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A Membrane Cytoskeleton from *Dictyostelium discoideum*. II. Integral Proteins Mediate the Binding of Plasma Membranes to F-Actin Affinity Beads

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ABSTRACT In novel, low-speed sedimentation assays, highly purified, sonicated *Dictyostelium discoideum* plasma membrane fragments bind to F-actin beads (fluorescein-labeled F-actin on antifluorescein IgG-Sepharose 4B beads). Binding was found to be (a) specific, since beads containing bound fluorescein-labeled ovalbumin or beads without bound fluorescein-labeled protein do not bind membranes, (b) saturable at ~0.6 µg of membrane protein per microgram of bead-bound F-actin, (c) rapid with a t½ of 4–20 min, and (d) apparently of reasonable affinity since the off rate is too slow to be measured by present techniques. Using low-speed sedimentation assays, we found that sonicated plasma membrane fragments, after extraction with chaotropes, still bind F-actin beads. Heat-denatured membranes, proteolysed membranes, and *D. discoideum* lipid vesicles did not bind F-actin beads. These results indicate that integral membrane proteins are responsible for the binding between sonicated membrane fragments and F-actin on beads. This finding agrees with the previous observation that integral proteins mediate interactions between *D. discoideum* plasma membranes and F-actin in solution (Luna, E. J., V. M. Fowler, J. Swanson, D. Branton, and D. L. Taylor, 1981, *J. Cell Biol.*, 88:396–409). We conclude that low-speed sedimentation assays using F-actin beads are a reliable method for monitoring the associations between F-actin and membranes. Since these assays are relatively quantitative and require only micrograms of membranes and F-actin, they are a significant improvement over other existing techniques for exploring the biochemical details of F-actin–membrane interactions.

Using F-actin beads as an affinity column for actin-binding proteins, we show that at least 12 integral polypeptides in *D. discoideum* plasma membranes bind to F-actin directly or indirectly. At least four of these polypeptides appear to span the membrane and are thus candidates for direct transmembrane links between the cytoskeleton and the cell surface.

Interactions between actin filaments and eucaryotic cell membranes are believed to be involved in endocytosis, chemotaxis, and regulation of surface receptor topography, as well as cell-cell and cell-substrate adhesion. Evidence for this involvement originates from microscopic observations of actin filaments localized at the plasma membranes of cells engaged in these processes, from the co-isolation of actin with membrane preparations, and from rates of lateral diffusion obtained from photobleaching experiments (reviewed in references 24, 34, 53, 79, 80, and 82). Although transmembrane linkages between F-actin and cell surface receptors are generally presumed to play a role in endocytosis, chemotaxis, and adhesion, the molecular details of such linkages are unknown. The human erythrocyte membrane (reviewed in references 6, 28, and 30) and the intestinal microvillar membrane (19, 26, 32, 47) are the only systems in which the sequences of polypeptides linking F-actin to the cell surface are understood with any certainty. However, these model systems are not notable for their endocytic, chemotactic, or adhesive properties.

The cellular slime mold, *Dictyostelium discoideum*, on the other hand, is a well-known model system for the study of endocytosis, chemotaxis, and cell-cell adhesion (41). In adi-
tions, various techniques for isolating D. discoideum plasma membranes are available (12, 61; others reviewed in references 51 and 57) and many cytoplasmic cytoskeletal proteins have been identified, isolated, and characterized (reviewed in references 15, 66, 71). Since it is well-established that F-actin co-isolates with the D. discoideum plasma membrane (14, 18, 25, 35, 43, 58, 65), this system is ideal for the study of the cytoskeleton–membrane interactions involved in phagocytosis, chemotaxis, and cell-cell attachment. Integral membrane proteins have been implicated as agents directly responsible for binding F-actin to D. discoideum plasma membranes (43), but the polypeptides involved have not been identified.

Our general lack of biochemical information about F-actin–membrane interactions is traceable to the paucity of qualitative and quantitative assays for the study of these interactions. Until recently, the only biochemical method for studying F-actin–membrane binding was the co-sedimentation of purified F-actin with isolated membranes (9, 17, 35, 43, 54, 70). Although this technique can detect qualitative differences in F-actin binding capabilities, sensitivity and quantification are hindered by the paucity of qualitative and quantitative assays. In this and the accompanying paper (27), we introduce a new biochemical technique for monitoring F-actin–membrane interactions. This technique is sensitive, relatively quantitative, and readily adaptable to the study of interactions between F-actin and integral membrane proteins, such as intact membrane vesicles.

