How Actin Binds and Assembles onto Plasma Membranes from *Dictyostelium discoideum*

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Abstract. We have shown previously (Schwartz, M. A., and E. J. Luna. 1986. *J. Cell Biol.* 102: 2067–2075.) that actin binds with positive cooperativity to plasma membranes from *Dictyostelium discoideum*. Actin is polymerized at the membrane surface even at concentrations well below the critical concentration for polymerization in solution. Low salt buffer that blocks actin polymerization in solution also prevents actin binding to membranes. To further explore the relationship between actin polymerization and binding to membranes, we prepared four chemically modified actins that appear to be incapable of polymerizing in solution. Three of these derivatives also lost their ability to bind to membranes. The fourth derivative (EF actin), in which histidine-40 is labeled with ethoxyformic anhydride, binds to membranes with reduced affinity. Binding curves exhibit positive cooperativity, and cross-linking experiments show that membrane-bound actin is multimeric. Thus, binding and polymerization are tightly coupled, and the ability of these membranes to polymerize actin is dramatically demonstrated. EF actin coassembles weakly with untreated actin in solution, but coassembles well on membranes. Binding by untreated actin and EF actin are mutually competitive, indicating that they bind to the same membrane sites. Hill plots indicate that an actin trimer is the minimum assembly state required for tight binding to membranes. The best explanation for our data is a model in which actin oligomers assemble by binding to clustered membrane sites with successive monomers on one side of the actin filament bound to the membrane. Individual binding affinities are expected to be low, but the overall actin–membrane avidity is high, due to multivalency. Our results imply that extracellular factors that cluster membrane proteins may create sites for the formation of actin nuclei and thus trigger actin polymerization in the cell.

ACTIN filament assembly at membrane surfaces has been observed in many biological systems. The elongation of actin filaments in intestinal brush border microvilli (Mooseker et al., 1982), the extension of the *Thyone* sperm acrosomal process (Tilney and Inoué, 1982), and the elongation of actin bundles during *Limulus* spermatid differentiation (Tilney et al., 1981) all involve the addition of actin monomers at the membrane-associated ends of actin filaments. Similarly, actin polymerization in fibroblast lamellipodia appears to occur preferentially at the cytoplasmic surface of the plasma membrane (Wang, 1985; Svitkina et al., 1986). Although these microscopic observations suggest a spatial correlation between biological membranes and actin assembly sites, the mechanism of actin filament assembly at membrane surfaces is not understood. As part of our ongoing effort to understand the molecular basis for actin–membrane interactions, we are investigating the mechanism of actin assembly onto the surfaces of highly purified plasma membranes isolated from the cellular slime mold, *Dictyostelium discoideum*.

Isolated *D. discoideum* plasma membranes bind preassembled actin filaments as measured by low shear viscometry (Luna et al., 1981) and F-actin affinity chromatography (Luna et al., 1984). Most of the binding between actin and these plasma membranes appears to involve the sides, rather than the ends, of the actin filaments (Bennett and Condeelis, 1984; Goodloe-Holland and Luna, 1984). Ponticulin, an integral membrane glycoprotein with a subunit molecular weight of 17,000, appears to be responsible for much of this binding (Wuestehube and Luna, 1987).

We recently have extended these observations by measuring the binding of radiolabeled actin to plasma membranes in a sedimentation assay (Schwartz and Luna, 1986). Because actin polymerizes, actin binding to membranes is non-saturable (Cohen and Foley, 1980; Jacobson, 1980). However, actin binding to membranes approaches saturation in the presence of gelsolin, a protein that cuts and caps actin filaments (Yin and Stossel, 1980). By limiting the size of the actin filaments, gelsolin allows us to distinguish between actin bound directly to membrane sites and actin bound indirectly by copolymerization. Gelsolin-capped actin binds to membranes with positive cooperativity. Half-maximal bind-

ing is observed at 8–18 μg/ml (0.2–0.4 μM) actin. At saturation, 80–200 μg of actin is bound per mg of membrane protein (Schwartz and Luna, 1986).

A possibility raised by our previous work is that the assembly of actin into filaments might be closely coupled to actin binding to membranes. Fluorescence microscopy with rhodamine-phalloidin and chemical cross-linking of actin bound to membranes both indicate that membrane-bound actin is polymeric, even at actin concentrations well below the critical concentration for actin polymerization in solution. Conversely, low salt conditions that inhibit actin polymerization completely inhibit actin binding to membranes.

The close relationship between actin polymerization and actin–membrane binding is of considerable interest since it provides a possible physical basis for the observed coupling between signals generated at the plasma membrane and increases in cytoplasmic F-actin (Painter and McIntosh, 1979; Laub et al., 1981; Fox and Phillips, 1983; Lewis, 1984; Pfeiffer et al., 1985; Carson et al., 1986; Newell, 1986; Omann et al., 1987). However, the close relationship between actin binding and actin polymerization at membrane surfaces also greatly complicates quantitative analyses of these interactions. Since binding and assembly appear to occur concurrently, the analysis of actin binding independent of assembly appears to be impossible. In an effort to dissociate binding and polymerization, we have made four chemically modified actins which appear not to polymerize in solution. Three of these modified actins also cannot bind to membranes. A fourth modification, ethoxyformylation, produces a modified actin that binds to the same sites on isolated plasma membranes that bind unmodified actin. Interestingly, ethoxyformylated (EF)[1] actin also appears to regain the ability to polymerize at the plasma membrane surface. Because EF actin does not polymerize appreciably in solution and because no capping (or nucleating) protein is needed in binding assays, this modified actin can be used to determine the cooperativity of the binding between actin and membranes. EF actin binding to membranes is highly cooperative with an apparent Hill coefficient of ∼2.9.

