1986-06-01

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*Et al.*

# Binding and Assembly of Actin Filaments by Plasma Membranes from *Dictyostelium discoideum*

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*Abstract.* The binding of native, $^{125}$I-Bolton-Hunter–labeled actin to purified *Dictyostelium discoideum* plasma membranes was measured using a sedimentation assay. Binding was saturable only in the presence of the actin capping protein, gelsolin. In the presence of gelsolin, the amount of actin bound at saturation to three different membrane preparations was 80, 120, and 200 μg/mg of membrane protein. The respective concentrations of actin at half-saturation were 8, 12, and 18 μg/ml. The binding curves were sigmoidal, indicating positive cooperativity at low actin concentrations. This cooperativity appeared to be due to actin–actin associations during polymerization, since phalloidin converted the curve to a hyperbolic shape. In kinetic experiments, actin added as monomers bound to membranes at a rate of 0.6 μg $ml^{-1}$ $min^{-1}$, while pre-polymerized actin bound at a rate of 3.0 μg $ml^{-1}$ $min^{-1}$. Even in the absence of phalloidin, actin bound to membranes at concentrations well below the normal critical concentration. This membrane-bound actin stained with rhodamine–phalloidin and was cross-linked by m-maleimidobenzoyl succinimide ester, a bifunctional cross-linker, into multimers with the same pattern observed for cross-linked F-actin. We conclude that *D. discoideum* plasma membranes bind actin specifically and saturably and that these membranes organize actin into filaments below the normal critical concentration for polymerization. This interaction probably occurs between multiple binding sites on the membrane and the side of the actin filament, and may be related to the clustering of membrane proteins.



THE connections between the plasma membrane and the underlying microfilament network are involved in essential cell activities such as the determination of cell shape, mechanical properties, interactions with other cells and with extracellular matrix or solid substrata, cell motility, and the distribution and mobility of membrane proteins (reviewed in references 21, 32, 46, 64, 66, 69). However, despite a wealth of information from cellular and microscopic studies, detailed information about these phenomena at the molecular level is sparse. This lack of information is due to the fact that both microfilaments and membranes are large, multimeric structures. Thus, quantitative analysis of actin–membrane interactions in sedimentation binding assays is complicated by actin–actin associations which cause the binding to be nonsaturable (8, 9, 31).

To overcome the complications inherent in the study of interactions between large, multimeric structures, two experimental strategies have been used. First, both structures may be left in their native state although, in this case, the interactions between the two structures can be measured only qualitatively through solution viscosities or by other indirect measurements (3, 17). Thus, low-shear viscometry is used to monitor the interactions between intact membranes and long actin filaments (18, 19, 39). In the second strategy, one of the structures is limited in extent so that it behaves as though it were monomeric in binding assays with its large, multimeric partner. For example, membranes can be restricted in size by sonication or solubilization with detergents. Thus, sedimentation assays with F-actin affinity beads can be used to study interactions between these sonicated or detergent-solubilized membranes and long actin filaments sequestered in the pores of the gel filtration beads (22, 40).

These techniques have been used to study the conditions under which actin binds to highly purified plasma membranes isolated from *Dictyostelium discoideum*. *D. discoideum* plasma membranes increase the viscosity of solutions containing F-actin (39) and bind specifically and saturably to F-actin affinity beads (40). Both the increases in viscosity and the binding to F-actin beads are eliminated by proteolysis or heat denaturation of the membranes, suggesting that actin filaments bind to membrane proteins. Since extraction of peripheral membrane components with chaotropes does not decrease binding in either assay, integral membrane proteins are thought to be responsible for much of the binding (39, 40). Fractionation of detergent-solubilized membranes on F-actin affinity columns demonstrates that at least twelve integral membrane polypeptides specifically bind to F-actin either directly or indirectly (40). Recently, Stratford and Brown ha-

reported that short, sonicated, glutaraldehyde–cross-linked actin filaments bind saturably to crude *D. discoideum* membranes (58). Several polypeptides in these membranes bind $^{125}$I-labeled actin in overlays of polyacrylamide gels (52, 58).

In this paper, we describe a new sedimentation binding assay in which short filaments of native, $^{125}$I-labeled actin are bound by highly purified *D. discoideum* plasma membranes in the form of large sheets. To limit the size of the actin filaments in this assay, we include the actin capping protein, gelsolin (70). In the presence of micromolar calcium ions, gelsolin binds tightly to the fast-growing (barbed) end of actin filaments (26, 35, 65, 72), prevents further addition of monomers at this end (26, 65), and severs existing actin filaments (26, 71, 72). When the actin filament length is limited by gelsolin, the binding of $^{125}$I-labeled F-actin to membranes is saturable, can be competed by unlabeled actin, and can be analyzed by classical biochemical methods.

In this paper, we not only use short actin filaments to define basic physical parameters describing the interactions between *D. discoideum* plasma membranes and F-actin, but we also present evidence that these membranes can provide potent signals for the assembly of actin filaments at actin concentrations well below the normal critical concentration for polymerization. This assembly activity may reflect a mechanism whereby actin–membrane associations regulate, or are regulated by, transmembrane interactions involved in cell adhesion, motility, or morphogenesis.

## Materials and Methods

### Chemicals

$Na^{125}I$ in pH 7–11 sodium hydroxide was purchased from Amersham Corp. (Arlington Heights, IL). Chloramine T was from Kodak (Rochester, NY). ATP, bovine serum albumin (BSA), and phalloidin were from Sigma Chemical Co. (St. Louis, MO). Bolton-Hunter reagent and m-maleimido benozyl *N*-hydroxysuccinimide ester (MBS)[1] were from Pierce Chemical Co. (Rockford, IL). Rhodamine-phalloidin was from Molecular Probes (Junction City, OR). L-3-Carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(3-methyl)butane (EP475), an inhibitor of calcium-activated protease (24, 48, 59, 62), was the generous gift of Dr. K. Hanada, Taisho Pharmaceutical Company (Saitama, Japan). All other chemicals were of reagent grade.

