Isolation of an Actin-binding Protein from Membranes of Dictyostelium discoideum

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ABSTRACT   We prepared a probe of radiolabeled, glutaraldehyde cross-linked filamentous actin (F-actin) to study binding of actin to membranes of Dictyostelium discoideum. The probe bound to membranes or detergent extracts of membranes with a high affinity and in a saturable manner. The binding could be reduced by boiling of either the actin probe or the membranes, or by addition of excess native F-actin, but not by addition of an equivalent amount of bovine serum albumin, to the assay. The probe labeled several proteins when used to overlay sodium dodecyl sulfate gels of Dictyostelium membranes. One of these labeled proteins was a 24,000-mol-wt protein (p24), which was soluble only in the presence of a high concentration of sodium deoxycholate (5%, wt/vol) at room temperature or above. The p24 was purified by selective detergent extraction and column chromatography. When tested in a novel two-phase binding assay, p24 bound both native monomeric actin (G-actin) and F-actin in a specific manner. In this assay, G-actin bound p24 with a submicromolar affinity.

The interaction of actin with membranes is thought to be involved in such cell activities as motility, adhesion, receptor rearrangement, and endocytosis (reviewed in references 12, 25, 39). Evidence for an association between actin filaments and the plasma membrane comes from a variety of experimental approaches. Biochemical studies show that actin is present in tight association with isolated plasma membranes (9, 15, 21, 34). Electron microscopic analysis demonstrates that actin filaments attach to the plasma membrane with a distinct polarity in several eucaryotic cell types (2, 10, 23, 24). Studies in which the redistribution of cell surface molecules into discrete clumps termed "caps" causes a concomitant and parallel intracellular rearrangement of actin have led to postulation of the existence of linking proteins in the membrane that couple the arrangement of intracellular actin filaments to membrane surface events (4, 11, 35, 36).

In the current study, we have used the cellular slime mold Dictyostelium discoideum, an organism that has been used for a number of studies of eucaryotic cell motility, to investigate the possible existence of membrane proteins that bind actin. We present evidence that there are indeed several membrane-associated proteins that bind filamentous actin (F-actin)[1] in Dictyostelium. We also report the purification of

one of these proteins, a 24,000-mol-wt protein (p24), which is soluble only in the presence of high concentrations of detergent.

MATERIALS AND METHODS

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[1] Abbreviations used in this paper: DOC, sodium deoxycholate; DTT, dithiothreitol; F-actin, filamentous actin; G-actin, monomeric actin; LLT, 10 mM Tris-HCl, pH 7.6, 0.02% (wt/vol) sodium azide, 30%

a lysis buffer described by Luna et al. (21) with the addition of 2 mM N-α-p-tosyl-L-lysyl chloromethyl ketone. It contains 10 mM Tris-HCl, pH 7.6, 0.02% (wt/vol) sodium azide, 30% (wt/vol) sucrose, 40 mM sodium pyrophosphate, 2 mM EDTA, 0.2 mM DTT, 5 mM 1,10-phenanthroline, 0.1 mg/ml phenylmethylsulfonyl fluoride, 2 mM N-carbobenzoxy-L-phenylalanine, 2 mM N-α-p-tosyl-L-lysyl chloromethyl ketone, 0.5% ethanol.

Actin Preparation: Actin was purified from *Dictyostelium discoideum* by the method of Uyemura et al. (38) and stored as F-actin in 10 mM imidazole, pH 6.5, 50 mM KCl, 1 mM MgCl₂, 1 mM ATP, 1 mM DTT, 0.05% sodium azide. Before use, the actin was recycled by pelleting (30 psi, 20 min in a Beckman Airfuge; Beckman Instruments, Inc., Fullerton, CA), sonication into G-buffer, and incubation on ice for 1 h. The actin was then centrifuged (30 psi, 10 min) and the resulting supernatant was used as monomeric actin (G-actin). ^{35}S-labeled actin was labeled in vivo (32) and purified as above.

Preparation of Membranes from Dictyostelium discoideum: Membranes were prepared using a modification of the method of Luna et al. (21). *Dictyostelium discoideum* amebae (strain Ax-3, grown in axenic culture in HL-5 medium) at a concentration of 0.6–1 × 10⁷ cells/ml were collected by centrifugation at 3,000 g for 5 min. This and all further steps were carried out at 4°C, unless otherwise indicated. The cells were washed in 10 mM Tris-HCl, pH 7.4, weighed, and stirred into an equal volume (milliliter per gram of cell pellet) of LLT buffer. The cell suspension was frozen in bottles suspended in liquid nitrogen, then thawed by short bursts of microwave radiation (Toshiba America, Inc., Torrance, CA), homogenized by five strokes in a Wheaton homogenizer (Wheaton Scientific, Millville, NJ), and layered onto a discontinuous sucrose gradient (35%/55% [wt/vol] sucrose in G-buffer). After centrifugation at 160,000 g_{max} in a Beckman SW-40 rotor for 1 h, the membrane band at the 35%/55% sucrose interphase was collected, resuspended in ~8 vol of G-buffer, and centrifuged at 27,000 g_{max} for 20 min. Membrane pellets were resuspended at a concentration of 4–10 mg protein/ml in G-buffer, and stored at −70°C.

In some preparations of membranes, the initial steps of the procedure were altered, with no appreciable difference in the protein composition of the resulting membrane preparation, as assessed by SDS PAGE. In these preparations, a low-speed membrane pellet was prepared as described (38), except that lysis buffer included 5 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.04 mg/ml aprotinin, 0.001 mg/ml pepstatin, and 0.01 mg/ml leupeptin, and was stored frozen at −70°C. The membrane pellets were then resuspended in an equal volume (milliliter per milliliter) of LLT buffer, layered onto a discontinuous sucrose gradient, and processed as described above.

SDS PAGE: SDS PAGE was carried out on 11% vertical slab gels with 5% stacking gels, using the discontinuous buffer system of Laemmli (18). Before electrophoresis, samples were mixed with solubilizing buffer and heated in a water bath at 50°C for 30 min.

Solubilization of Dictyostelium discoideum Membranes: Frozen membranes were quickly thawed and diluted to a protein concentration of 4 mg/ml with TED buffer, divided into 5-ml aliquots, and centrifuged at 125,000 g_{max}, 30 min. Each pellet was resuspended in 5 ml each of 0.1% (wt/vol) DOC in TED buffer by sonication at 4°C (maximum power setting, 2 30-s bursts). The mixture was then centrifuged as above and the resulting supernatant (S1) removed. This procedure was repeated on the resulting pellets using sequentially increasing concentrations of DOC: 0.5% (S2), 2% (S3), 5% (S4), and 5% (S5). The pellet (P5) was then resuspended by sonication into 0.1 the original volume (0.5 ml) of 5% DOC in TED buffer at room temperature. The suspension was aliquoted into 0.2-ml portions in Airfuge tubes, warmed to 40°C, then sonicated (2 15-s bursts, setting 3, Kontes cell disrupter [Kontes Co., Vineland, NJ]), and centrifuged in a Beckman Airfuge (30 psi, 10 min). The resulting supernatants (S6) were used for further purification of p24.