Using sedimentation binding and competition assays, we show that highly purified, sonicated D. discoideum plasma membrane fragments bind saturaingly, rapidly, and specifically to F-actin on beads and that this binding is mediated, at least in part, by integral membrane proteins. Initial fractionation of Triton-extracted membranes on F-actin affinity columns implicates twelve integral membrane proteins as agents involved in direct or indirect associations with F-actin. Of particular interest are four polypeptides (apparent molecular weights of 180,000, 130,000, 86,000, and 77,000) that are candidates for transmembrane, F-actin binding proteins. Thus, these polypeptides may belong to a rapidly expanding class of integral membrane proteins that are suspected of mediating interactions between cell surfaces and actin-containing cytoskeletal structures (8, 10, 19, 21, 36, 39, 50, 52, 60, 62, 64).

This work was presented in a preliminary form at the Annual Meeting of the American Society for Cell Biology in November, 1982 (44).

MATERIALS AND METHODS

Chemicals

ATP and phalloidin were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Tris, phenylmethylsulfonyl fluoride (PMSF), BSA, diisopropylfluorophosphate, dithiothreitol (DTT), Triton X-100, N-carboxybenzoxypenlyalanine, and 1,10-phenanthroline monohydrate were purchased from Sigma Chemical Co. (St. Louis, MO). Three times crystallized concanavalin A (Con A) was obtained from Miles-Yeda Ltd. (Israel). SDS and other reagents used for SDS PAGE were purchased from Bio-Rad Laboratories (Richmond, CA).

Plasma Membrane Fragments

Cell Culture: D. discoideum amebae (strain Ax3, a gift from Dr. Richard Kessin, Columbia University) were grown in HL-5 medium (16) and were harvested at a concentration of approximately 1 × 10^6 cells/ml. The cells were disrupted by centrifugation at 1,500 g for 2 min and were washed twice with 14.6 mM KH2PO4, 2.0 mM NaHPO4, pH 6.1, (Sorensen’s buffer) at 650 g for 2 min at 20-22°C.

Con A Stabilization: Con A-stabilized plasma membrane fragments containing associated actin and myosin were isolated using a modification of the procedures described by Parish and Müller (58) and Condeelis (18). Washed cells were resuspended to approximately 2 × 10^6 cells/ml and stirred for 8 min at 20-22°C with ~50 µg/ml Con A. At this point, the actomyosin-dependent patching and capping process is, at most, 25% complete (18). After centrifugation at 1,000 g for 2 min, the cells were washed once with Sorensen's buffer at 2°C and resuspended to a concentration of about 5 × 10^6 cells/ml with ice-cold Buffer 1 (40 mM sodium pyrophosphate, 0.4 mM DTT, 0.1 mg/ml PMSF, 2 mM EDTA, 1 mM EGTA, 5 mM 1,10-phenanthroline, 2 mM N-carboxybenzoxypenlyalanine, 0.02% sodium azide, 0.05% ethanol, 10 mM Tris-HCl, pH 7.6). The cell suspension was then made 0.2% in Triton X-100, swirled on ice for 1 min, and centrifuged at 4,000 g for 10 min at 2°C. After aspiration of the supernatant, the flocculent top layer of the pellet was collected, gently homogenized into fresh Buffer 1 with a few strokes of a Dounce homogenizer (Kontes Co., Vineland, NJ), and centrifuged at 14,500 g for 5 min at 2°C. Again, the supernatant and the tightly packed bottom layer of the pellet were discarded; the flocculent part of the pellet was washed once with 1 mM EGTA, 5 mM Tris-HCl, pH 7.6, at 14,500 g for 10 min at 2°C and resuspended into this buffer with gentle homogenization. Then, the membranes were layered onto 40-60% sucrose gradients (first sucrose gradients) containing 20 mM sodium phosphate, 0.02% sodium azide, pH 6.8 (Buffer 2), and centrifuged at 120,000 g for 1 h at 2-4°C. The dense membrane band at ~50% sucrose was collected, washed once with Buffer 2 at 27,000 g for 10 min at 2°C, and resuspended with ~1-2 vol of this buffer.