### Proteins

Actin, isolated from rabbit skeletal muscle according to Spudich and Watt (56), was column-purified (41) on Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.2 mM dithiothreitol (DTT), 0.4 mM ATP, 2 mM Tris-HCl, pH 8.0. *D. discoideum* actin was purified as described by Uyemura et al. (63). Purified actin was stored in the G-form at concentrations >2 mg/ml by dialysis against depolymerization buffer (50 μM $CaCl_2$, 1 mM ATP, 1 mM DTT, 0.02% sodium azide, 2 mM Tris-HCl, pH 8.0). After 2 wk, the actin was cycled by polymerization and depolymerization, was used for an additional 2 wk, and then was discarded. Highly purified rabbit plasma gelsolin was prepared using a protocol graciously provided by Dr. Carl Frieden (Frieden, C., unpublished observations). Human plasma gelsolin was a generous gift from Dr. Helen Yin and anti-fish actin was generously provided by Dr. K. Fujiwara.

The concentrations of actin, gelsolin, and total membrane protein were determined in the presence of 1% SDS by the procedure of Lowry et al. (37); BSA was used as a standard.

### Actin Labeling

Bolton-Hunter reagent (4) was iodinated by first mixing 10 μg chloramine T in 5.0 μl of 50 mM sodium phosphate, pH 7.0, with 5.0 μl (0.5 mCi) of $Na^{125}I$, and then adding 0.5 μg Bolton-Hunter reagent in 1.0 μl acetone. The reaction was quenched immediately by adding 1.0 μl of 1 M sodium thiosulfate. 200 μg of G-actin were added quickly, mixed, and incubated 1 h on ice. $^{125}$I-Bolton-Hunter–labeled G-actin was separated from unreacted reagents by chromatography on Sephadex G-25 (Pharmacia Fine Chemicals) in depolymerization buffer. In general, ~20% of the initial radioactivity was covalently coupled to the protein and ~10% of the actin was labeled. After chromatography, the actin was polymerized by adding the appropriate volume of 5× polymerization buffer (0.50 M KCl, 10 mM $MgCl_2$, 0.50 M sodium phosphate, pH 7.0) and by incubating 40 min at room temperature. The polymerized actin was centrifuged 20 min at 30 psi (178,000 *g*) in a Beckman airfuge (Beckman Instruments, Inc., Palo Alto, CA) and the supernatant was discarded. The F-actin pellet was resuspended in depolymerization buffer, sonicated 10 s in a bath sonicator (Laboratory Supply Co., Hicksville, NY), dialyzed for 24 h at 4°C against depolymerization buffer, and re-centrifuged in the airfuge to remove residual polymerized actin. Radiolabeled actin was diluted with unlabeled actin so that the ratio of unlabeled to labeled actin used in binding experiments was at least 50:1. $^{125}$I-labeled actin was stored as G-actin in dialysis against depolymerization buffer for no longer than 2 wk before use in binding assays.

The critical concentration for polymerization of $^{125}$I-Bolton-Hunter–labeled actin was determined by sedimentation as described by Yin et al. (71). Actin at 1 mg/ml was polymerized for 1 h in polymerization buffer (50 mM KCl, 2 mM $MgCl_2$, 50 μM $CaCl_2$, 1.0 mM DTT, 0.4 mM ATP, 100 μM phenylmethylsulfonyl fluoride, 10 μM EP475, 5 mM sodium phosphate, pH 7.0) and then was diluted to several concentrations between 5 and 100 μg/ml. After equilibration at room temperature for 3 h, the actin was centrifuged 2 h in the airfuge at 178,000 *g*. The supernatant was removed and both pellet, and supernatant were counted for gamma radiation. The critical concentration then was determined by plotting the amount of actin in the supernatant against the initial actin concentration. In three separate determinations, these points varied by no more than 25%.

### Gelsolin Labeling

Gelsolin was radioiodinated as described by Harris (25). Briefly, ~20 μg gelsolin in 20 μl 0.1 M sodium borate, pH 8.4, was reacted at 0°C for 3.5 h with 100 μCi of $^{125}$I-Bolton-Hunter reagent (New England Nuclear, Boston, MA). Residual Bolton-Hunter reagent was reacted with 25 μl 0.1 M glycine, 0.1 M sodium borate, pH 8.4, for 2 h and separated from labeled gelsolin by chromatography on Sephadex G-25 equilibrated with 0.1 M NaCl, 0.1 mM EGTA, 0.02% $NaN_3$, 1 mg/ml gelatin, 10 mM Tris-HCl, pH 6.8. Assuming a 50% recovery of gelsolin, its specific activity was 2,000 cpm/ng and ~4% of the gelsolin was labeled. Labeled gelsolin bound to actin filaments in high-speed sedimentation assays and appeared to be stable for at least 2 wk when stored on ice in the chromatography buffer.

### Plasma Membranes

Unless otherwise stated, highly purified plasma membranes were used in our experiments. These highly purified plasma membranes were prepared as described previously (40). Briefly, log-phase *D. discoideum* amebae were incubated with concanavalin A (Con A; Miles-Yeda, Rehovot, Israel) to initiate patching and capping of membrane proteins (11, 47). The cells were disrupted with 0.2% Triton X-100 and membranes were isolated on 40–60% sucrose density gradients. The dense plasma membrane–enriched fraction was incubated with 1.5 M alpha-methyl-D-mannoside for 12–25 h to remove most of the Con A. Then, the membranes were washed and dialyzed against a low ionic strength buffer for 48–72 h to remove endogenous actin and myosin. After the removal of Con A, actin, and myosin, the plasma membranes had a much lower protein-to-lipid ratio. Thus, they banded at a lower density on the second sucrose gradient and were easily separated from dense contaminants. For a few experiments, these membranes were extracted with 0.1 N NaOH, 1 mM DTT, pH 12.5, as described previously (39, 40).

Plasma membranes prepared by this method appear as a homogeneous preparation of large, open sheets in electron micrographs and contain no residual actin or myosin detectable on SDS polyacrylamide gels (39, 40). Immunoblotting with anti-fish actin (6), detected no endogenous actin. Using purified *D. discoideum* actin, the limit of sensitivity of this technique was ~30 ng, or 0.06% of the membrane protein.