Purification of p24 from S6 Membrane Extract: The S6 supernatant (2–4 ml) was loaded onto a Bio-Gel A1.5m column (1.5 × 45-cm, 80-ml bed volume), which was previously equilibrated with 5% DOC in TED buffer at room temperature. Fractions were screened for presence of p24 by SDS PAGE. The fractions containing pure p24 were pooled, diluted 1:1 with TED buffer, and concentrated using Centricon 10 microconcentrators (Amicon Corp., Scientific Systems Div., Danvers, MA).

Preparation of Cross-linked F-actin: Filamentous *Dictyostelium* actin was cross-linked with glutaraldehyde, using a modification of the method described by Lehrer (19). F-actin was dialyzed overnight against 200 vol of 5 mM HEPES/0.2 mM ATP/0.1 M KCl, pH 7.5, then diluted to a protein concentration of 1 mg/ml using the same buffer. Glutaraldehyde was added to a final concentration of 2 mM while the solution was vigorously mixed on a vortex mixer. The mixture was incubated at room temperature for 20 h. Concentrated Tris-HCl, pH 7.4, was then added to a final concentration of 0.1 M, to block any remaining glutaraldehyde, and the actin was pelleted by centrifugation at 70,000 g_{max} for 2 h at 5°C. The resulting pellet was sonicated

(two 30-s bursts, 4°C) into two times the original volume of a buffer appropriate for the radiolabeling procedure to be used, as described below.

For labeling by [³H]N-ethylmaleimide, the cross-linked F-actin was suspended in and dialyzed against G-buffer overnight to remove glutaraldehyde and to depolymerize any actin that was not cross-linked. The actin was then loaded onto a Sephadex G-150 column equilibrated with 10 mM imidazole, pH 7.5, 0.2 mM ATP, to separate cross-linked actin from G-actin. The resulting fractions were assayed for protein, and the leading protein peak (void volume) was collected. [³H]N-ethylmaleimide was added to the actin pool to give a final concentration of 2 × 10⁻⁷ M (3% vol/vol pentane), and the mixture was incubated at 0°C for 2 h. To separate labeled actin from unreacted [³H]N-ethylmaleimide, the sample was loaded onto a Sephadex G-150 column equilibrated with G-buffer plus 0.01% sodium azide, and fractions were assayed for radioactivity. A pool was made of the peak of radioactivity at the void volume of the column. Most preparations of labeled actin had a specific activity of approximately 10,000 cpm/μg of protein.

For iodination of the cross-linked actin, the glutaraldehyde-treated actin pellet was sonicated into 50 mM Na-phosphate buffer, pH 7.5, 0.2 mM DTT, 0.2 mM ATP, and dialyzed overnight against the same buffer. The dialyzed cross-linked actin was loaded onto a Sephadex G-150 column equilibrated with the same buffer, and the void fractions containing the cross-linked F-actin were pooled, then dialyzed against 50 mM Na-phosphate buffer, pH 7.5. Iodination was carried out on 60 μl of the cross-linked actin (0.2 mg/ml) by addition of lactoperoxidase (final concentration 16 μg/ml), 1 mCi of ¹²⁵I (carrier-free), and hydrogen peroxide (final concentration 9.8 × 10⁻⁷ M). The reaction was carried out at room temperature for 1 min, then terminated by addition of sodium azide (final concentration 10 mM). The iodinated protein fraction was separated from free iodine on a Sephadex G-25 column previously treated with BSA and washed extensively with 50 mM Na-phosphate buffer, pH 7.5. The iodinated cross-linked actin usually contained ~4 × 10⁶ cpm/μg protein.

¹²⁵I-G-actin was prepared using the same iodination conditions as above, except that 100 mM KCl and 0.5 mM MgCl₂ were added so that actin was polymerized during iodination. The actin was then recycled to G-actin as described earlier in this section into 5 mM HEPES, 0.2 mM ATP, pH 7.5, and passed through a Sephadex G-25 column. The peak of radioactivity in the void volume was collected, and concentrated by dialysis against dry Sephadex. Some of it was treated with glutaraldehyde (as above, except that the KCl was omitted to avoid polymerizing the actin) to make cross-linked G-actin. Only intramolecular cross-links were introduced only when this procedure was followed (19).

Co-sedimentation Binding Assay: Membranes (3–5 mg of protein/ml) were diluted in 10 vol of 0.6 M KI in G-buffer to remove endogenous actin (34) and incubated for 30 min on ice. The mixture was centrifuged at 20,000 g_{max} for 15 min. The membrane pellet was washed by resuspension in 10 vol of G-buffer and centrifugation as before. The resulting pellet was resuspended in the original volume of G-buffer. The KI-treated membranes were mixed with G-buffer, BSA (final concentration, 0.1 mg/ml), and KCl (final concentration, 0.1 M) plus ³H-cross-linked F-actin in a final volume of 150 μl. A 50-μl aliquot of the mixture was removed for verification of total radioactivity added. Incubation was for 5 min at room temperature, followed by pelleting of the membranes in a Beckman Airfuge (10 min, 30 psi). Radioactivity associated with supernatants and pellets was determined by counting in a Beckman LS9000 scintillation counter.

Two-phase Binding Assay: This assay was developed based on a method described by Albertsson (1) for fractionating hydrophobic proteins. It does not depend upon co-sedimentation of the actin probe with membranes, and is therefore useful in assessing binding of actin to detergent extracts of membranes. KI-treated membranes, detergent-solubilized membrane extract, or purified protein was mixed with the following components (final concentrations) in a volume of 0.2 ml: Triton X-100 (0.4%), KCl (0.1 M), radiolabeled cross-linked F-actin or ³⁵S-labeled F-actin, and G-buffer. In experiments employing ³⁵S-G-actin, the KCl was omitted. After 5 min at room temp, 0.2 ml of dextran (31.5%, wt/vol), 0.1 ml of PEG (28%, wt/vol), and 20 μl of a thick slurry of Sephadex G-150 were added to the samples. The solutions were mixed by vigorous vortexing, then centrifuged for 10 min at 5,000 g in a Fisher microfuge (Fisher Scientific, Pittsburgh, PA) at room temperature. This procedure results in a partitioning of the PEG and dextran phases. Membrane proteins were observed by gel electrophoresis to concentrate at the interface between the two phases. The Sephadex also concentrated at the interface, and served to mark this region for collection. The three regions (PEG upper phase, interface (50 μl), and dextran lower phase) were collected, placed in scintillation vials with 1 ml of TS-1 (Research Products International, Mount Prospect, IL), and incubated for 30 min at 50°C. Samples were neutralized by addition of 50 μl of acetic acid before addition of scintillation fluid (ACS, Amersham, 10 ml).