Con A Removal: The resuspended membranes were incubated at 0°C with an equal volume of 0.5 M α-methyl-mannoside, 0.02% sodium azide, 100 mM Tris-HCl, pH 8.5. After 12-24 h, the suspension was diluted with a few volumes of Buffer 2, and centrifuged at 39,000 g for 30 min at 2°C. The pellet was resuspended with 6-8 vol of 0.5 M α-methyl-mannoside, 0.02% sodium azide, 100 mM Tris-HCl, pH 8.5, 1 mM EGTA, and 1 mM MgATP. Clumps of the pelleted membranes were disrupted by gentle homogenization. After an additional 12-24 h on ice, the membranes were diluted with Buffer 2, centrifuged at 39,000 g for 30 min at 2°C, and washed once with this buffer.

Removal of Actin and Myosin: Actin and residual myosin were removed by low ionic strength extraction as described previously (43). The membranes were resuspended with gentle homogenization in a minimal volume of Buffer 2, and were layered onto 26-51% linear sucrose gradients (second sucrose gradients) containing 50 mM glycine, pH 8.5 at 22°C over a cushion of 64% sucrose in 50 mM glycine, pH 8.5 (61). After centrifugation at 120,000 g for 2 h at 2°C, most of the plasma membrane sheets were concentrated in a band at 36-38% sucrose; variable amounts of material were recovered from the bottom of the gradient. These purified plasma membranes—now depleted of bound Con A and essentially devoid of actin and myosin—were washed twice with Buffer 2 at 39,000 g for 45 min at 2°C, resuspended, and stored at 0°C in this buffer. All clumps of membranes were disrupted by gentle homogenization.

Membrane Extractions: Purified plasma membranes were extracted with 0.5 N NaOH, 1 mM DTT, pH 12.5, as previously described (43). During the 30-min incubation at 0°C, the extraction mixture was sonicated with two 5-s bursts (43). NaOH-extracted plasma membranes were collected by centrifugation in an HB-4 rotor (E. I. Du Pont de Nemours & Co., Wilmington, DE) at 27,900 g for 20 min at 4°C. The pellet was immediately washed with Buffer 2 and then resuspended and stored in this buffer. KCl, area-extracted membranes, proteolytically digested membranes, heat-denatured membranes, and D. discoideum vesicles were resuspended and used as controls for the purified plasma membranes by published procedures (43). Before use, all membranes and lipid vesicles were sonicated as described below.

Proteolytically digested membranes were adjudged free of residual, active protease if no proteolysis of the actin band on SDS polyacrylamide gels was observed after 0.8 mg/ml of actin and 0.0-0.14 mg/ml of membrane protein was incubated with 5 µl of residual, active protease. Residual protease was measured for pronase-digested membranes treated with both diisopropylfluorophosphate and PMSF (43). However, both pronase-digested membranes treated with...
PMSE only or papain-digested membranes treated with 7.4 mM iodoacetate acid cleaved actin in solution. In competition assays (see below), membranes with residual, active protease prevented the co-sedimentation of 125I-labeled membrane fragments with F-actin beads, no doubt owing to the proteolysis of bead-associated actin (not shown). 

**Sonication:** Membranes were sonicated with 15- to 30-s bursts at -1.3 A with a model S75 Branson sonifier (Branson Sonic Power Co., Danbury, CT) for a total of 6 min, or until the solution opalescence diminished dramatically. During sonication, the tube containing the membranes was immersed in an ice-water bath; 10- to 20-s pauses between bursts of sonication prevented significant heating.

**Radioiodination:** Sonicated plasma membrane fragments, sonicated NaOH-extracted membrane fragments, and sonicated D. discoideum lipid vesicles were radioiodinated with [125I]Bolton-Hunter reagent (reference 5; 2,000 Ci/mmol, Amersham Corp., Arlington Heights, IL) in 20 mM sodium phosphate, pH 7.8, for 16- to 20-h. Labeled membranes were separated from unreacted reagent by chromatography on a column of Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) equilibrated with 50 mM KCI, 1 mM MgCl2, 0.02% sodium azide, 20 mM Tris-acetate, pH 7.0, at 20°C. The specific activities of unextracted plasma membrane fragments were 69 ± 37 mCi/g membrane protein; the specific activities of NaOH-extracted membrane fragments and D. discoideum lipid vesicles were 125 ± 50 mCi/μg phospholipid phosphorus.