For a few experiments, *D. discoideum* plasma membranes were purified without using Con A by the method of Das and Henderson (12, 23). Briefly, cells were lysed in 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glycine, pH 8.5, by passage through 5-μm filters (Nucleopore Corp., Pleasanton, CA). The lysate was centrifuged for 18 h on a 0.75–1.5 M sucrose gradient in 50 mM glycine, pH 8.5, formed over a cushion of 1.8 M sucrose in the same buffer. Fractions enriched in plasma membranes were washed with 20 mM sodium phosphate,

1. *Abbreviations used in this paper:* Con A, concanavalin A; DTT, dithiothreitol; EP475, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(3-methyl)butane; MBS, m-maleimido benzoyl *N*-hydroxysuccinimide ester.

pH 6.8, and stored on ice in this buffer plus 0.02% $NaN_3$. Electron micrographs, SDS polyacrylamide gels, lectin blots, and surface labeling of plasma membranes prepared by this method suggest that these membranes are representative of the intact plasma membrane and are reasonably pure. However, they are not as highly purified as are the membranes prepared with the Con A, Triton-extraction procedure (23). After dialysis against low ionic strength buffer for 48–72 h, these Das–Henderson membranes also appear to be free of endogenous actin and myosin. All membranes were stored at 0°C as suspensions in 0.2% $NaN_3$, 20 mM sodium phosphate, pH 6.8.

### *Actin–Membrane Binding Assays*

Binding was carried out in 50 μl of polymerization buffer. Samples contained 100 μg/ml membrane protein, 1 mg/ml ovalbumin, and varying amounts of actin and gelsolin. After incubation for 1 h at room temperature, the mixtures were layered onto 350 μl of 10% sucrose in polymerization buffer in 400-μl polypropylene centrifuge tubes. After centrifugation for 20 min at 11,600 rpm (8,700 $g_{max}$) in a Beckman microcentrifuge with a horizontal rotor, the tubes were frozen. The tips containing the pellets were cut off, and the radioactivity in both the pellets and supernatants was measured in a gamma counter.

### *Gelsolin–Membrane Binding Assays*

Binding was done in 50 μl of assay buffer containing 50 mM KCl, 2 mM $MgCl_2$, 100 μM phenylmethylsulfonyl fluoride, 10 μM EP475, 50 μM $CaCl_2$, and 21.2 mM $NaPO_4$, pH 6.8. (Also, 13.6 mM NaCl, 10 μM EGTA, and trace amounts of Tris-HCl, ATP, DTT, gelatin, and $NaN_3$ were contributed by the addition of gelsolin, actin, and membranes to the assay mixtures. Appropriate solutions without these proteins were used in controls.) Samples contained 150 μg/ml membrane protein, 1 mg/ml ovalbumin, and varying amounts of mixtures of $^{125}$I-labeled human plasma gelsolin, unlabeled rabbit plasma gelsolin, and F-actin. To ensure constant stoichiometries of gelsolin and actin, the proteins were mixed in a concentrated solution before dilution into assay buffer containing membranes. Complete assay mixtures were incubated for 1 h at room temperature and the amount of membrane-bound, $^{125}$I-labeled gelsolin was determined as described above for actin–membrane binding assays.

### *Rhodamine–Phalloidin Staining*

*D. discoideum* plasma membranes were diluted to 5.0 μg/ml in 1.0 ml of polymerization buffer with or without 5 μg/ml unlabeled actin and without gelsolin. After incubation at room temperature for 1 h, the samples were centrifuged 20 min at 11,600 rpm (8,700 $g_{max}$) in a Beckman microcentrifuge and the supernatants were removed. The pellets were resuspended in 1 ml of 3.7% formaldehyde in polymerization buffer and were incubated 30 min at room temperature. The pellets were centrifuged as before, washed once with phosphate-buffered saline (PBS: 0.15 M NaCl, 10 mM sodium phosphate, pH 7.0), and resuspended in 50 μl PBS containing 30 μM rhodamine–phalloidin. After 20 min at room temperature, the samples were diluted to 1 ml with PBS, centrifuged, and resuspended in 10 μl PBS. The membranes were examined using a Zeiss Photomicroscope III equipped with phase contrast and epifluorescence optics. Phase contrast and fluorescent images were recorded on Kodak Tri-X film with the automatic camera of the photomicroscope at ASA 2000. The film was developed with Edwal FG7 developer as described by the manufacturer (Edwal Scientific Products, Mountainside, NJ).

### *Chemical Cross-linking*

Samples with or without 100 μg/ml plasma membranes were prepared in 50 μl of a modified polymerization buffer (50 mM KCl, 2 mM $MgCl_2$, 0.4 mM ATP, 0.02% Tween 20, 5 mM sodium phosphate, pH 7.0). The addition of Tween 20 was necessary to prevent nonspecific adsorption of actin onto the sides of the tube. $^{125}$I-labeled actin, without gelsolin, was added at 2.5 μg/ml (with a ratio of unlabeled to labeled actin of 10:1). To promote polymerization (10, 16) in the positive control, 5 μM phalloidin was added to modified polymerization buffer containing 5 μg/ml labeled actin and 100 μg/ml unlabeled actin. To inhibit polymerization in the negative control, actin was added to a low salt buffer containing 0.4 mM ATP, 1 mM sodium phosphate, pH 7.0. After incubation at room temperature for 1 h, 1 μl of 5 mM MBS (a noncleavable cross-linker) dissolved in 10% aqueous dimethylformamide was added to a final concentration of 100 μM. Samples were incubated 40 min and then the membranes were pelleted by centrifugation for 10 min at 11,600 rpm in a Beckman microfuge. The supernatants were removed, the pellets were resuspended in 50 μl modified polymerization buffer, and both supernatants and pellets were counted in a gamma counter. 30% of the total radioactivity pelleted in the sample containing native membranes, while only ~5% pelleted in the samples without membranes or with denatured membranes. The samples were normalized for total radioactivity when analyzed on gels, except for the pellets from the tubes without membranes and with denatured membranes. Since so little radioactivity pelleted in these tubes, all of the pellet was loaded. Samples were electrophoresed on a 5–10% gradient SDS polyacrylamide gel (36), fixed, dried, and autoradiographed on Kodak XAR-5 film with a Dupont Cronex Lightening Plus screen at −80°C for 2 d.

## *Results and Discussion*

### *Radiolabeled Actin*

Actin radiolabeled with $^{125}$I-Bolton-Hunter reagent polymerizes and depolymerizes normally, indicating that the labeling procedure does not dramatically alter actin association or dissociation. Actin radiolabeled with this reagent also retains its ability to bind and to inhibit the activity of DNase I (55), indicating that the DNase binding site also is relatively unaffected by this labeling procedure. The critical concentration of $^{125}$I-Bolton-Hunter–labeled actin is 5–8 μg/ml (~0.15 μM). This value is consistent with critical concentrations determined by other workers for both native, unlabeled actin and for actin derivatized with another lysine-directed probe (5, 13, 35, 42, 61, 67, 68). In the presence of human plasma gelsolin, the critical concentration of $^{125}$I-labeled actin rises to ~30 μg/ml (~0.7 μM). This value compares favorably with the critical concentration of unlabeled actin in the presence of both platelet and macrophage gelsolin (30–40 μg/ml; references 14 and 35) and is consistent with the hypothesis that, by blocking the barbed end, gelsolin raises the net critical concentration to the critical concentration of the pointed end of the filament (27, 45, 50). Analysis of a complete cyanogen bromide digest of $^{125}$I-labeled actin on an 18% SDS polyacrylamide gel shows that many fragments are radiolabeled (data not shown). This result suggests that the Bolton-Hunter reagent reacts with many different lysines and agrees with the broad specificity found by others for lysine-directed reagents reacted with actin (13, 28, 38, 43).