¹²⁵I-Actin Overlay of SDS Gels: Our procedure was a modification of that described by Snabes et al. (33). Protein fractions were electrophoresed on SDS gels as described above. The unstained gels were soaked in 50% methanol/10% acetic acid for 30 min, then soaked in 10% ethanol overnight.

Gels were then washed in 10 mM triethanolamine, pH 7.5, 0.02% sodium azide until the pH of the washing solution stabilized at ~7.0. The gels were transferred to a blocking solution of 3% (wt/vol) BSA in 10 mM triethanolamine, 0.02% sodium azide, pH 7.5, and soaked for 2 h at room temperature. They were then placed on a solid platform in a humidified box at 4°C, blotted of excess moisture, and overlaid with iodinated cross-linked F-actin, iodinated G-actin, or cross-linked G-actin in a buffer containing 10 mM triethanolamine, pH 7.5, 3% BSA, 50 mM KCl, 0.01% sodium azide. (KCl was omitted in experiments using G-actin or cross-linked G-actin.) After 18–20 h of incubation in the overlay solution, the gels were washed extensively in several changes of 10 mM triethanolamine, 100 mM KCl, 0.02% sodium azide. Variations from this procedure are indicated in figure legends. Gels were dried on filter paper and placed on X-ray film (Kodak XAR-5, Eastman Kodak Co., Rochester, NY) for autoradiography at −70°C using intensifying screens.

Protein Determination: Protein concentrations were determined by the method of Bradford (5) or Peterson (28), using BSA as standard.

RESULTS

Characterization of the Cross-linked F-actin Probe

To study the binding of F-actin to membrane proteins, we wanted a probe with the following characteristics. First, we wanted short filaments, because native filaments of 10 μm or more would be unwieldy and subject to breakage in a binding assay. Furthermore, short filaments would have more filament ends. This was desirable because filaments may bind to membranes via their ends (as suggested by electron microscopy (2, 10, 23, 24)). Second, the short filaments needed to be stable, so that they neither depolymerized nor assembled into longer filaments. Third, the short, stabilized filaments had to retain the binding properties of native F-actin.

Cross-linking F-actin with glutaraldehyde (19) gave us a probe with the desired characteristics. Several methods were used to assess the length of the cross-linked filaments: SDS PAGE showed that most of the probe was at least tetramer, but was small enough to enter the (11% polyacrylamide) separating gel. Because the probe was prepared as the void volume fraction of a Sephadex G-150 column (see Materials and Methods), we would expect to have eliminated monomers and dimers. Most of the probe did not sediment when centrifuged 10 min at 30 psi (180,000 g_{max}) in an Airfuge (Fig. 1), suggesting that the filaments were decamer length or less (40). Electron microscopy revealed short filaments of ~70 nm (± 50 nm standard deviation, n = 30). This is almost certainly an overestimate of the average filament length, inasmuch as filaments shorter than ~20 nm are difficult to recognize. A decamer would be ~30 nm in length.

Stability was demonstrated by gel electrophoresis, which showed that the actin is not dissociated to monomer by SDS, and is therefore covalently cross-linked. We also found no change in the viscosity or sedimentability of the probe whether it was incubated in an actin-polymerizing or depolymerizing buffer, suggesting that it is stable in length. Lehrer (19) showed that glutaraldehyde cross-linking stabilizes the actin against heat and EDTA denaturation.

The cross-linked probe resembled native F-actin in a number of ways. Lehrer (19) showed that crosslinking F-actin under conditions similar to those used in the present study did not interfere with the ability of the filaments to bind heavy meromyosin. We found that the cross-linked actin probe binds tritiated cytochalasin B, as demonstrated by the method of Carter-Su et al. (8), and that the binding was efficiently blocked by excess unlabeled cytochalasin D (0.1 mg/ml of probe was labeled with 5 × 10⁻⁷ M [³H]cytochalasin B; 10⁻⁵ M cytochalasin D gave a 71% reduction in labeling). Also, the probe accelerated actin assembly when added to G-actin under polymerizing conditions (1.5 μg/ml of probe added to
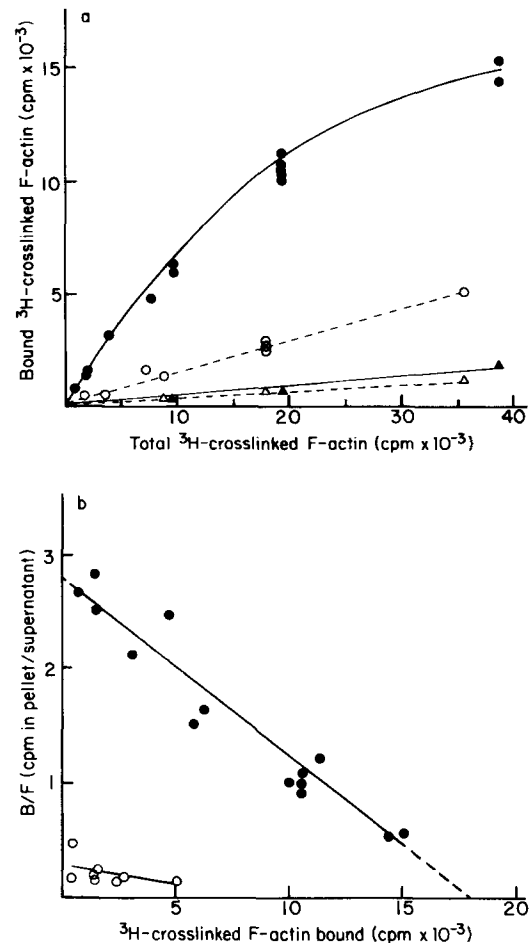

FIGURE 1 Binding of cross-linked F-actin probe to membranes of *Dictyostelium discoideum*. (*a*) *Dictyostelium discoideum* membranes (3.5 mg protein/ml) were prepared and treated with KI as described in Materials and Methods. Membranes (20 μl) were mixed with various amounts of ³H-cross-linked F-actin (●) or boiled cross-linked F-actin (○) in the presence of 0.1 M KCl, 0.1 mg/ml BSA, in G-buffer in a final volume of 150 μl. Incubations were also carried out in the absence of membranes with cross-linked (▲) and boiled cross-linked (△) F-actin. Pellet-associated radioactivity is plotted as a function of cross-linked F-actin added. Points represent singlet determinations, pooled from three separate experiments. Specific activity of cross-linked F-actin, 11,000 cpm/μg of protein. (*b*) Scatchard plots of the same data. ●, binding of cross-linked F-actin to membranes; ○, binding of boiled cross-linked F-actin to membranes; B/F, ratio of bound cross-linked F-actin to free cross-linked F-actin.