These results strengthen and extend our previous observation of the potency of the signal for actin filament formation present in *D. discoideum* plasma membranes and provide strong evidence that tight binding of actin to membranes requires the assembly of the membrane-bound actin into trimers, or higher oligomers. In conjunction with published information about actin filament structure and the established predilection of these membranes for binding to the sides of actin filaments, our data suggest a model by which factors that cluster actin-binding membrane proteins can generate membrane-associated actin nuclei in the cell.

Materials and Methods

Materials

Ethoxyformic anhydride (EFA) was purchased from Aldrich Chemical Co., Milwaukee, WI or from Sigma Chemical Co., St. Louis, MO. Succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB) was obtained from Pierce Chemical Company, Rockford, IL. All other reagents were as described in Schwartz and Luna (1986). Actin was isolated from rabbit muscle by the pro-

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1. *Abbreviations used in this paper*: EFA, ethoxyformic anhydride; EF, ethoxyformylated; SMPB, succinimidyl 4-(p-maleimidophenyl)butyrate.

cedure of Spudich and Watt (1971) and gel filtered on Sephadex G-150 according to Uyemura et al. (1978). Gel-filtered actin was iodinated using ^{125}I-labeled Bolton–Hunter reagent purchased from New England Nuclear, Boston, MA or prepared as described by Schwartz and Luna (1986).

Plasma membranes were isolated according to Luna et al. (1984). Briefly, cells were treated with concanavalin A to initiate patching and capping of cell surface receptors and a concanavalin A-enriched, dense membrane fraction was isolated on sucrose gradients. Then, the concanavalin A and endogenous actin and myosin were removed and a less dense, plasma membrane–derived fraction was isolated from a second set of sucrose gradients.

Preparation of EF Actin

EFA dissolved in ethanol was added to 3–5 mg/ml G-actin in 0.2 mM $CaCl_2$, 0.2 mM ATP, 2 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.5, such that the mole ratio of EFA to actin was 12:1 and the final concentration of ethanol was <2% (vol/vol). After 20 min on ice, 0.1 vol of a 10× polymerization buffer was added such that, after dilution, the solution contained 50 mM KCl, 2 mM $MgCl_2$, 10–25 mM Pipes, pH 7.0. After 1 h at 20–25°C, polymerized actin was sedimented either for 30 min at 30 psi (199,000 g_{max}) at a fixed angle of 18° in an airfuge (Beckman Instruments, Inc., Fullerton, CA) or for 2.5 h at 45,000 rpm (243,000 g_{max}) in a swinging bucket rotor. Radiolabeled EF actin was prepared similarly by EFA treatment of [^{125}I]Bolton–Hunter–labeled G-actin (Schwartz and Luna, 1986). Note that, if the radiolabeled EF actin is centrifuged for too short a time or is less concentrated than ∼1.5 mg/ml after centrifugation, residual polymerizable actin can cause artifactually high values in binding assays (see below).

The concentrations of actin and total membrane protein were determined in the presence of 1% SDS by the method of Lowry et al. (1951); BSA was used as a standard.

Critical Concentration of EF Actin

EF actin, prepared as described, was chilled to 0°C and then concentrated quickly by overlaying about 600 μl of EF actin in polymerization buffer onto ∼260 mg of dry Sephadex G-25 (Sigma Chemical Co.) in a 1.5-ml polypropylene tube with a small hole in the bottom. The concentrated EF actin was immediately centrifuged into an intact 1.5 ml tube at 2,520 g_{max} for 5 min at 20–25°C in a high speed centrifuge (Savant Instruments, Inc., Hicksville, NY). EF actin at concentrations as high as 6.4 mg/ml was recovered. EF actin was then diluted with polymerization buffer at 0°C, warmed to 20–25°C for 45 min, and resedimented for 30 min at 30 psi (199,000 g_{max}) at a fixed angle of 18° in an airfuge.

Viscosity Measurements

Viscosity was measured using a low shear, falling ball viscometer (Griffith and Pollard, 1978; Fowler et al., 1981). The assay buffer contained 56 mM KCl, 2.25 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM ATP, 1% vol/vol ethanol, 1.8 mM 2-(N-morpholino)-ethanesulfonic acid, 4.7 mM tris(hydroxymethyl)-aminomethane, 22.5 mM Pipes, pH 7.0. Gel-filtered actin at 170 μg/ml (4.0 μM) was mixed at 0°C with varying amounts of untreated actin or freshly prepared EF actin. Samples were incubated at 28°C for 1 h before assay.

Actin–Membrane Binding Assays

Since the ethoxyformyl group on actin hydrolyzes with a half-life of 55 h at pH 7 (Melchior and Farney, 1970), all binding experiments were performed within 1 h of ultracentrifugation. Binding assays with radioiodinated actin were carried out in 50 μl of assay buffer (50 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM dithiothreitol, 0.1–0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 10–25 mM Pipes, pH 7.0) containing 100 μg/ml membrane protein, 1 mg/ml ovalbumin, and varying amounts of actin. After incubation for 1 h at room temperature, membranes were pelleted through a sucrose cushion and the amounts of bound actin were determined as described in Schwartz and Luna (1986). Since binding reaches a steady state by 10 min (data not shown), with an initial rate of binding of ∼0.9 μg/ml per min (0.02 μM/min) with EF actin at 750 mg/ml, these conditions are assumed to yield equilibrium values.