**Cell Surface Labeling**

Cell surfaces were labeled using a modification of the procedure of Hubbard and Coln (33). About 4 x 109 cells were washed and resuspended in 2 ml of cold Sorensen's buffer. To the cell suspensions were added, in order, 10 μl of 0.5 mM AT, 0.5 M glucose, 10 μl of 0.25 M NaPO4, pH 7.5. The iodination reaction was initiated by the addition of 2 μl of 20 U/ml glucose oxidase, 20 U/ml horseradish peroxidase (Sigma Chemical Co.) and 300 μl of carrier-free Na125I (Amersham Corp.). The reaction was quenched with the addition of 12 μl of 20 U/ml glucose oxidase (Sigma). After 15 min on ice with gentle agitation, the reaction was quenched with 20 μl of 0.1 M KI. Cells were pelleted and washed three times in cold Sorensen's buffer. They were resuspended to 2 x 109 cells/ml and plasma membrane fragments were isolated as described above up to the first sucrose gradient. For each labeling experiment, two control experiments were also performed. In one control experiment, the reaction mixture lacked lactoperoxidase; in the second, the mixture lacked glucose.

**F-Actin Affinity Beads**

**Anti-fluorescein IgG Antibody:** Anti-fluorescein IgG was prepared and isolated from latex immune rabbit antiserum as described previously (45, 78). The r-value of each antibody preparation, a measure of the molar ratio of tightly bound fluorescein to antibody, was determined as described by Kranz and Voss (37). Antibody preparations with r-values of at least 0.2 were required for the preparation of F-actin affinity beads.

**BEAD PREPARATION:** Sepharyl S-1000 beads (300-400 nm porosity, Pharmacia Inc.) were activated with cyanogen bromide (46) and conjugated to rabbit antifluorescein antibody (IgG) as described previously (45). F-actin labeled at Cys-374 with 5-iodoacetamidofluorescein (AF-actin) was prepared by the method of Wang and Taylor (77), as modified by Luna et al., (45). AF-actin was bound to antifluorescein IgG-Sepharyl S-1000 beads essentially as described previously (45). However, to increase the final concentration of bead-bound actin, we added a 60-μg aliquot of AF-actin per milliliter of bead solution in the first two binding cycles concomitant with overnight binding at 3°C. To stabilize the increased amounts of bead-bound actin, we increased the amount of phallolidin added at the end of each cycle to approximate the molarity of the actin added in that cycle. Also, ATP and DTT concentrations during each cycle were increased from 0.5 to 1.0 mM. Antifluorescein IgG-Sepharyl S-1000 beads containing bound AF-actin (F-actin beads) were stored on ice at 25% suspension in 0.5 mM ATP, 0.5 mM DTT, 50 mM KCl, 1 mM MgCl2, 20 μM phallolidin, 0.02% NaN3, 20 mM Tris-acetate, pH 7.0. The average amount of actin associated with F-actin beads was 2.7 mg/μl of packed beads; the concentration range was 1.58-5.09 mg of actin per milliliter of packed beads and was dependent on the amount of actin added during the binding cycles. The capacity of these F-actin beads was as high as that previously described for AF-actin on antifluorescein IgG-Sepharose 4B beads (i.e., the ratio of functional to total actin was at least 0.2 as assayed by the binding of 125I-heavy meromyosin; see reference 45). Similarly, the association between AF-actin and antifluorescein IgG-Sepharyl S-1000 beads was as stable as that described previously (45). The concentration of free actin in the bead supernatant averaged only 80 μg/ml, even after a few cycles of repeated resuspensions into buffer with 25% sucrose, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 1 mM MgCl2, 0.1 M sucrose, pH 7.0. After F-actin beads supernatants were centrifuged at 234,000 g for 2 h at 2°C, we concluded that the actin in the bead supernatants was primarily F-actin that had been released from the beads, perhaps by filament shortening during bead resuspension or by leaching of IgG from the bead matrix (20, 81). The site of F-actin release is probably not the antifluorescein IgG link with AF-actin since incubation of F-actin beads with 1% SDS at 20-25°C for several hours failed to release all bead-bound fluorescein.

**Sedimentation Binding Assays:** Low-speed sedimentation assays were performed as described (45) except that it was not necessary to cut disposable pipette tips in assays with Sephacryl S-1000 beads. Unless otherwise specified, our incubation buffer (total volume, 110 μl) consisted of 50 mM KCI, 1 mM MgCl2, 2.3 μM phallolidin, 45 mM ATP, 45 μM DTT, 0.45 mg/ml ovalbumin (Worthington Biochemical Corp., Freehold, NJ), 20 mM Tris-acetate, pH 7.0. Except where noted, assay components were added in rapid succession to tubes on ice and then incubated, with shaking, at 21-23°C for 2 h.