200 μg/ml of actin decreased the half-time for assembly from 22 to 3.5 min, as assayed by Ostwald viscometry). We conclude from these two experiments that the cross-linked actin probe retains assembly-competent filament ends.

Binding of Cross-linked F-actin to Membranes of Dictyostelium discoideum

The next step was to investigate whether the probe could be used to demonstrate binding of F-actin to membranes. Fig. 1 *a* shows that tritiated cross-linked F-actin co-sediments with *Dictyostelium* membranes in a saturable manner and that boiling of the probe reduces this binding. Fig. 1 *b* shows a Scatchard plot of the same data. Using the following assumptions, these data give estimates of binding stoichiometry and

affinity. If we assume that each monomeric subunit of the cross-linked actin interacts with a membrane protein(s), maximal binding is 540 pmol actin monomer per milligram of membrane protein, with an apparent affinity (K_d) of 9×10^{-8} M. If we then assume that the average molecular weight of all proteins present in the membrane is 50,000, we calculate that ~2.5% of all membrane proteins are involved in binding actin. If we assume the other extreme, that there is one decameric cross-linked actin filament per membrane binding-protein molecule, the calculated B_{max} is 54 pmol actin decamer per milligram of membrane protein, with an apparent K_d of 9×10^{-9} M. In this case, 0.25% of all membrane proteins are estimated to be involved in actin binding.

Several approaches were taken to evaluate the significance of the binding. First, we asked whether heat denaturation destroyed the interaction. Boiling the membranes before they were used in the assay reduced binding of the actin probe by 70% (Table I). Likewise, boiling of some preparations of the actin probe reduced its binding to membranes by 78% (Table I). However, with other cross-linked actin preparations, boiling sometimes had less effect on binding to membranes. We attribute this variability in the ability of boiling to destroy binding to variations in the degree of intramolecular cross-linking of the actin probe in different preparations. As mentioned above, Lehrer (19) reported that the glutaraldehyde treatment protected actin from heat denaturation, presumably by preventing unfolding of the molecule.

Next, we looked for evidence that the cross-linked F-actin was binding to the same sites on the membranes as native F-actin. We found that 13-fold excess native F-actin reduced binding of the cross-linked actin probe by 40% (corrected for background; Table I). There was little binding of boiled cross-linked F-actin probe in this experiment, and native F-actin had no effect on this small amount of binding (Table I). If instead of adding native F-actin, we increased the amount of BSA added to the assay, little or no effect on the binding of the probe to membranes was observed (Table I). Binding was reduced 75% by treating the membranes with either DOC (0.5%, wt/vol) or Triton X-114 (2%, wt/vol) (data not shown), indicating either solubilization of binding site(s) from the membrane pellet or interference with binding by detergent.

(We favor the former possibility; see below.)

We also used a two-phase binding assay for binding of actin to membranes. Because this second assay did not depend on the sedimentation of membranes, it could be used with detergent extracts of membranes as well. When this assay was used with whole KI-treated membranes, or with Triton X-100 extracts of membranes (Table II), the membrane proteins partitioned at the PEG/dextran interface (see below). The cross-linked F-actin probe also redistributed to this interface in the presence of membranes or membrane extract, suggesting that it binds to the membrane proteins present at the interface. This binding was reduced to background in the presence of a 33-fold excess of native unlabeled F-actin but was not reduced appreciably in the presence of an equivalent amount of BSA (Table II). (This assay suggests that Triton does not interfere with interactions between actin and membrane proteins, as opposed to the possibility proposed in the previous paragraph.) In a separate experiment (not shown), membranes were mixed with native actin or BSA, and the three fractions—PEG, interface, and dextran—were electrophoresed on SDS gels. Essentially all of the membrane protein was found at the interface. Actin, but not BSA, became concentrated at the interface in the presence of membranes. In the absence of membranes, neither actin nor BSA was concentrated at the interface; instead, they were distributed evenly in both phases.

Fractionation of Dictyostelium Membranes by Selective Detergent Solubilization

After having satisfied ourselves that the cross-linked F-actin probe could be used to demonstrate binding of actin to membranes, we turned to a modification of the gel overlay procedure described by Snabes et al. (33) to identify the membrane proteins responsible for this binding. Several proteins were labeled by crosslinked F-actin (Fig. 2b). Initial attempts to fractionate these proteins by column chromatography of the detergent extract were unsatisfactory. Therefore, a number of detergents were tested for their ability to selectively extract proteins from Dictyostelium membranes. Of the detergents tested (octylglucoside, DOC, Brij, Triton X-114, Triton X-100, SDS, 3-3-cholamidopropyl-dimethylammonio-1-propane sulfanate), DOC showed the greatest selectivity of protein extraction when supernatants and pellets of detergent-extracted membranes were compared on SDS gels (data not shown). Therefore, DOC was used to fractionate the proteins of Dictyostelium membranes. Fig. 2a shows a typical

TABLE I

Binding of Cross-linked F-actin to Dictyostelium Membranes in the Co-sedimentation Assay

Cross-linked F-actin	Membranes	BSA	Native F-actin	Percent of total counts in pellet
		mg/ml	mg/ml	
Untreated	−	0.1	0	7
	+	0.1	0	53
	+	1.3	0	50
	+	0.1	0.15	35
Boiled	−	0.1	0	4
	+	0.1	0	12
	+	0.1	0.15	16
Untreated	+	1.0	0	38
	+ (boiled)	1.0	0	12

KI-treated membranes were mixed with untreated or boiled ³H-cross-linked F-actin (25,000 cpm total; 0.012 mg/ml, final concentration) as described in Materials and Methods. In a separate experiment (last two lines of Table), untreated vs. boiled membranes were mixed with the same concentration of cross-linked F-actin.