Chemical Cross-linking

Samples with or without 350 μg/ml plasma membranes and with or without 5 μM phalloidin were prepared in 20 μl of polymerization buffer with 0.02% Tween 20. Then, either ^{125}I-labeled EF actin or ^{125}I-labeled untreated actin

(without gelsolin) was added to a final concentration of 480 μg/ml or 50 μg/ml, respectively. After incubation at room temperature for 40 min, 0.5 μl of 40 mM SMPB (a noncleavable cross-linker) dissolved in dimethylformamide was added to a final concentration of 1.0 mM. Samples were incubated 20 min and then the membranes were pelleted by centrifugation as described (Schwartz and Luna, 1986). The samples were normalized for total radioactivity and run in 6% polyacrylamide-SDS gels, using the discontinuous system of Laemmli (1970). Gels were 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

Derivatized Actins

A number of chemical treatments have been described that block the polymerization of actin. Using published procedures, we have prepared actin in which lysine-61 is labeled by FITC (Burtnick, 1984), actin in which tyrosine-53 is reacted with 5-diazonium-(1H)-tetrazole (Bender et al., 1976), and actin in which histidine and tryptophan are photooxidized (Mühlrád et al., 1968). After chemical modification, polymerization, and centrifugation as described above, 50–90% of each of these derivatized actins is rendered nonsedimentable. Under the same conditions, only ∼3% of untreated actin fails to sediment. Each of the supernatants containing derivatized actin has been assayed for the ability to bind to *D. discoideum* plasma membranes by direct binding of radiolabeled, derivatized actin in sedimentation assays. Binding also was assayed by the ability of unlabeled, derivatized actin to compete with radiolabeled, underivatized actin for sites on membranes. None of these actin derivatives shows any detectable membrane-binding activity (data not shown), a result consistent with the idea that actin polymerization and actin–membrane binding are coupled under these conditions (Schwartz and Luna, 1986).

EF Actin

A fourth method for generating nonpolymerizable actin by reacting histidine residues with EFA has been described by Muhlrád et al. (1969) and Hegyi et al. (1974). G-actin has four accessible histidine residues of apparently equal reactivity. A concentration of EFA sufficient to modify an average of one histidine per G-actin renders 25% of the actin nonpolymerizable, derivatization of two histidines per actin blocks polymerization of 50% of the actin, and so on. By contrast, only three histidine residues are accessible to EFA in F-actin, and EFA-treated F-actin polymerizes normally. Analysis of tryptic fragments from [¹⁴C]EFA-labeled F- and G-actin indicates that histidine-40 is protected from EFA in F-actin and that this same amino acid is the major ¹⁴C-labeled residue in nonpolymerizable, EFA-treated G-actin. Therefore, it appears that modification of histidine-40 renders actin incapable of polymerization while modification of any of the other three histidines has no effect on actin polymerizability.

Hegyi et al. (1974) have reported that, at a 12:1 mole ratio of EFA to G-actin, two of the reactive histidines in G-actin become labeled. We have confirmed this observation by measuring the increased absorbance at 240 nm due to the histidine ester reaction product (Myles, 1977). We have found that, under these conditions, 2.1 histidines per actin are modified and 40–60% of the EFA-treated G-actin is rendered nonpolymerizable. Since this EF actin appears to interact
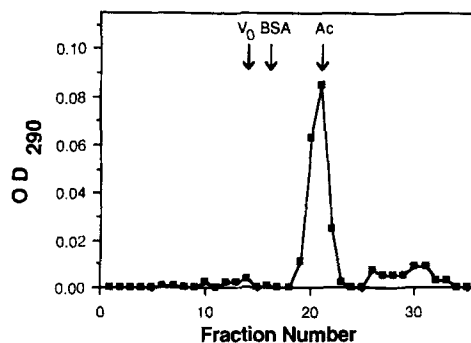

Figure 1. Gel filtration of EF actin. About 200 μg EF actin in 100 μl polymerization buffer containing 0.4 mM ATP was applied to a 21-ml column of Sephadex G-100 and was eluted at 1.5 ml/h with polymerization buffer containing 0.2 mM ATP-1.0 mM dithiothreitol. Fractions of 0.52 ml were collected and read at OD₂₉₀. Arrows denote the void volume (V_o), the elution position of BSA in polymerization buffer (*BSA*), and the elution position of G-actin in depolymerization buffer (*Ac*).

with *D. discoideum* plasma membranes, we have characterized this actin derivative further and have examined in detail its interaction with plasma membranes.

Nonsedimenting EF actin was chromatographed on Sephadex G-100 to determine whether it is monomeric or whether it exists in the form of higher oligomers that do not sediment under our experimental conditions. As shown in Fig. 1, a single major peak elutes at the position of monomeric actin. Less than 6% of the total protein elutes at the position of actin dimers or higher oligomers.

Polymerizability of EF Actin

One measure of actin–actin affinity is the critical concentration necessary for actin filament assembly in solution. Normal, untreated actin has a critical concentration of 4–8 μg/ml, or 0.1–0.2 μM (Bonder et al., 1983; Kurth et al., 1983; Wegner, 1982; Wegner and Isenberg, 1983). In contrast, EF actin at concentrations as high as 1.8 mg/ml (42 μM) does not sediment upon ultracentrifugation (Fig. 2), suggesting that the critical concentration for polymerization of EF actin is at least 200 times higher than that of untreated actin.