**Affinity Columns:** For each column, 400 μl of a 25% suspension of Sephacryl S-1000 beads was tested. Beads were separated from unreacted reagent by chromatography on a column of Sephadex G-25 (Sigma) as a standard. The site of F-actin release is probably not the antifluorescein IgG link with AF-actin since incubation of F-actin beads with 1% SDS at 20-25°C for several hours failed to release all bead-bound fluorescein.

**PAGE and Protein Blotting**

6-16% linear gradient SDS slab gels were prepared and run according to the method of Laemmli (38). Details were as previously described (43).

We prepared blots by transferring electrophoretically separated proteins to nitrocellulose paper (Schleicher and Schuell, Inc., Keene, NH) according to the method of Towbin et al., (72). Blots were soaked overnight at 4°C with 2% BSA (wt/vol) in PBS, pH 7.4. They were stained for 2.5 h at room temperature with -10 ml of 4% BSA (wt/vol), 150 mM NaCl, 0.025% Triton X-100, 50 mM Tris-HCl, pH 7.2, containing 200,000 cpm 125I-Con A per ml of staining solution (3-5 μg Con A). Controls for 125I-Con A binding to blots were performed in the presence of 24 mM α-methyl-D-mannoside, a sugar which specifically binds to Con A. The labeled blots were washed for 2 h with 4 changes of 150 mM NaCl, 0.02% Triton X-100, 50 mM Tris-HCl, pH 7.2, and for 1 h with 4 changes of 0.1% Triton X-100, 50 mM Tris-HCl, pH 7.2. Blots were air-dried on filter paper before a 1-2 d exposure of Kodak XAR-2 film.

**Assays**

Total membrane protein was determined in the presence of 1% SDS by the procedure of Lowry, et al., (42); BSA was used as a standard. Bead-bound protein was estimated as described (27).

Organic solvent-extractable phosphorus was measured as an estimate of phospholipid phosphorus. 50-μl aliquots of membranes were extracted with water-methanol-chloroform according to the procedure of Bhig and Dyer (4). The chloroform layer from the final centrifugation was assayed for total phosphorus by the method of Ames (1) with dipalmitoyl phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL) as a standard.

Viscosity of actin--membrane mixtures was measured using a low-shear, falling ball viscometer (29) as described previously (22, 43).
Electron Microscopy

Samples were processed for transmission electron microscopy by a modification of the method of McDonald (48). Samples were fixed in 1% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA), 2% tannic acid (Mallincrodt Inc., St. Louis, MO), 50 mM sodium cacodylate (Ted Pella Co., Tustin, CA), pH 7.0, for 30 min at room temperature (2). After three rinses in 0.1 M sodium cacodylate, pH 7.0, and a 30-min wash in buffered 0.8% K4Fe(CN)6 (Fisher Scientific Co., Springfield, NJ) at 0°C, samples were post-fixed in 0.5% OsO4 (PolySciences Inc., Warrington, PA), 0.8% K4Fe(CN)6, 50 mM sodium cacodylate, pH 7.0, for 30 min at 0°C. After a buffer rinse and two or three rinses with distilled water, samples were stained for 1-3 h in 2% aqueous uranyl acetate, dehydrated in acetone, and embedded in Spurr’s standard resin (67). Thin sections (60-70 nm) were cut on an LKB Ultratome III (LKB Instruments, Inc., Gaithersburg, MD) with a diamond knife. Grids were stained briefly with 4% uranyl acetate in 40% ethanol followed by Reynold’s lead citrate (63) and were viewed in a JEOl 100C transmission electron microscope.

To minimize error, we measured the sizes of sonicated membrane fragments by two methods. First, diameters of vesicles or lengths of fragments with clear membrane profiles were measured from electron micrographs of thin sections. Measurements in millimeters were rounded to the next highest whole millimeter. Vesicles sectioned obliquely were not measured. Second diameters were measured from micrographs of whole-mount samples negatively stained with 2% aqueous uranyl acetate.

RESULTS

Plasma Membrane Preparation

Previous work has demonstrated that preparations enriched in D. discoideum plasma membranes induce large increases in viscosity when incubated with rabbit skeletal muscle F-actin (43). To verify that this activity is indeed associated with plasma membranes, and not with one or more contaminants, we modified existing protocols (18, 58, 61) to obtain a plasma membrane preparation that contains no contaminants detectable by electron microscopy. Although essentially all membrane fragments in this preparation originate from the plasma membrane, all domains of the intact plasma membrane are not necessarily present.