### *Actin Binding to Plasma Membranes*

As shown in Fig. 1, actin binding to highly purified *D. discoideum* plasma membranes is saturable in the presence of gelsolin, but is not saturable in the absence of gelsolin.[2] Binding appears to be specific since it is inhibited by preincubating the membranes with excess unlabeled actin (Fig. 1 *a*). Binding also is independent of the method used to isolate the membranes since membranes isolated without Con A, using the procedure of Das and Henderson (12), bind significant amounts of actin. However, since the extent of binding to membranes prepared from Con A–stabilized regions of the plasma membrane is 2–4-fold greater, the Con A–membranes are used in the rest of our experiments. Native membrane proteins are required for the binding of $^{125}$I-labeled actin to membranes since binding is virutally eliminated by protein denaturation. Neither heat-pretreated membranes (Fig. 1 *a*) nor membranes denatured by reduction with DTT followed by alkylation with *N*-ethylmaleimide (not shown) bind $^{125}$I-labeled actin. Also, little binding is observed if the assay is done in a low salt actin depolymerizing buffer (Fig. 1 *a*).

2. Since we find that binding curves of rabbit actin to *D. discoideum* membranes resemble binding curves generated with *D. discoideum* actin qualitatively and quantitatively, all experiments presented herein were done with the more easily isolated rabbit actin.

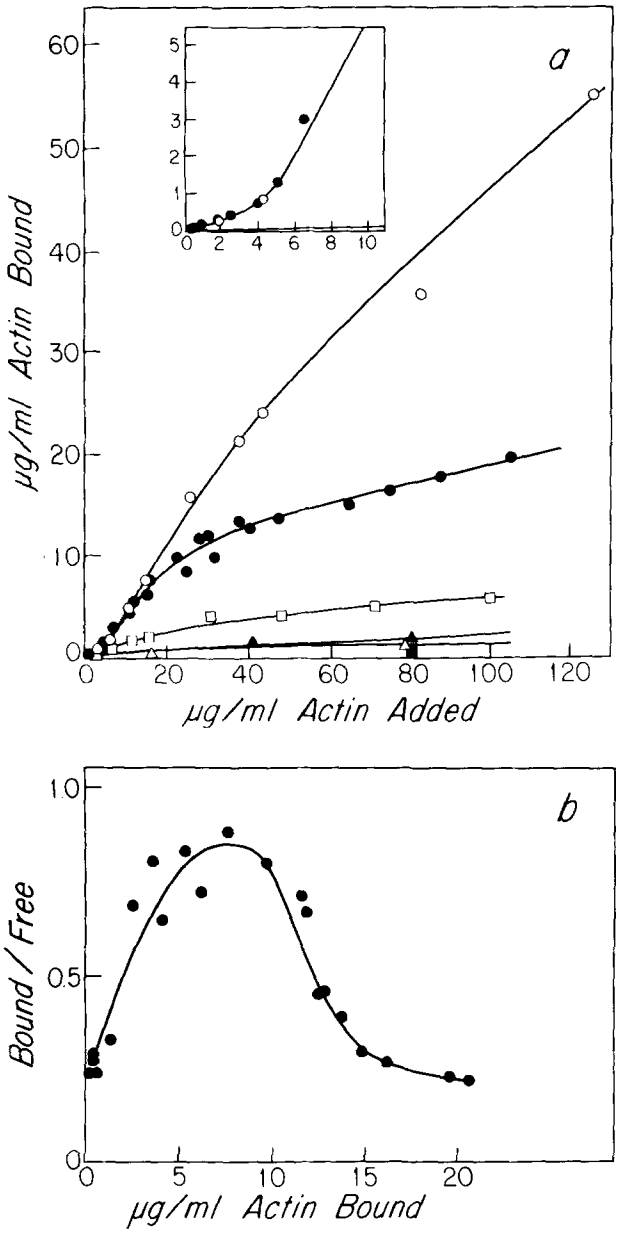


*Figure 1.* (a) Binding of $^{125}$I-Bolton-Hunter-labeled actin to *D. discoideum* plasma membranes. Membranes prepared using Con A were incubated with $^{125}$I-actin without (○) and with (●) gelsolin at a 15:1 mole ratio of actin to gelsolin. Membranes prepared without Con A treatment also bind $^{125}$I-actin in the presence of a 15:1 mole ratio of actin to gelsolin (□). Controls: Binding to membranes in the presence of 2.5 mg/ml unlabeled actin with a 15:1 mole ratio of actin/gelsolin (▲); binding to membranes pre-heated to 95°C for 10 min (△); and binding in low salt buffer (■). (*Inset*) Data at low actin concentrations are displayed on an expanded scale. For comparison, the line obtained for binding in control experiments is extended from the points shown in *a* (lower line). (*b*) Scatchard (51) analysis of actin binding to membranes prepared using Con A in the presence of a 15:1 mole ratio of actin/gelsolin.

Actin binding to membranes does not appear to be mediated by residual membrane-bound actin. First, no endogenous actin or myosin can be detected on polyacrylamide gels of highly purified membranes (39, 40) or on immunoblots with anti-actin (not shown). Since these assays could detect actin if it constituted 0.06% or more of the membrane protein, it seems unlikely that residual actin is responsible for the observed binding of ~100 μg of actin in short filaments per milligram of membrane protein. Second, membranes stripped of peripheral proteins with sodium hydroxide bind similar amounts of actin (data not shown). Third, significant binding occurs well below the critical concentration for actin polymerization (Fig. 1 *a*, *inset*). Fourth, we performed binding experiments with membranes which were dialyzed against low ionic strength buffer for only 16–24 h; in these membranes, ~0.5% of the membrane protein is residual, endogenous actin as measured on immunoblots. These preparations bind approximately the same amounts of actin as do exhaustively dialyzed membranes.