Another measure of the affinity of an EF actin monomer for other actin molecules is the ability of EF actin to interact with untreated actin. Although EF actin has essentially no
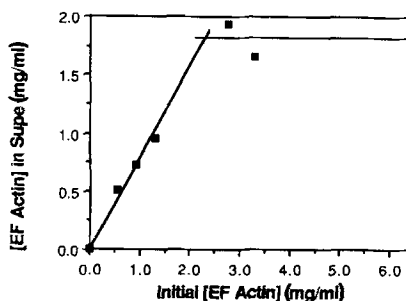

Figure 2. Critical concentration of EF actin. Protein concentrations of aliquots before and after centrifugation for 30 min at 199,000 g_{max} were determined as described in Materials and Methods.
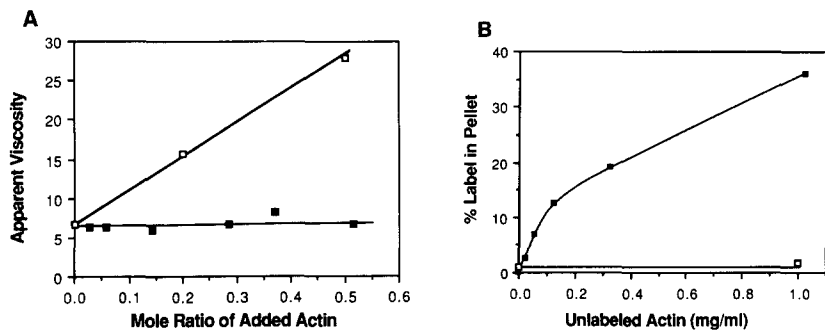
Figure 3. (A) Apparent viscosity (centipoise) vs. mole ratio of added actin. Varying amounts of gel-filtered EF actin (■) or gel-filtered untreated actin (□) were copolymerized with 170 μg/ml (4.0 μM) of gel-filtered untreated actin. Data points are averages of duplicate determinations for each sample. (B) Cosedimentation of radiolabeled EF-actin (■) or protein A (□) with unlabeled untreated actin. ^{125}I-labeled EF actin (25 μg/ml, 8.9 cpm/ng) or ^{125}I-labeled protein A (equal counts) was mixed with varying amounts of unlabeled protein in polymerization buffer, incubated at room temperature for 1 h, and centrifuged for 30 min at 199,000 g_{max}. The radioactivity in both the supernatants and the pellets was measured in a gamma counter.

effect on the low shear viscosity of untreated actin (Fig. 3 A), a specific, although weak, interaction between EF actin and untreated actin is observed in cosedimentation experiments (Fig. 3 B). Extrapolating from the data shown in Fig. 3 B, the concentration of untreated actin at which 50% of the EF actin cosediments is ∼1.6 mg/ml (37 μM). For untreated actin with a critical concentration of ∼6 μg/ml (0.14 μM), 50% sedimentation occurs at only 12 μg/ml (0.28 μM), a value ∼100 times lower than for EF actin. Thus, while the measured affinity of EF actin for other actin molecules is somewhat dependent on the method used, it is at least two orders of magnitude weaker than that observed for untreated actin.

Binding of EF Actin to D. discoideum Plasma Membranes

In polymerization buffer, EF actin binds specifically and saturably to D. discoideum plasma membranes (Fig. 4 A). Under low salt depolymerizing conditions, EF actin, like untreated actin (Schwartz and Luna, 1986), does not bind membranes (data not shown). Also, as is observed with ^{125}I-labeled untreated actin (Schwartz and Luna, 1986), ^{125}I-labeled EF actin binds neither heat-pretreated membranes nor membranes denatured by reduction with dithiothreitol followed by alkylation with N-ethylmaleimide (data not shown).

The membrane-binding activity of radiolabeled EF actin competes with either excess nonradioactive EF actin (data not shown) or excess nonradioactive untreated actin (Fig. 4 A). Conversely, the binding of ^{125}I-labeled untreated actin to membranes competes with high concentrations of both nonradioactive EFA-treated and untreated actins (see below). These experiments indicate that EF actin and untreated actin bind to the same sites on the membranes.

As compared with the binding of EF actin, untreated actin binds with a much higher apparent avidity (Fig. 4 B). Half-maximal binding occurs at 8–18 μg/ml (0.2–0.4 μM) for untreated actin as opposed to 100–200 μg/ml (2.3–4.6 μM) for EF actin. While different membrane preparations have different avidities, the concentration of half-maximal binding for EF actin is always ∼10 times greater than for untreated actin. Another major difference in the binding of these two actins is that saturable binding in assays with untreated actin can be observed only in the presence of a filament capping protein, like gelsolin (Fig. 4 B; Schwartz and Luna, 1986). In sharp contrast, the binding curve for EF actin plateaus at high concentrations in the complete absence of capping protein (Fig. 4 A). This saturability of EF actin binding curves is consistent with the observation that EF actin polymerizes poorly in solution.

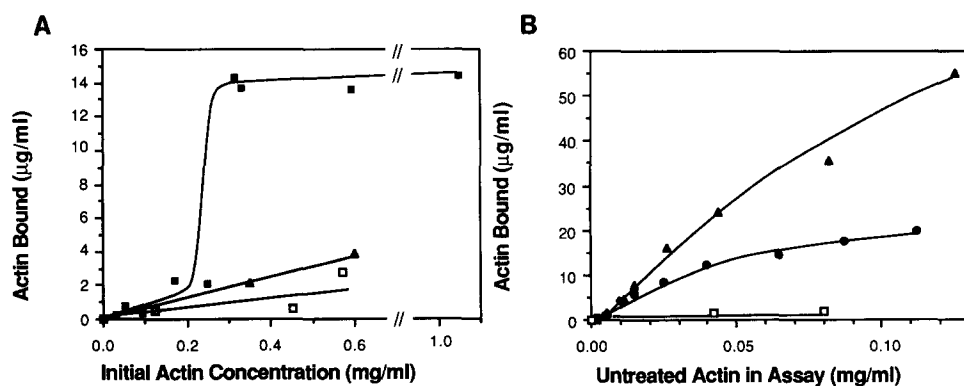The ability of EF actin to copolymerize onto membranes