TABLE II

Binding of Cross-linked F-actin to Triton X-100 Extract of Dictyostelium Membranes in the Two-phase Binding Assay

Triton extract	Cross-linked F-actin (0.018 mg/ml)	BSA (0.6 mg/ml)	Native F-actin (0.6 mg/ml)	Percent of total counts at interface
−	+	−	−	12
+	+	−	−	61
+	+	+	−	54
+	+	−	+	7

Membranes (3 mg protein/ml) were treated with KI, extracted for 1 h with 2 vol of 1% wt/vol Triton X-100 in 10 mM Tris-Cl, pH 8, 1 mM EDTA, 0.5 M NaCl, then centrifuged at 30 psi for 10 min in an Airfuge. The supernate (Triton extract; 90 μl/200 μl of total volume) was mixed with the above components and assayed as described in Materials and Methods. Total radioactivity added to assay, 8,620 cpm.
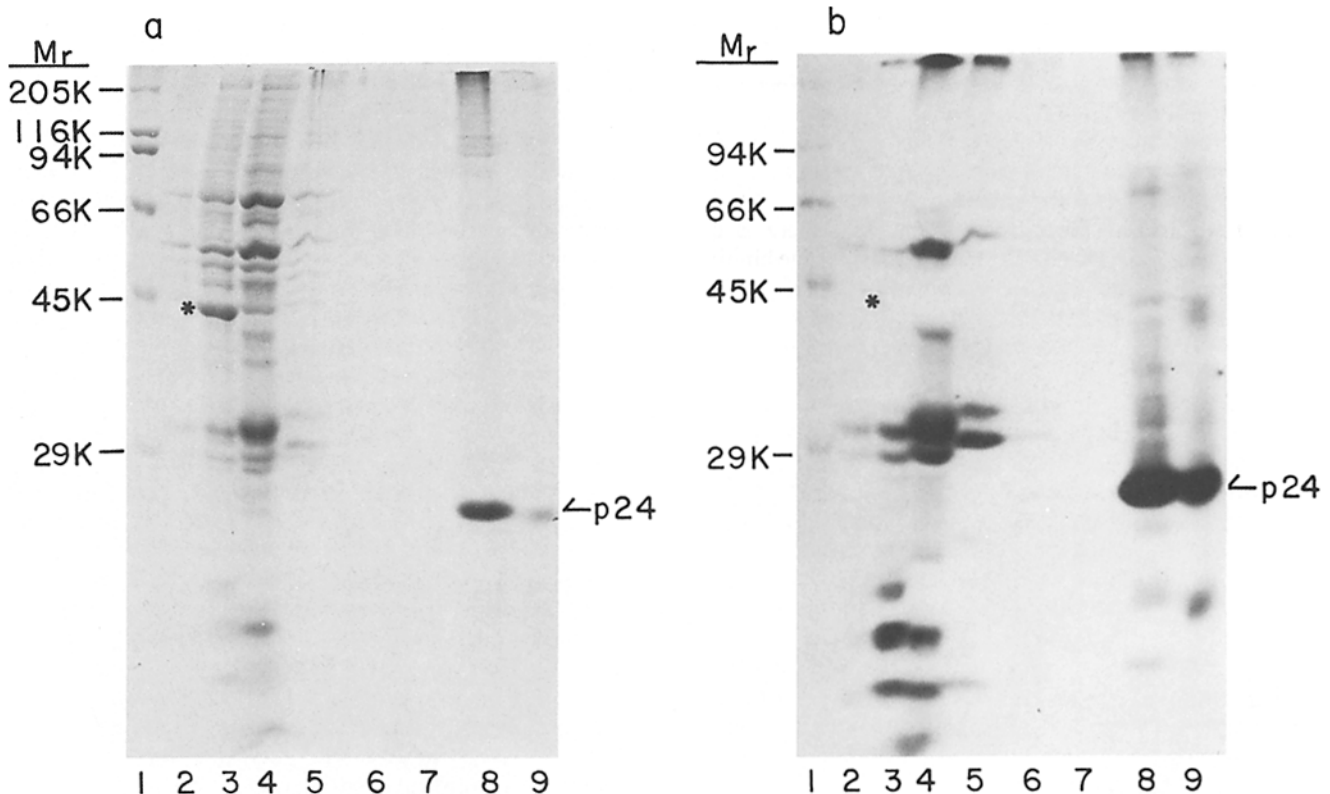
FIGURE 2 ^{125}I-cross-linked F-actin overlay of DOC solubilized fractions from *Dictyostelium* membranes (see Materials and Methods). (a) Coomassie-stained 11% SDS gel. (b) Autoradiograph of duplicate gel overlaid with ^{125}I-cross-linked F-actin, as described in Materials and Methods. Lanes and volumes applied to gels: (1) molecular weight standards; (2) whole membranes (3 mg of protein/ml), 1 μl; (3) S1 (0.1% DOC), 10 μl; (4) S2 (0.5% DOC), 20 μl; (5) S3 (2% DOC), 40 μl; (6) S4 (5% DOC), 40 μl; (7) S5 (second 5% DOC), 40 μl; (8) S6 (5% DOC at 40°C), 40 μl; (9) P6 (final pellet, resuspended in the same volume as in 8), 40 μl. See Materials and Methods for details of preparation of these fractions. Actin is indicated by star. Molecular weight standards (K, thousands). 205K, myosin; 116K, B-galactosidase; 94K, phosphorylase b; 66K, BSA; 45K, ovalbumin; 29K, carbonic anhydrase. ^{14}C-molecular weight standards were used in b.

sequential extraction of proteins from membranes with increasing concentrations of DOC (S1–S6), as described in Materials and Methods. Many of the major membrane-associated proteins, including most of the actin, were extracted when membranes (4 mg of protein/milliliter) were treated with 0.1% DOC (S1); however, a differential extraction of several proteins by 0.5% (S2) and by higher concentrations of the detergent was also apparent. Heating the extraction mixture to 40°C at a concentration of 5% DOC (S6) resulted in the solubilization of a number of proteins that were not previously solubilized by 5% DOC at 4–10°C, including a prominent 24,000-mol-wt protein.

Fig. 2b shows an overlay of the same gel with ^{125}I-cross-linked F-actin. Although the actin probe bound to several of the major membrane proteins (e.g., the 30,000-mol-wt band that is most prominent in S2), a selectivity of binding was also apparent. For example, actin (indicated by star in Fig. 2, a and b), a major component of the S1 fraction, did not bind the cross-linked F-actin probe. Another obvious example is the prominent 70,000-mol-wt protein band in S2, which also can be seen not to bind the probe.

Characterization of p24

We chose to study further the prominent actin-binding 24,000-mol-wt protein (p24) of the S6 fraction, because of its relative purity (~50% pure) after the differential extraction procedure. The S6 fraction was used to further characterize

the binding of cross-linked actin to p24 in gel overlays. Fig. 3 demonstrates that the binding of the actin probe could be blocked by pretreating with 500-fold excess unlabeled cross-linked F-actin. In contrast, a lower molecular weight protein on the same gel (indicated by star in Fig. 3) showed no reduction in radiolabeled cross-linked F-actin binding after pretreatment with excess unlabeled probe. Thus the binding of cross-linked F-actin to p24 is largely specific. Further experiments with native and cross-linked ^{125}I-G-actin indicated that p24 also binds these species in the gel overlay assay (Fig. 4). The binding of cross-linked G-actin could be blocked more effectively than that of native G-actin with excess unlabeled actin. A possible explanation for this observation is that native G-actin is more susceptible to denaturation, and therefore may have a larger nonspecific component in its binding. Cross-linking of G-actin had no apparent effect on the amount of labeling seen. This is another piece of evidence that cross-linked actin can be legitimately used in place of native actin.