### *Interpretation of the Binding Curve*

In the presence of gelsolin, the binding curve of actin to membranes is sigmoidal (Fig. 1 *a*, *inset*). The inflection point is always observed but varies between 0.5 and 5 μg/ml, depending upon the membrane preparation. This sigmoidal binding curve indicates that actin binds to membranes with positive cooperativity (53) and is consistent with the suggestion that actin polymerization is involved in actin binding to membranes.

Binding of actin to membranes does not appear to correspond to a simple association–dissociation equilibrium, since binding also may involve actin–actin and actin–gelsolin associations, ATP hydrolysis, or multiple interactions between membrane sites and a given actin filament (57). The Scatchard (51) plot of the binding data shown in Fig. 1 *b* clearly illustrates this complexity. The plot shows convex curvature at low concentrations, indicating positive cooperativity, and concave curvature at high concentrations, indicating negative cooperativity, site heterogeneity, and/or the binding of more than one membrane protein to a given actin filament. Thus, the binding cannot be described by a simple affinity constant. However, the high affinity of the binding can be inferred from the fact that with three different batches of membranes, the actin concentrations at half maximal binding were 8, 12, and 18 μg/ml. The amounts of actin bound to 1 mg of membrane protein at saturation were 80, 120, and 200 μg, respectively.

To explore the relationship between actin assembly and the observed positive cooperativity of binding, actin binding to membranes was measured in the presence of phalloidin (Fig. 2). Since phalloidin stabilizes actin filaments and reduces the

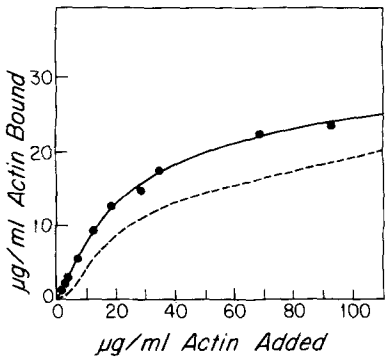


*Figure 2.* Binding of $^{125}$I-labeled actin to membranes in the presence of 5 μM phalloidin (●). Otherwise, conditions were identical to those in Fig. 1. For comparison, the curve for binding of actin with gelsolin is reproduced from Fig. 1 (dashed line).

critical concentration to undetectably low levels (10, 16), we reason that phalloidin should eliminate any effect of actin assembly equilibria on binding to membranes. As seen in Fig. 2, phalloidin converts the actin binding curve from a sigmoidal to a hyperbolic shape. Phalloidin also slightly increases the extent and apparent affinity of binding. This result further supports the hypothesis that actin polymerization is involved in actin binding to membranes.

### *Homogeneity of Labeled Actin*

To determine whether the actin labeling procedure affects the ability of $^{125}$I-labeled actin to bind to plasma membranes, we incubated $^{125}$I-labeled actin with membranes under conditions such that ~30% of the actin bound. The membranes then were sedimented and the actin in the supernatant was reassayed for membrane binding. As shown in Fig. 3, this free actin binds to membranes to the same extent as does unfractionated actin.

### *Assay Conditions*

Although little binding of $^{125}$I-labeled actin to membranes is observed in a low salt actin depolymerizing buffer, many protein interactions are sensitive to low salt. Therefore, we did additional experiments (not shown) to determine which buffer components are necessary for actin–membrane binding. Binding is significantly decreased when assayed in buffer containing 2 mM $MgCl_2$, but no KCl. In assay buffer containing 50 mM KCl without $MgCl_2$, the binding of $^{125}$I-labeled actin to membranes is essentially the same as that observed in the presence of both KCl and $MgCl_2$. Also, no difference is observed between the binding of ADP–actin (49) and ATP–actin (not shown). These results suggest that the mechanism of binding and polymerization onto membranes may differ somewhat from the mechanism of actin polymerization described in solution studies (20, 34, 49).

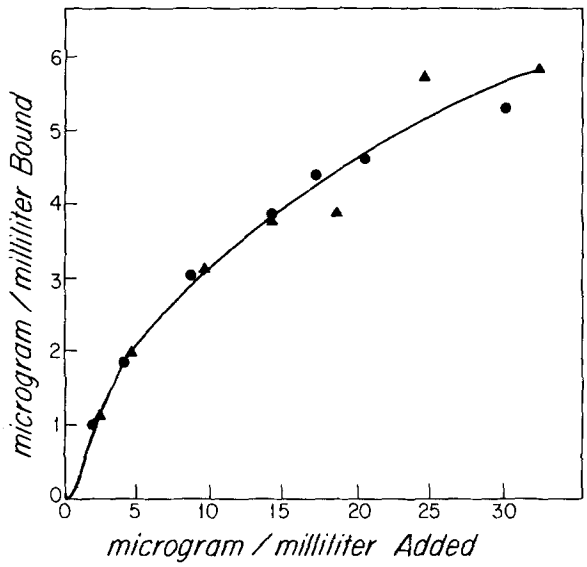


*Figure 3.* Homogeneity of radiolabeled actin. $^{125}$I-labeled actin was incubated with membranes in polymerization buffer for 1 h, and the bound actin (30%) was removed by sedimenting the membranes. The binding of the remaining actin to membranes was compared with the binding of untreated actin as in Fig. 1. Untreated actin (●); actin in the supernatant after depletion by membranes (▲).

### *Role of Gelsolin*

The dependence of binding on the mole ratio of actin to gelsolin is shown in Fig. 4*a*. At both subsaturating and saturating concentrations of actin, binding is essentially independent of gelsolin concentration over about a 10-fold range of actin-to-gelsolin mole ratios. As the actin-to-gelsolin ratio decreases from infinity (no gelsolin) to ~50:1, the amount of bound actin decreases steadily, then plateaus and remains nearly constant until the actin-to-gelsolin ratio reaches ~5:1, when binding to the membranes decreases again. A mole ratio of 15:1, about the center of the plateau, was used for all actin binding assays. For the binding assays with $^{125}$I-labeled gelsolin and unlabeled actin, a mole ratio of 40:1 was used to ensure an excess of actin.