Figure 4. (A) Binding of ^{125}I-labeled EF actin to 100 μg/ml D. discoideum plasma membranes in the absence (■) and presence (□) of 2 mg/ml unlabeled untreated actin. As a control for residual polymerizable actin, binding also was monitored for comparable volumes of the supernatant from ^{125}I-labeled untreated actin (▲). ^{125}I-labeled untreated actin was polymerized and centrifuged in parallel with ^{125}I-labeled EFA-treated actin. The supernatant was added to the assay at the same dilution as the EF actin supernatant. Thus, the data point at 0.6 mg/ml for the untreated actin supernatant was obtained with supernatant that had been diluted by the same factor as had EF actin at a final concentration of 0.6 mg/ml, although its concentration was much lower. (B) Binding of ^{125}I-labeled untreated actin to 100 μg/ml plasma membranes in the absence (▲) and presence (●) of gelsolin at a 1:15 mole ratio of gelsolin to actin. Binding in the presence of 2 mg/ml unlabeled actin with gelsolin at the same ratio (□).
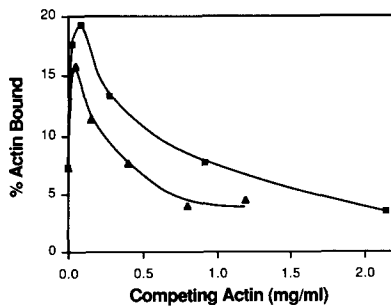
Figure 5. Binding of ^{125}I-labeled untreated actin (7 μg/ml; 0.18 μM) to plasma membranes (100 μg/ml) in the presence of unlabeled EF actin (▲) and unlabeled untreated actin (■).

with untreated actin was tested by measuring the binding of a low concentration of radiolabeled untreated actin in the presence of unlabeled EF actin or untreated actin (Fig. 5). With low concentrations of each nonradioactive actin, the binding of ^{125}I-labeled actin increases, up to 218% of the initial value for EF actin and to 265% for untreated actin. As more competing actin is added, the binding of ^{125}I-labeled actin decreases to background levels with the extent of competition at high concentrations about the same for both actins. These results provide further evidence that actin binding to membranes is cooperative and show that membranes facilitate the copolymerization of EF actin with untreated actin.

Controls

The preparation of EF actin involves a step in which actin capable of polymerizing in solution is removed by sedimentation. Since untreated actin has a finite critical concentration, a small amount remains after ultracentrifugation. To eliminate the possibility that residual polymerizable actin is responsible for the observed EF actin binding to membranes, we performed control experiments with supernatants from untreated actin (Fig. 4 *A*). Also, we performed binding experiments with EF actin centrifuged for 2 h instead of 30 min in the airfuge, long enough to sediment dimers according to our calculations. EF actin prepared in this way bound membranes as usual (not shown). These experiments indicate

that, under our experimental conditions, only a small amount of the observed binding is directly due to the subcritical concentration of polymerization-competent actin.

Fig. 6 *A* shows that EF actin is homogeneous with respect to its membrane-binding activity. EF actin which has been previously incubated with *D. discoideum* plasma membranes binds to membranes as competently as EF actin that is being added to membranes for the first time. Since 10% of the EF actin was removed by the preincubation step, this result rules out the possibility that the 2–5% of the EF actin which binds in our assays is due to a subset of actin molecules with a disproportionately high membrane-binding activity. This conclusion is supported by experiments in which we have prepared EF actin in the presence of phalloidin. This treatment lowers the critical concentration of polymerization-competent actin to unmeasurably low levels (Coluccio and Tilney, 1984), so that only EF actin will remain in solution. After centrifugation, the supernatant contains 37–43% of the total EFA-treated actin (a percentage only slightly less than that observed in the absence of phalloidin). The binding of this supernatant to plasma membranes is essentially identical to that of EF actin prepared without phalloidin (data not shown). We conclude that the small amount of polymerizable actin in our standard preparations of EF actin contributes very little to the observed membrane-binding activity.

Another possible artifact is that EFA treatment directly alters the affinity of actin for the plasma membrane, apart from its effect on actin polymerization. For instance, one of the three histidines that are accessible in F-actin might be involved in binding to the membranes. As one test of this possibility, we have prepared EF actin in which as little as 16% of the treated actin is nonpolymerizable. This EF actin, presumably derivatized only at histidine-40, binds to membranes with the same affinity and cooperativity as more highly derivatized EF actin (data not shown). As another test of the possibility that accessible histidines affect membrane binding, we have polymerized ^{125}I-labeled actin before reaction with EFA. This actin is modified by EFA, but its polymerization is unaffected since histidine-40 is protected (Mühlrád et al., 1969). As shown in Fig. 6 *B*, EFA-treated F-actin binds to membranes with an avidity that is almost indistinguishable from that of untreated actin. This result, and similar experiments with resuspended pellets from EF actin
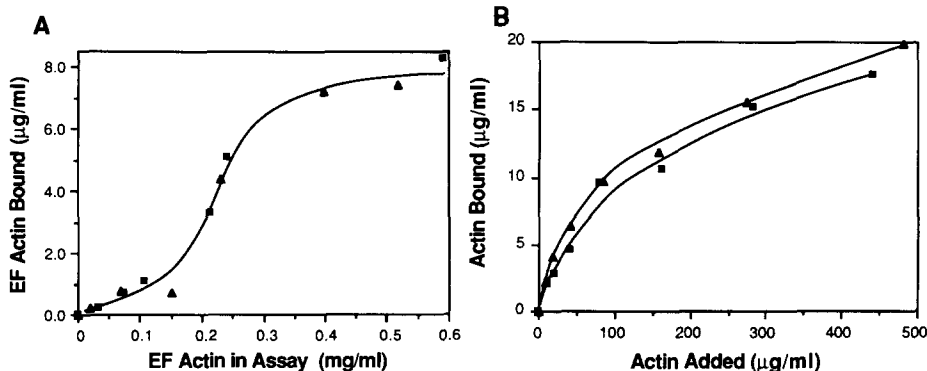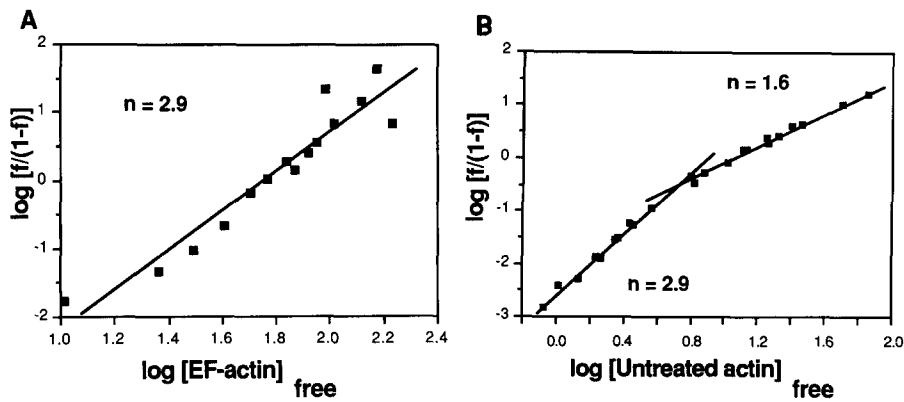