Binding of actin to p24 could also be demonstrated with the two-phase assay. To use this assay, it was first necessary to purify p24 further. This was accomplished by chromatography of S6 on a Bio-Gel A1.5m column at room temperature in the presence of 5% DOC (see Materials and Methods). In a typical chromatography profile, ~30% of the p24 fractionated with a group of high molecular weight proteins in the void volume of the column. This result may indicate that some of the p24 is present in an aggregated form, possibly co-
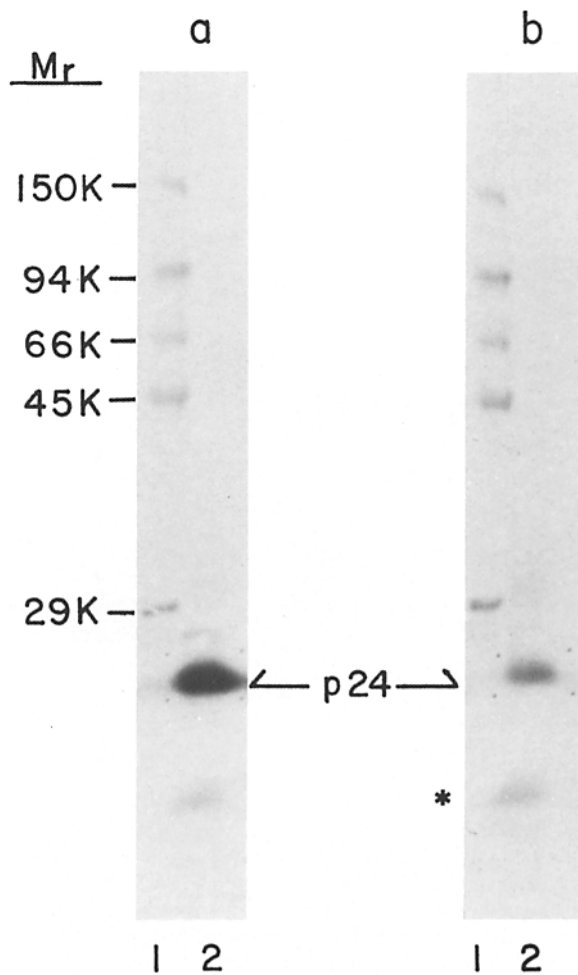
FIGURE 3 Binding of ^{125}I-cross-linked F-actin to p24. ^{14}C-molecular weight standards (1) or S6 fractions containing p24 (2) were electrophoresed on 11% SDS gels and overlaid for 9 h with ^{125}I-cross-linked F-actin (0.4 µg/ml) after 18 h of preincubation without (a) or with (b) unlabeled cross-linked F-actin (0.2 mg/ml) in 100 mM KCl, 10 mM triethanolamine, pH 7.5, 0.01% azide, 3% wt/vol BSA. After autoradiography of the dried gels, p24 bands were excised using the autoradiograph as a guide, and radioactivity was determined in a gamma-counter: (a) 4,070 cpm; (b) 1,587 cpm. Starred band is a low molecular weight polypeptide that nonspecifically binds cross-linked actin. K, thousands.

aggregating with the other, higher molecular weight proteins. The remainder of the p24 ran as a single peak whose elution volume was consistent with p24 migrating as a monomer or dimer (data not shown). Fig. 5 compares S6 and column-purified p24.

Column-purified p24 was concentrated, and dialyzed extensively against 10 mM Tris, 0.2 mM DTT, pH 8, to remove DOC. This resulted in some aggregation of the p24; therefore, it was sonicated immediately before use in the two-phase binding assay. This assay was used to test binding of native ^{35}S-labeled G-actin and F-actin to p24. SDS electrophoresis demonstrated that most of the p24 concentrated at the interface of the two phases (data not shown). Fig. 6 shows that in the presence of p24, both G-actin and F-actin redistributed to the interface, indicating that they bind to p24. Excess unlabeled native G-actin and F-actin, respectively, blocked this binding. In contrast, an equivalent amount of BSA did not block the binding. As might be expected, a larger amount
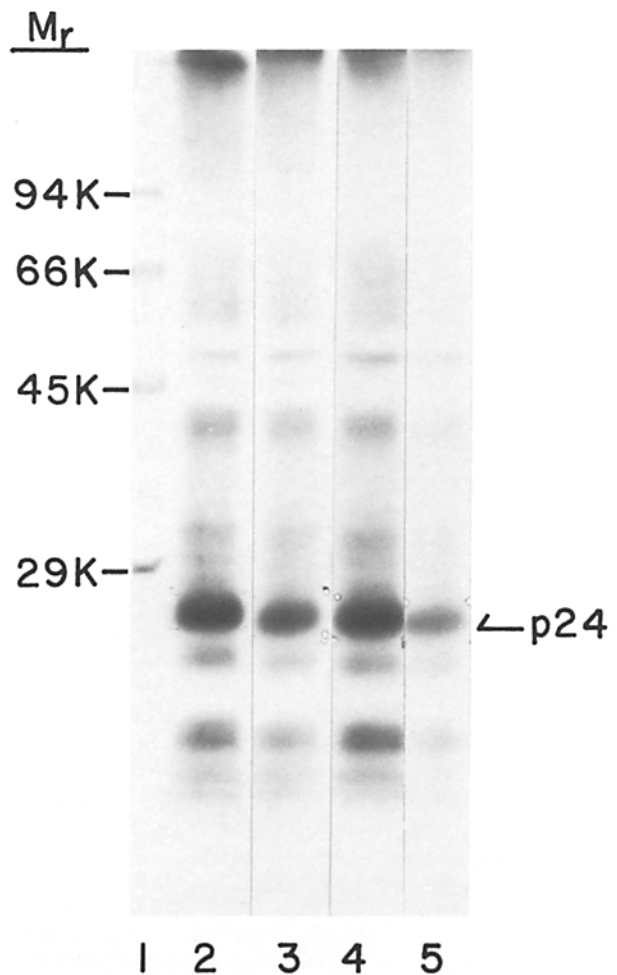

FIGURE 4 Binding of ^{125}I-G-actin and ^{125}I-cross-linked-G-actin to p24 in S6 fraction. S6 fractions containing p24 were electrophoresed on 11% SDS gels, overlaid with buffer containing BSA (lanes 2 and 4) or excess unlabeled actin (lanes 3 and 5) for 4.5 h, then overlaid with ^{125}I-labeled G-actin (lanes 2 and 3) or cross-linked G-actin (lanes 4 and 5 in the same solution for 14 h. Lane 1, ^{14}C-molecular weight standards as described in Fig. 2. K, thousands.

of F-actin than G-actin bound to p24 in this experiment, presumably because it is polymerized.

