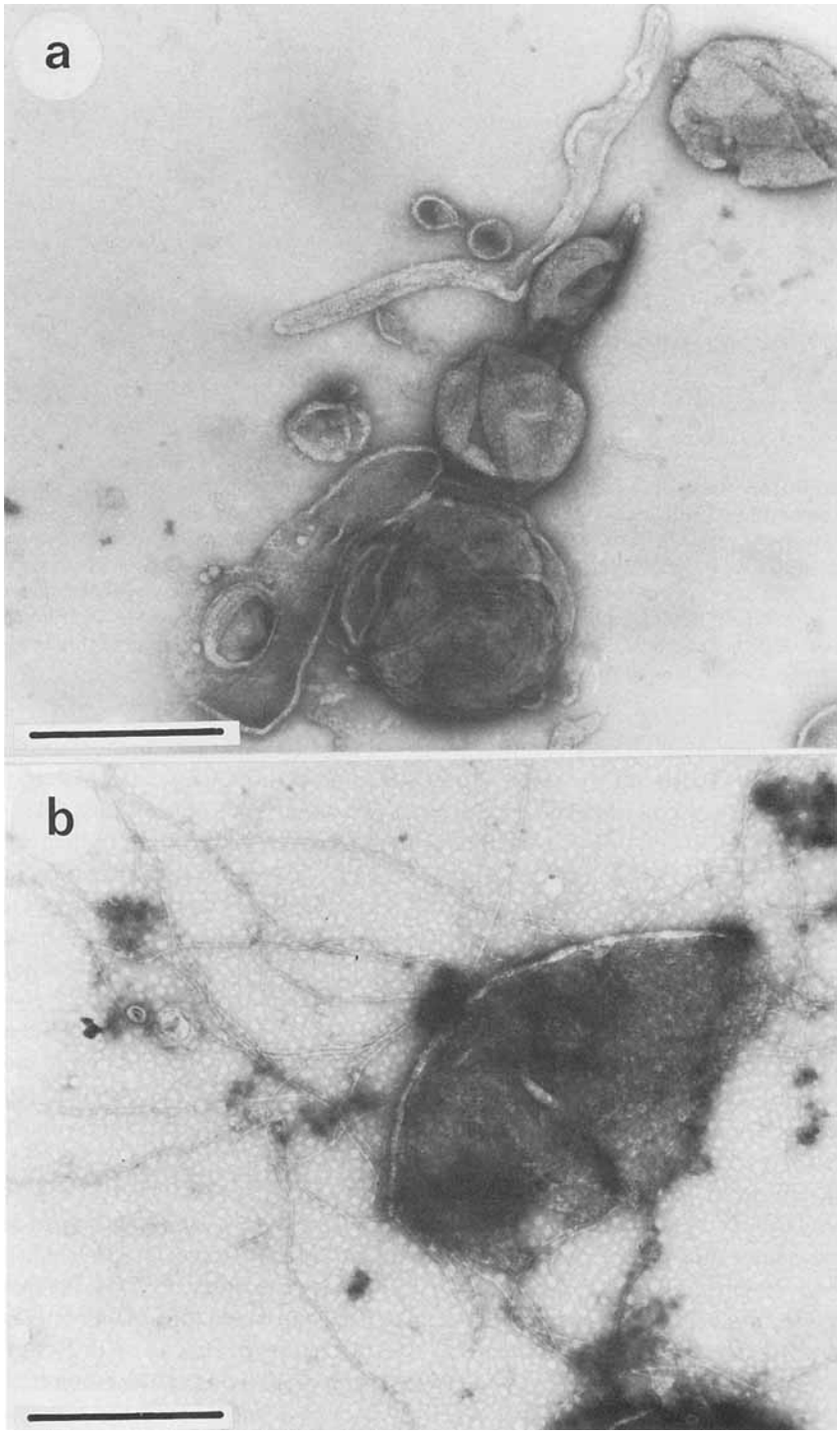

Fig. 5. Inside-out vesicles (a) or inside-out vesicles reconstituted with crude spectrin as described in Table II (b) were incubated for 60 minutes with 50 μg/ml ^{3}H-G-actin, and sedimented into 20% w/v sucrose as described in Methods. The vesicle pellet was gently resuspended, applied to a Formvar coated grid and stained with 1% uranyl acetate. Bar is 0.5 μm; magnification = × 54,000.

that the actin–membrane association is a direct result of actin filament formation. This implies that some component or components at the cytoplasmic surface, present in crude spectrin, serve as a nucleation site or initiator of actin polymerization.

Spectrin itself seems a likely candidate for this function particularly in view of recent observations [20] showing that purified phosphorylated spectrin (bands 1 and 2) can initiate polymerization of G-actin in solution. However, we have found that purified spectrin (bands 1 and 2, prepared by the method of Bennett and Branton [17], from freshly drawn blood) does not have the ability to stimulate G-actin binding to inside-out vesicles or to inhibit binding to ghosts [C.M. Cohen, P.L. Jackson, and D. Branton, in preparation] in the way that crude spectrin does. While it is possible that preparation of pure spectrin from crude spectrin induces subtle alterations in phosphorylation or conformation which abolish the spectrin–actin interaction, it is equally plausible that proteins in the crude spectrin (Fig. 2C) other than bands 1 and 2 are responsible for the observed effects. Such a protein could be red cell actin, some fraction of which could already exist in the F form in crude spectrin. We consider this to be a real possibility since we have found that preformed F-actin will bind to inside-out vesicles and, when bound, will stimulate the subsequent binding of G-actin in a manner analogous to that seen with crude spectrin [C.M. Cohen, P.L. Jackson, and D. Branton, in preparation]. This suggests that there may be F-actin present in the crude spectrin which binds to the membrane and is responsible for the stimulation of G-actin binding, possibly by acting as a nucleation site for filament elongation in the same way as F-actin fragments in solution can induce the explosive polymerization of G-actin [34, 35]. Although the protein composition of reconstituted inside-out vesicles prior to incubation with G-actin (Fig. 2d) reveals no apparent increase in actin content, the amounts of F-actin involved may be too low to detect above the high background staining in this region of the gel.

The relationship of the observations presented here to the state of endogenous actin on the red cell membrane is difficult to assess, especially since it is still unknown whether actin on the red cell membrane is filamentous or monomeric. While Tilney and Detmers [13] have proposed that red cell actin exists in a nonfilamentous form in vivo, it has also been shown that phosphorylated spectrin can induce filament formation of skeletal muscle actin in vitro [20]. Nevertheless, our demonstration that ghost membranes can bind large amounts of actin over and above that already present on the membrane and that this bound actin is in the filamentous form indicates that several types of actin–membrane association may be possible. Further, our observation that ghosts may possess nucleation sites for actin polymerization suggests that they may serve as a valuable model system for the study of actin–membrane associations. Similar studies should be possible with more complex cells using methods specifically designed to expose their cytoplasmic membrane surface [36, 37].

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