Our results suggest that the only important effect of gelsolin on actin binding to membranes is to render actin binding saturable by limiting the length distribution of actin filaments. First, below the critical concentration for polymerization,

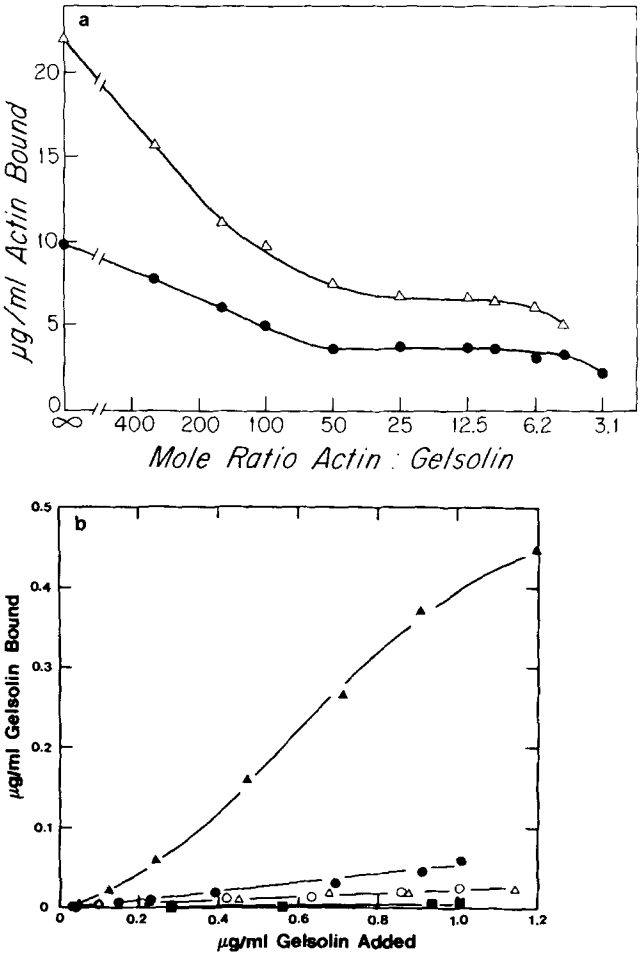


*Figure 4.* (*a*) Binding of radiolabeled actin at 20 μg/ml (●) or 80 μg/ml (△) to membranes prepared using Con A in the presence of variable mole ratios of gelsolin. (*b*) Binding of $^{125}$I-Bolton-Hunter-labeled human plasma gelsolin to membranes. Sodium hydroxide-extracted membranes were incubated with $^{125}$I-gelsolin without (●) and with (▲) actin at a 40:1 mole ratio of actin to gelsolin. Controls: Binding of $^{125}$I-gelsolin premixed with a 120-fold excess of unlabeled rabbit plasma gelsolin without (○) and with (△) actin at a 1:3 mole ratio of actin to gelsolin; and $^{125}$I-gelsolin sedimenting in the absence of membranes (■).

gelsolin has no effect on the binding of actin to plasma membranes (Fig. 1 *a*). Second, above the critical concentration, actin binding is constant over a wide range of actin-to-gelsolin mole ratios (Fig. 4 *a*). Third, the extent of binding is the same if gelsolin is omitted during the incubation and added just before centrifugation (not shown). Finally, the binding of $^{125}$I-labeled gelsolin to membranes is highly dependent on the presence of actin (Fig. 4 *b*). In the absence of actin, binding is low and mostly nonspecific. When actin is added, the binding is stimulated about sevenfold, and is 95% competed for by unlabeled gelsolin. Thus, nearly all of the binding of gelsolin to membranes appears to be mediated by actin. However, a small amount of gelsolin binds specifically to membranes in the absence of exogenous actin.

### *Kinetics of Actin Binding to Membranes*

The rate of actin binding to membranes was measured by adding 25 μg/ml of $^{125}$I-labeled actin to membranes, incubating for various times at room temperature, and then sedimenting through sucrose. This assay was done both with actin initially added as G-actin in depolymerization buffer ($G_i$-actin) and with actin initially added as pre-formed F-actin in polymerization buffer ($F_i$-actin). As shown in Fig. 5, $G_i$-actin and $F_i$-actin bind membranes to approximately the same extent. The initial first order reaction rates are 0.6 μg/ml $min^{-1}$ for $G_i$-actin and 3 μg/ml $min^{-1}$ for $F_i$-actin. However, since the rate for $F_i$-actin is close to the time resolution of the technique, this value must be taken as an approximation. Nevertheless, it appears that the initial rate of binding is substantially faster for $F_i$-actin. These results are consistent with the idea that membrane-bound actin is in a polymerized state.

### *Role of Actin Assembly*

One of the striking features of the binding data is that substantial binding to membranes is observed below the critical concentration for actin polymerization (Fig. 1). For example, when 1.25 μg/ml $^{125}$I-labeled $G_i$-actin is included in the assay, 20% of it binds to membranes even though, in the presence of gelsolin, the critical actin concentration is ~30–40 μg/ml (14, 35). These results imply either that G-actin, as well as F-actin, binds to membranes or that the membrane binding sites promote actin assembly into filaments. To determine whether the actin bound to membranes at concentrations below the critical concentration is monomeric or filamentous, we analyzed the membrane-bound actin in two different ways.

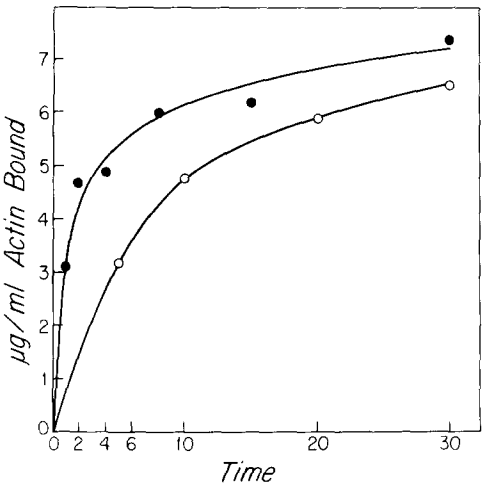


*Figure 5.* Time course of actin-membrane binding. $^{125}$I-labeled actin at 400 μg/ml without gelsolin was pre-incubated for 1 h, either in polymerization buffer ($F_i$-actin) or in low salt depolymerization buffer ($G_i$-actin). Actin from each mixture was diluted to 25 μg/ml in polymerization buffer containing membranes. After the indicated times, the samples were spun through 10% sucrose and the radioactivity in the pellets was counted. $G_i$-actin (○); $F_i$-actin (●). Time, in minutes.