Figure 6. (*A*) Binding to *D. discoideum* plasma membranes of ^{125}I-labeled EF actin without (▲) and after (■) preadsorption against a different aliquot of these membranes. Preadsorption was carried out by incubating EF actin (96 μg; 2.2 μM) with 50 μg of *D. discoideum* plasma membranes for 1 h at room temperature. The membranes and ∼10% of the EF actin were removed by sedimentation at ∼12,000 g_{max} for 15 min. (*B*) Binding of ^{125}I-labeled, polymerization-competent, EFA-treated (■) and untreated (▲) F-actin to plasma membranes. Polymerization-competent, EFA-treated actin was prepared by reacting prepolymerized F-actin with EFA, as described in Materials and Methods.

Figure 7. (A) EF actin–membrane binding data plotted according to the Hill (1910) equation, log $(f/1-f)$ vs. log [EF-actin]$_{\text{free}}$, where f is the fraction of membrane sites occupied by actin and [EF-actin]$_{\text{free}}$ is the difference between the initial concentration of EF actin and the concentration of EF actin bound to membranes (100 μg/ml). The binding of EF actin was corrected for background binding by subtracting values obtained for comparable volumes of a supernatant from untreated actin, centrifuged under identical conditions as described in the legend for Fig. 4 A. The correlation coefficient for the line shown is 0.95. (B) Untreated actin–membrane binding data were plotted according to the Hill (1910) equation, as in Fig. 7 A. The binding between membranes (25 μg/ml) and untreated actin was monitored in the presence of a 1:10 mole ratio of gelsolin to actin and was corrected for background binding by subtracting values obtained with plasma membranes denatured by reduction with dithiothreitol and alkylation with N-ethylmaleimide. The correlation coefficient for each of the lines in this figure is 0.99.

prepared in the usual way (data not shown), indicate that the membrane-binding activity of EF actin is not substantially affected by derivatization of the three accessible histidines.

Cooperativity of Binding

Both EF actin and untreated actin bind to membranes in a highly cooperative fashion even though the concentrations at which binding occurs differ by an order of magnitude. When binding data for EF actin are plotted according to the Hill (1910) equation, linear plots are observed with slopes (n) of 2.3–2.9 (Fig. 7 A). Hill plots of binding data for untreated actin are biphasic with slopes of 2.9–3.3 for data obtained

with actin concentrations below ~10 μg/ml (approximately the critical concentration for actin polymerization in solution); slopes of 1.5–1.6 are observed for data obtained at actin concentrations >10 μg/ml (Fig. 7 B). Because preincubation of untreated actin with the polymerization-inducing drug, phalloidin, results in a Hill plot with a slope of unity (data not shown), we conclude that the positive cooperativity reflects changes in the association state of actin.

Under conditions in which binding is not complicated by actin polymerization in solution (i.e., with EF actin or untreated actin below its critical concentration), the values obtained for n suggest that at least three actin monomers are involved in the initial association with the membrane sur-
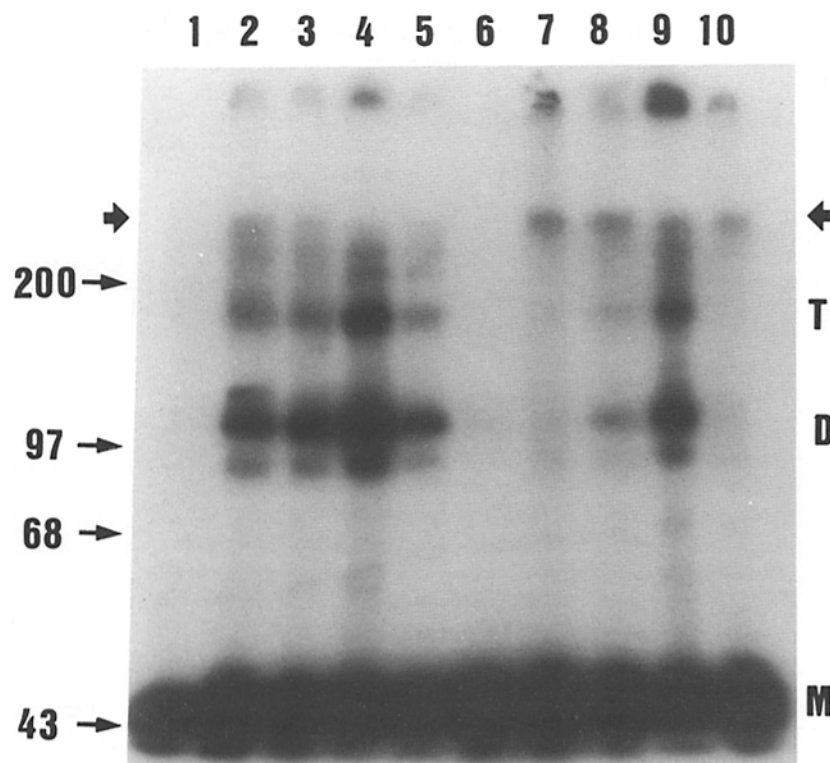