Fig. 7 shows a Scatchard plot of data from an experiment in which various concentrations of G-actin were tested for binding to p24 in the two-phase assay. The shape of this curve suggests the existence of both high- and low-affinity components to the binding. If we generate regression lines through the first several points of the curve, we estimate K_d's in the range of $1.8-3.5 \times 10^{-7}$ M. Therefore, we conclude that the high-affinity component is in the submicromolar range. B_{max} is likewise estimated as $\sim 10^{-11}$ mol actin per 2×10^{-10} mol p24.

DISCUSSION

Although it is clear that actin interacts with membranes, the molecular basis of the interaction has not been established. There are several possibilities: actin might interact with lipids (26, 37), and/or indirectly with the membrane via another protein (such as spectrin [7]). A third possibility is that actin interacts directly with an integral membrane protein. Evidence has been presented for several examples of this latter
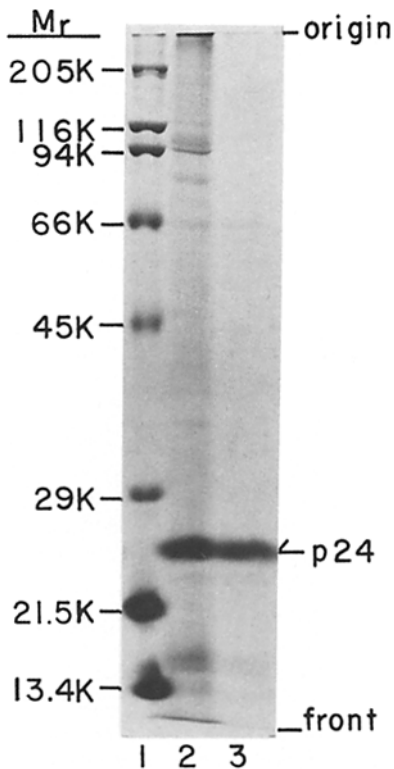
FIGURE 5 Purification of p24 from S6 extract. Comparison of S6 extract and concentrated column purified p24. (Lane 1) Molecular weight standards (K, thousands) described in Fig. 2 with addition of 21.5K, soybean trypsin inhibitor, 13.4K, cytochrome c; (lane 2) S6, a 5% DOC (40°C) extract of membranes; (lane 3) concentrated purified p24.

type of interaction (6, 13, 14, 29, 30).

Several laboratories are investigating the question of actin–membrane interactions in *Dictyostelium*. Jacobson (17) has shown that both F-actin and G-actin will bind to the cytoplasmic surface of *Dictyostelium* membranes, and that binding can be abolished by pretreating the membranes with trypsin. Luna et al. (21) have demonstrated that membranes can increase the low shear viscosity of actin, and these investigators present evidence that the membranes do this by binding, and thus cross-linking, the filaments. They believe that this interaction is mediated by integral membrane proteins, because the effect can be blocked by proteolysis or heating, but not by treatment with chaotropes. Luna et al. (22) have demonstrated specific, saturable binding of *Dictyostelium* membranes to F-actin attached to beads, and they present evidence that this binding is responsible for the effects on low shear viscosity of actin that they reported earlier.

In this paper, we show that binding of actin to membranes can also be demonstrated using a cross-linked F-actin probe. By using this system, we find that the binding is saturable and can be blocked by excess unlabeled cross-linked actin. We obtain a submicromolar affinity of actin for 0.25–2.5% of the total membrane protein in *Dictyostelium*. By using this cross-linked actin probe in a modification of the gel overlay procedure of Snabes et al. (33), we can demonstrate binding to several membrane-derived proteins. We have purified one of these proteins (p24) and have confirmed by using the two-phase assay that the isolated protein binds actin.

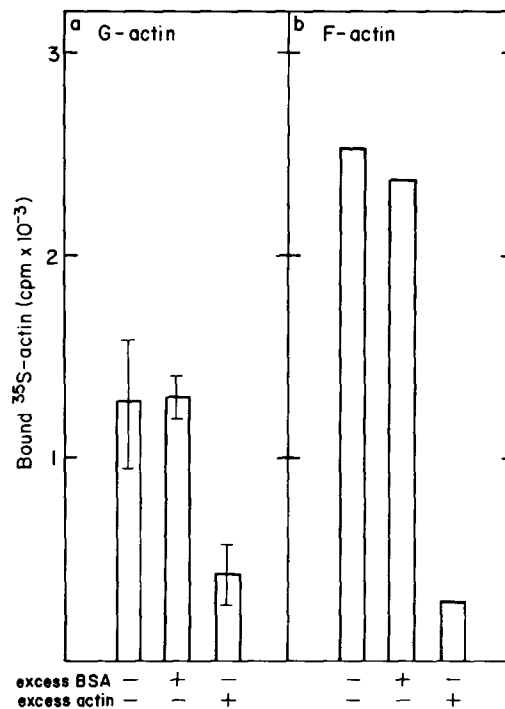As described in Results, we have tested the cross-linked

FIGURE 6 Binding of G- and F-actin to p24 in two-phase assay. (a) Purified, dialyzed p24 (final concentration, 0.025 mg/ml) was mixed with ^{35}S-G-actin (final concentration, 0.04 mg/ml) in the absence or presence of BSA or excess unlabeled G-actin (final concentration, 1 mg/ml) in G-buffer in the two-phase binding assay described in Materials and Methods. Bound ^{35}S-actin is that collected at the interface, corrected for background of 912 cpm present at interface in absence of p24. Shown are mean and SEM of $n = 3$ pooled from two separate experiments. Total radioactivity added to assay, 13,220 cpm. (b) Conditions and concentrations identical to those in a, except that ^{35}S-F-actin and unlabeled F-actin were used and 100 mM KCl was present in the assay. Background of 1,792 cpm subtracted from all values. Data are from singlet determinations. Total radioactivity added to assay, 12,100 cpm.
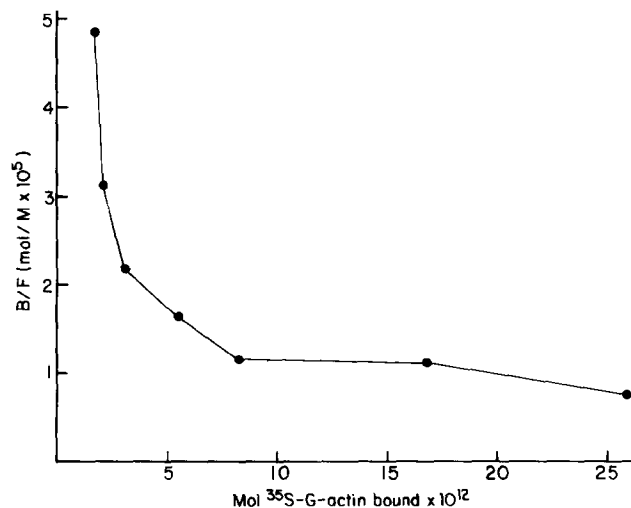