### *Staining with Rhodamine–Phalloidin*

First, membranes incubated with or without 5 μg/ml actin were examined for staining by rhodamine–phalloidin. Since phalloidin binds F-actin, but not G-actin (16), it can be used to distinguish actin monomers from filaments. Fig. 6 shows that membranes incubated with actin stain brightly, while untreated membranes show essentially no fluorescence. Membranes incubated with as little as 1 μg/ml actin show dim, but very distinct, fluorescence. Rhodamine–phalloidin staining is blocked by excess unlabeled phalloidin, and heat-denatured membranes incubated with 20 μg/ml actin do not stain (not shown). These results suggest that actin bound to membranes is in an assembled state even below its normal critical concentration. However, these results do not rule out entirely the possibilities that monomeric actin bound to membranes is in a conformation sufficiently like F-actin to allow it to bind phalloidin, or that the formaldehyde-fixed actin still can be induced to polymerize by the rhodamine–phalloidin.

### *Chemical Cross-linking*

To analyze directly the state of assembly of the bound actin, we used MBS, a noncleavable chemical cross-linker (33). This reagent forms cross-links between subunits in F-actin by reacting through its maleimide moiety with Cys-374 and by simultaneously reacting with Lys-191, Lys-213, or Lys-215 via the succinimide ester (60; residues designated as in reference 34). When added to solutions containing actin, MBS cross-links F-actin into multimers with high efficiency while leaving G-actin essentially unaltered (Fig. 7). When 2.5 μg/ml $G_i$-actin is incubated with membranes, it is cross-linked by MBS into multimers with the same pattern observed for F-actin. Such multimers are not observed in parallel samples with heat-denatured membranes, in samples without membranes, or in the unbound actin from any of these samples. This result demonstrates that the membranes specifically induce the polymerization of bound actin at concentrations below the normal critical concentration for polymerization.

Cross-linking within the actin filament evidently is much more efficient than cross-linking of actin to membrane proteins since we detect no complexes at molecular weights other than those of actin multimers. Actin multimers as large as octamers can be observed bound to membranes. However, membranes have no detectable effect on the critical concentration of the actin which is free in solution. These results provide strong evidence for the polymeric nature of membrane-bound actin and suggest that *D. discoideum* plasma membranes stabilize assembled actin.

The observed stabilization of membrane-bound actin filaments might be due to the existence of clustered actin-binding sites along the membrane which may bind to several actin

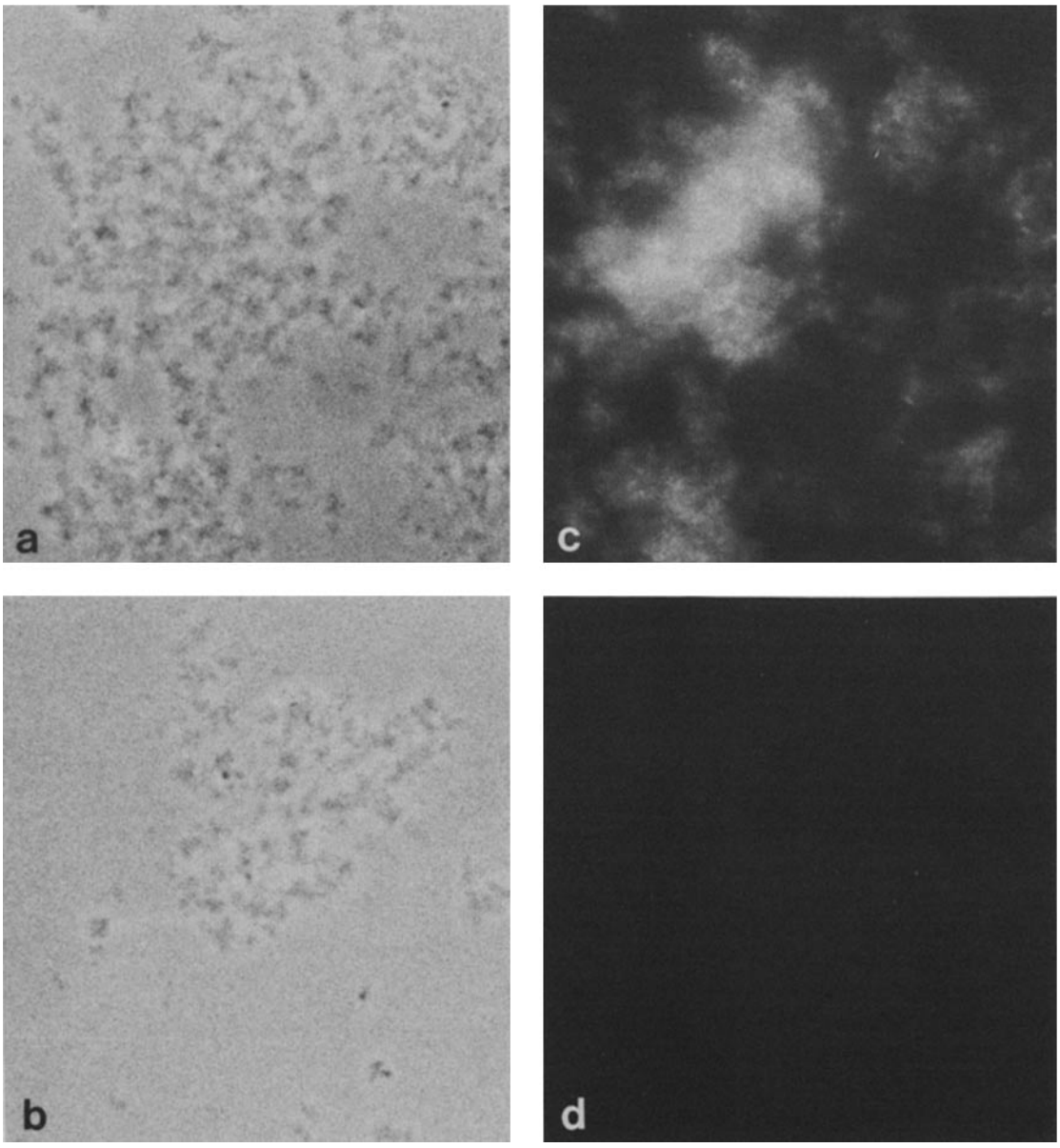


*Figure 6.* Rhodamine–phalloidin staining of membrane-bound actin. Membranes, incubated with (*a* and *c*) or without (*b* and *d*) 5 μg/ml unlabeled actin, were fixed and then stained with rhodamine–phalloidin. Samples were viewed with phase contrast (*a* and *b*) and with fluorescence (*c* and *d*) optics. The fluorescence photographs were taken and printed using identical exposure settings.

monomers simultaneously. These clustered actin-binding sites may raise the local actin concentration above the critical concentration and, thus, may nucleate actin filaments. Alternatively, in a mechanism analogous to that proposed for phalloidin (10, 16), actin-binding sites on membranes might stabilize spontaneously nucleated actin filaments by preventing monomer dissociation. Whatever the mechanism, it is likely that the binding we see represents an interaction between the membrane and the sides of the actin filaments.