Figure 8. Chemical cross-linking with SMPB of 50 μg/ml (1.1 μM) untreated actin (lanes 1–5) and 480 μg/ml (11 μM) EF actin (lanes 6–10). Samples were incubated with ^{125}I-labeled actin in polymerization buffer, cross-linked with SMPB, and processed as described in Materials and Methods. Lanes 1 and 6, actin in solution without cross-linker. Lanes 2 and 7, actin in solution with 1 mM SMPB. Lanes 3 and 8, actin in solution with 1 mM SMPB and 5 μM phalloidin. Lanes 4 and 9, SMPB cross-linking of actin bound to sedimented plasma membranes. Lanes 5 and 10, SMPB cross-linked actin in the supernatants corresponding to the membrane pellets in lanes 4 and 9, respectively. Numbers on the left refer to molecular mass standards (Bethesda Research Laboratories, Gaithersburg, MD). Letters on the right denote the migration positions of actin trimers (T), dimers (D), and monomer (M). Arrows denote the top of the resolving gel. The electrophoretically distinct forms of actin dimer and trimer are believed to result from different extents of unfolding as a consequence of different intramolecular cross-links (Mockrin and Korn, 1981; Gilbert and Frieden, 1983).

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face. However, since Hill plots may underestimate the size of the cooperative unit (Dahlquist, 1978), the true cooperative unit may be even larger than a trimer.

Chemical Cross-linking

To analyze directly the state of assembly of membrane-bound actin, we used SMPB, a noncleavable chemical cross-linker. By analogy to the known reaction site of chemically similar, shorter chain-length cross-linkers (Elzinga and Phelan, 1984; Sutoh, 1984), SMPB probably cross-links subunits in F-actin by reacting through its maleimide moiety with cysteine-374 in one subunit and through the succinimide ester with lysine-191 in an adjacent subunit. When added to solutions containing ^{125}I-labeled, but otherwise untreated F-actin, SMPB efficiently cross-links it into dimers; small amounts of trimers and higher oligomers also are observed (Fig. 8, compare lane 2 with lane 1). Although phalloidin increases the extent of cross-linking (Fig. 8, lane 3), D. discoideum plasma membranes increase the extent of cross-linking even further (Fig. 8, lane 4). No enrichment in actin multimers is seen in the supernatant from this sample (Fig. 8, lane 5).

As is expected given the poor polymerizability of EF actin, SMPB does not appreciably cross-link ^{125}I-labeled EF actin in solution (Fig. 8, compare lane 7 with lane 6). The addition of phalloidin, which has only a marginal effect on the sedimentability of EF actin (see above), results in the appearance of only a small amount of cross-linked EF actin dimer (Fig. 8, lane 8). In contrast, a large fraction of EF actin bound to D. discoideum plasma membranes is cross-linked by SMPB into dimers, trimers, and even higher multimers (Fig. 8, lane 9). The supernatant from this sample is essentially devoid of cross-linked EF actin (Fig. 8, lane 10). Thus, EF actin bound to membrane surfaces is polymerized and trimers and higher oligomers are directly observed.

Summary of Results

Actin filament assembly occurs at membrane surfaces under conditions that do not support actin polymerization in solution. Hill plots from binding data with untreated actin show positive cooperativity, with a cooperative unit of at least three below the critical concentration for polymerization in solution. Cross-linking data also indicate the existence of membrane-bound actin trimers and higher oligomers. Membrane binding requires K^+ or Mg^{++}, indicating that salt is required for binding and assembly onto membranes. Under all conditions tested, actin binding to membranes and actin filament assembly appear to be tightly coupled. EF actin, which polymerizes in solution about two orders of magnitude less efficiently than untreated actin, binds to membranes with ~10 times lower avidity. It coassembles on membranes with untreated actin and binds to the same membrane sites. EF actin also binds tightly only as multimers with a minimum cooperative unit of three.

A Model for Actin Assembly at Membrane Surfaces

The model for actin–membrane binding that we believe explains our data most simply and completely is shown in Fig. 9. Adjacent actin monomers along one side of a filament bind to two or more membrane sites which are stably associated in the plane of the membrane. An actin trimer bound to two membrane sites is the smallest stable complex. Elongation
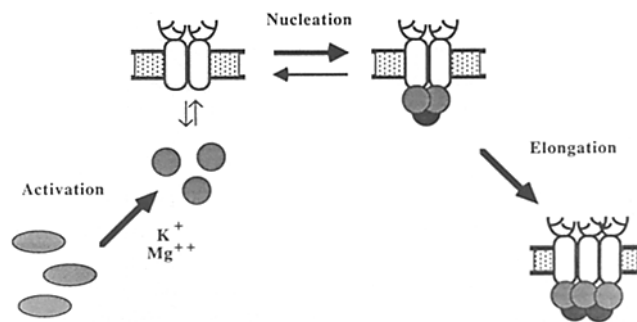

Figure 9. A diagrammatic model for the assembly of actin filaments on plasma membranes. Clustered membrane sites, perhaps activated by extracellular factors, bind tightly to two or more monomers along the side of a short actin filament. Individual actin monomers bind, if at all, with low affinity. Membrane-bound actin exists in the form of oligomers with both ends free. Elongation at the membrane surface occurs by addition of units consisting of one membrane site and two actin monomers. Elongation away from the membrane surface occurs only under conditions permitting filament growth in solution.

along the membrane occurs preferentially by addition of one membrane site and two actin monomers (Fig. 9). Elongation away from the membrane may occur if conditions permit actin polymerization in solution (not shown).