FIGURE 7 Scatchard plot of binding of ^{35}S-G-actin to p24 in two-phase binding assay. Purified, dialyzed p24 (final concentration, 0.02 mg/ml) was mixed with various concentrations of ^{35}S-G-actin in the two-phase binding assay under the conditions described in Fig. 6a. Bound ^{35}S-G-actin is that collected at the interface in the presence of p24, corrected for background of interfaces prepared without p24 in assay. B/F is the ratio of bound to free G-actin.

actin probe in several ways to be sure that it is a valid substitute for native F-actin. The probe binds heavy mero-myosin (19) and cytochalasin B, and accelerates actin assembly, demonstrating that both the ends and sides of the cross-linked filaments resemble those of native F-actin. In addition, the fact that the probe binds to membranes in a specific, saturable, high-affinity manner suggests that it labels authentic actin-binding sites. This binding can be blocked with excess unlabeled cross-linked F-actin, and less efficiently with native F-actin. It may not be surprising that the cross-linked filaments block more efficiently, inasmuch as they are much shorter than the native filaments.

Our finding that more than one membrane protein labels with actin (Fig. 2) is in agreement with the finding of Luna et al. (22) that a number of *Dictyostelium* membrane proteins bind to an F-actin affinity column. It is difficult to assess whether any of the proteins identified by our approach are the same as those identified by Luna et al. (22). There are several reasons that many of the proteins may not be the same. As these authors point out, some of their proteins may interact indirectly with actin. On the other hand, our approach of labeling proteins in SDS gels would identify only a subset of actin-binding proteins that can renature after SDS removal. It is known that some actin binding proteins, e.g., DNase I, cannot bind actin after similar treatment (33).

Schleicher et al. (31) have also used a gel overlay system to label *Dictyostelium* membranes with native ^{125}I-actin. They have observed binding to several proteins; one of 31,000 mol wt may be identical to the major protein labeled by the ^{125}I-cross-linked actin probe in our S2 fraction.

It seems plausible that there be a number of actin-binding proteins in membranes, just as there are a number of soluble actin-binding proteins. Most of the membrane proteins referred to above (6, 13, 29, 30) as putative actin-binding proteins are specialized in function, i.e., they are receptors for collagen or laminin, or are a viral membrane protein or membrane immunoglobulin. These would seem to be poor candidates for mediating all the interactions of actin with membranes (in endocytosis, cytokinesis, locomotion, and cell–cell attachment). We estimate that p24 could be responsible for only 25% or less of the binding seen with whole membranes, because 75% of the membrane activity is extracted by detergent conditions that do not extract p24. The other proteins seen by gel overlay are presumably responsible for at least a fraction of the remaining activity. The linear Scatchard plot for binding of cross-linked actin to whole membranes (Fig. 1*b*) suggests that if multiple proteins are responsible for the binding seen, most may have similar affinities for actin in the submicromolar range. The Scatchard plot for p24 (Fig. 7) appears less linear, and the assay differs in several ways, but there appears to be a submicromolar component to this binding, as well. The affinities obtained from both Scatchard plots are approximate values only, in that the assays used may perturb the equilibrium state, and thus lead to an underestimate of the true affinity.

It was easy to isolate p24 because of its unusual insolubility; it was necessary to use 5% DOC and to raise the temperature (40°C) to solubilize the protein. This makes it extremely unlikely that p24 is a soluble protein contaminant of the membrane preparation. On the other hand, this behavior is unusual even for an integral membrane protein, where stoichiometry and not absolute detergent concentration is thought to determine how much protein is solubilized, as long

as the concentration of detergent is above its critical micellar concentration (16). The DOC concentration used here greatly exceeds the critical micellar concentration; in fact, it is in a range where it may be affecting protein–protein interactions (16). Others have shown (20) that there is a selective resistance of some membrane-associated proteins to detergent extraction and have suggested an interaction of these proteins with the cytoskeleton. We have no further evidence bearing on the nature of the interaction of p24 with membranes at present, but would suggest that it is either an integral or peripheral membrane protein that is part of a detergent-insoluble protein network such as that proposed by Ben-Ze'ev et al. (3). We were unable to bind concanavalin A to p24 or to stain it with the periodic acid–Schiff procedure (p24 contains <9% sugar by comparison with peroxidase standards; unpublished results), either of which would have demonstrated that it was a glycoprotein and therefore likely to be an integral membrane protein. However, neither of these experiments rule out that p24 is a glycoprotein; more lectins or more sensitive assays for sugars could be tried. Approaches such as those used by Glenney and Glenney (14) might also be useful in determining whether p24 is integral.

We also found that a high DOC concentration was required not only for the solubilization but also for the maintenance of solubility of p24, inasmuch as it aggregated when the DOC was removed. This is not an unusual property for hydrophobic membrane proteins, but is unfortunate, because a high DOC concentration causes actin depolymerization. We were therefore unable to perform many of the assays (for effects on assembly rate or final viscosity, for example) that might ordinarily be used to examine interactions of p24 with actin.

The aggregation that occurred upon DOC removal made co-sedimentation with actin an unsatisfactory binding assay. Because p24 was aggregated to some degree in the two-phase assay that we used instead, the stoichiometry of binding in that experiment is expected to be low. The Scatchard plot of the binding of native G-actin to p24 (Fig. 7) suggests that there is a high-affinity component to the binding, although the shape of the curve also indicates the presence of a low-affinity binding component. The overlay data (Fig. 4) suggests that there may be more nonspecific binding with native G-actin than with cross-linked F-actin or G-actin; thus this may be the explanation for the low-affinity binding component in the two-phase assay.

In summary, we have obtained evidence that there are a number of actin-binding proteins in *Dictyostelium* membranes, and have purified one of these proteins (p24). Two different types of assays indicate that p24 interacts with actin. We are currently making antibodies to p24 in order to define its cellular localization and further characterize its interaction with actin.

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