## Discussion

The evidence presented here suggests that *D. discoideum* plasma membranes specifically bind F-actin and that these membranes induce actin polymerization under conditions that normally would not permit filament formation. This conclusion is consistent with equilibrium binding data, kinetic measurements, binding of rhodamine–phalloidin to membranes incubated with subcritical actin concentrations, and chemical cross-linking of membrane-bound actin. While some of these experiments are open to alternative explanations, it is difficult to conceive another interpretation that is consistent with all of our results. Our data also strongly suggest that binding occurs between multiple sites on the membrane and the sides of actin filaments. Otherwise, the membrane might nucleate assembly but would not be expected to stabilize filaments as large as octamers at low actin concentrations. Binding to the sides of actin filaments also is supported by the competitive inhibition of actin–membrane associations by myosin subfragment-1 (22) and by electron micrographs of actin bound to *D. discoideum* membranes (2, 22). Actin filaments may bind to membranes in an end-on configuration

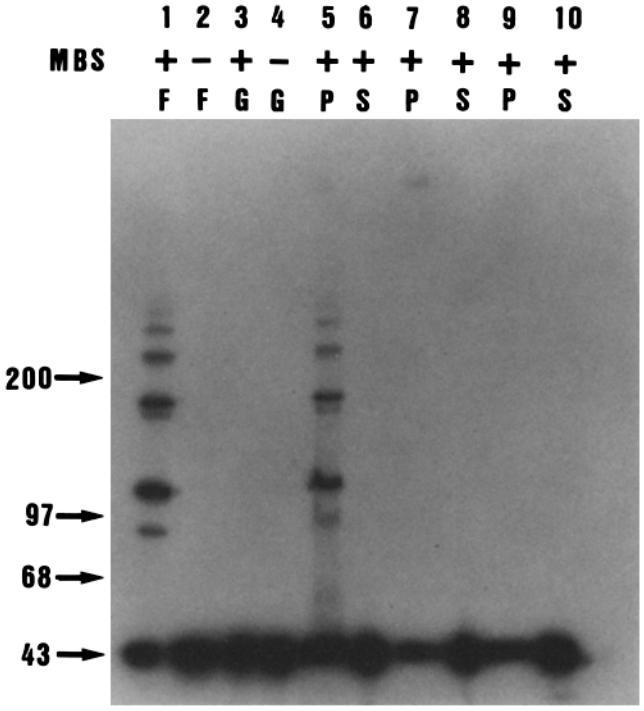


*Figure 7.* Chemical cross-linking of membrane-bound actin. Radiolabeled actin at 2.5 μg/ml was incubated either in a modified polymerization buffer or in low salt depolymerization buffer, with or without 100 μg/ml plasma membranes. The actin was cross-linked with MBS, samples were sedimented to pellet the membranes, and both pellets and supernatants were run on an SDS polyacrylamide gel and processed for autoradiography. Controls: Lanes *1* and *2*, actin in polymerization buffer containing 5 μM phalloidin (F-actin) either with MBS (lane *1*) or without MBS (lane *2*). Lanes *3* and *4*, actin in low salt depolymerization buffer (G-actin) with MBS (lane *3*) or without MBS (lane *4*). Experiment: MBS cross-linking of actin with membranes; pellet (lane *5*) and supernatant (lane *6*). MBS cross-linking of actin with heated membranes; pellet (lane *7*) and supernatant (lane *8*). MBS cross-linking of actin without membranes; pellet (lane *9*) and supernatant (lane *10*).

as well, but our assay probably is less sensitive to such binding, especially since it is done in the presence of gelsolin which binds the same end of the filament that binds to membranes (1, 15, 29, 30, 44, 54).

Since *D. discoideum* membranes apparently mediate both actin assembly and lateral binding of actin filaments, we suggest that the clustering of membrane proteins in the plane of the membrane may be a critical factor in the stabilization of actin filaments. The membranes used in this study were prepared from cells engaged in Con A–induced patching and capping, a process that induces large scale recruitment of actin to the regions of the membrane directly beneath the patches (7, 11). Preliminary freeze fracture electron microscopy shows that most of the large intramembraneous particles are found in large patches. Thus, membrane proteins in these membranes probably are highly clustered.

We also have examined the actin binding properties of membranes prepared without Con A, using the isolation procedure of Das and Henderson (12). These membranes bind actin at approximately the same concentrations, but the extent of binding is 2–4-fold lower (Fig. 1 *a*). These membranes form closed vesicles, so the difference in actin binding could be due to a difference in sidedness or sealing of the vesicles. Also, since these membranes are somewhat less pure than membranes prepared using Con A, part of the difference in extent of binding might be due to the presence of contaminants that do not bind actin. However, the possibility exists that the difference in actin binding is due to the fact that membrane proteins are less clustered in membranes prepared without Con A.

In summary, our data indicate that plasma membrane proteins can provide potent signals for actin binding and polymerization. This phenomenon may represent a fundamental aspect of the interaction of actin with membranes and also might play a role in transmembrane signalling processes thought to occur during cell growth, motility, and adhesion.

We thank Imelda Daley for her skilled technical assistance, Catherine M. Goodloe-Holland and Gail Barcelo for assistance with membrane preparation, Steven Proshan for assistance with actin purification, Dr. Keigi Fujiwara for providing the anti–actin antiserum, and Drs. Edward Bonder and Carl Frieden for helpful discussions. We are especially grateful to Dr. Helen Yin for her generosity in providing gelsolin and for helpful discussions.

This work was supported by American Cancer Society grant No. BC-433 to M. A. Schwartz, and by National Institutes of Health grant No. GM29699, March of Dimes grant Nos. 6-375 and 1-981, and a Faculty Research Award (No. FRA-289) from the American Cancer Society to E. J. Luna.

Received for publication 2 August 1985, and in revised form 20 February 1986.

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