This model is supported by a number of arguments. (a) It explains how actin binding to membranes both requires and enhances polymerization. The binding affinity of a single membrane protein for actin could be quite low—low enough that binding of actin monomers would be undetectable in our assays. Yet, the complex could be highly stable. Studies with antibodies and myosin fragments, for example, show that association constants for interactions with two sites of attachment are 400–600 times larger than for a single site (Greenbury et al., 1965; Greene and Eisenberg, 1980). Physically, this can be thought of as being due to the higher local concentration of ligands at the second site once the first site binds. In the case of EF actin, a decrease in the actin–actin affinity should lower the overall stability of the complex, but the same principles will apply.

(b) The model is consistent with observations indicating that these membranes bind primarily to the sides, rather than the ends, of actin filaments. Electron micrographs show many lateral associations between actin filaments and membranes (Goodloe-Holland and Luna, 1984; Bennett and Condeelis, 1984); myosin fragments, which bind only the sides of actin filaments, competitively inhibit most of the actin–membrane binding in this system (Goodloe-Holland and Luna, 1984; Luna and Goodloe-Holland, 1986). By contrast, binding is essentially independent of the concentration of gelsolin, a barbed-end capping protein, over a broad range of actin-to-gelsolin mole ratios (Schwartz and Luna, 1986).

(c) The model is consistent with geometric considerations. The actin filament (reviewed by DeRosier and Tilney, 1984; Pollard and Cooper, 1986) is a single-start helix in which each successive subunit is 2.73 nm above the previous subunit and is rotated clockwise by an angle that ranges from 156° to 176° (Egelman et al., 1982). Because of the large angle (166 ± 10°) between successive subunits in an actin filament, an actin dimer has the same effective valence for a

membrane surface as an actin monomer; i.e., one. By contrast, an actin trimer has two subunits, the first and third, which could bind membrane receptors. Thus, a trimer is the smallest actin multimer which could bind to membranes with higher affinity due to multiple interactions.

(d) The model is consistent with Hill plots that indicate a cooperative unit of about three, both for EF actin and for untreated actin below the critical concentration. Hill plots yield only a lower limit for the cooperative unit (Weber and Anderson, 1965). However, our observations of concomitant actin binding and assembly over a wide range of solution conditions and actin pretreatments suggest that monomer binding is virtually undetectable in this system. Thus, we suggest that n (Fig. 7 A) may approach the true cooperative unit which is likely to be three, or not much higher than three. The idea of an actin trimer is especially appealing in light of the geometric considerations.

Our model is based on equilibrium binding data and describes actin–membrane interactions at steady state. Thus, by definition, it cannot distinguish between the different kinetic pathways by which steady state may be reached. However, the observed membrane binding of untreated actin below the critical concentration and, especially, the binding activity of EF actin argue against a model in which stable actin polymers must form before membrane binding. Therefore, although membranes clearly do bind preassembled actin filaments (Luna et al., 1984; Schwartz and Luna, 1986), filament formation appears not to be an obligate first step in binding to membranes. Thus, actin assembly at membranes should involve either the binding and stabilization of transient nuclei formed in solution or the weak binding of salt-activated actin monomers that, by virtue of their proximity on the membrane, polymerize to form a stable structure.

Our model makes several testable predictions. First, it predicts that the actin-binding membrane proteins also are bound to each other; i.e., they cannot be clustered only by virtue of their interaction with an actin filament. Weak binding of actin monomers to mobile monomeric sites in the membrane theoretically could increase the local actin concentration at the membrane surface (Cohen and Eisen, 1977) and, thus, could promote actin polymerization. However, actin trimers are thought to be transient, inherently unstable structures in solution (reviewed in Pollard and Cooper, 1986). If membrane proteins were free to diffuse apart, actin trimers on the membrane would be no more stable than actin trimers in solution (Reynolds, 1979). Therefore, the actin-binding membrane proteins must be stably associated with each other after, if not before, actin binding.

The second prediction of our model is that actin binding to multiple sites on a surface will generate rotational strain in the filament since the normal angle between actin subunits is <180°. This strain could either limit the size of actin filaments bound directly to the membrane or result in "untwisted" actin filaments along the membrane surface.

Finally, our model has interesting implications for the mechanism by which extracellular signals influence actin assembly inside cells. Pollard and Cooper (1986) have suggested that profilin in cells binds most of the free actin in such a way that spontaneous nucleation is strongly suppressed but elongation onto available nuclei still occurs. Since plasma membranes can assemble actin into filaments at low concentrations, membranes could create nuclei. Thus, the creation of actin nuclei by clustering of membrane proteins could have a large effect on the state of actin polymerization in the cell. This nucleation differs from the nucleating activity of capping proteins (Cooper and Pollard, 1985; Coue and Korn, 1985; Weber et al., 1987), since both ends of membrane-associated nuclei will be free. This mechanism, which is analogous to previous theoretical proposals (Edelman, 1976; Brandts and Jacobson, 1983), could explain how external signals trigger large increases in actin polymerization during capping (Laub et al., 1981), chemotaxis (reviewed in Newell, 1986; Omann et al., 1987), phagocytosis (Painter and McIntosh, 1979), secretion (Pfeiffer et al., 1985), and cell shape changes (reviewed in Fox and Phillips, 1983; Lewis, 1984).

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