Cells incubated with Con A for a few minutes acquire a thick coat of Con A in patches covering at least half of their extracellular surfaces (Fig. 1, a and b). These cells also have initiated capping of the patched Con A receptors (18). Con A-patched regions of the plasma membrane are associated with actin and myosin, are resistant to solubilization by low concentrations of Triton X-100, and, because of their high protein to lipid ratios, have an apparent density of about 1.22 on sucrose gradients (18, 58). After lysis in the presence of a cocktail of protease inhibitors known to minimize proteinolysis in this system (43), preparations of Con A-stabilized membranes contain long sheets of plasma membrane with Con A still visibly bound to one surface, small membrane vesicles that sometimes appear to have Con A bound to their inner surfaces, and filamentous meshworks that are apparently associated with both the membrane sheets and the vesicles (Fig. 1 c and also Fig. 8 of reference 27). After successive removal of Con A, actin, and myosin, the plasma membrane fragments have smooth surfaces (Fig. 1 d). However, small amounts of a contaminant that appears to be the detergent-insoluble remnant of either rough endoplasmic reticulum or mitochondrial inner membranes remain in the preparation (Fig. 1 d). Centrifugation on a second sucrose gradient separates the plasma membrane fragments (Fig. 1 e) from dense contaminants and from variable amounts of membrane with tenuously bound actin and myosin. After the contaminants have been removed, the purified plasma membranes have a much lower protein to lipid ratio and an apparent density of about 1.16-1.17.

The SDS polyacrylamide gel patterns of the plasma membrane fragments (Fig. 2, left) are similar to the gel patterns reported previously for Con A-Triton plasma membranes (18, 43, 59). The major exceptions are prominent bands at 30,000, 32,000, 52,000, 55,000 and 58,000 daltons that co-isolate with plasma membranes through the first gradient (Fig. 2 left, lane J), but separate from the bulk of the membranes on the second gradient (Fig. 2 left, lanes 4 and 5). Although these polypeptides may be associated with plasma membrane domains with extremely high affinities for actin or myosin, the small and variable amount of material as well as the large proportion of contaminants in the fraction containing these polypeptides has discouraged further investigation.

Since large plasma membrane sheets pellet significantly under the centrifugal forces used in our sedimentation binding assays, the sheets are vesiculated by sonication before use. In thin sections, sonicated membrane vesicles have diameters that average 110 nm with a range of 25-500 nm (Fig. 1 f). We also observe small, apparently discoid, membrane segments that average 96 nm in cross section with a range of 25-225 nm (Fig. 1 f). In negatively stained images of sonicated membranes, vesicles and discoid membrane segments are apparently indistinguishable; diameters of membrane structures average 83 nm and vary from 40 to 200 nm (G. Q. Daley and E. J. Luna, unpublished observations).

Sonication does not appear to adversely affect the polypeptides responsible for the interactions between F-actin and plasma membranes. SDS polyacrylamide gel patterns of sonicated and unsonicated membranes are essentially identical (not shown) and sonicated membranes retain the ability to increase the viscosity of solutions containing actin (Fig. 2, right). The 5- to 10-fold lower concentration of sonicated, vs. unsonicated, membranes required for a given viscosity increase may be due to the increased number of vesicles (or "crosslinkers") per milligram of membrane protein that are generated during sonication. In addition, sonication may increase the proportion of membrane vesicles having an orientation conducive to interactions with actin filaments in vitro. As is observed with unsonicated membranes (43), heat denaturation abolishes the ability of sonicated membranes to induce viscosity increases in actin-containing solutions (not shown).

Membrane Binding to F-Actin Affinity Beads

In agreement with hypotheses suggested by low-shear viscosity experiments (Fig. 2 right and reference 43), [125I]Bolton-Hunter-labeled, sonicated D. discoideum plasma membranes bind to F-actin beads (Fig. 3). Specific membrane binding is maximized and variability minimized by using Sephacryl S-1000 as the bead matrix. This observation is probably attributable to the large exclusion limit of Sephacryl S-1000 (300-400 nm), an exclusion limit that is greater than the diameters of 97% of the sonicated membrane vesicles and greater than the largest measured discoid membrane segments. Specific binding of membranes to F-actin on less porous Sepharose 2B beads (Fig. 3 A) or Sepharose 4B beads (not shown) is low and varies greatly from one membrane preparation to the next. Unsonicated plasma membranes, although active in low-shear viscosity assays (see Fig. 2 right and reference 43), neither bind to F-actin-Sephacryl S-1000 beads in sediment-
FIGURE 1  Electron micrographs of amebae before (a) or after (b) incubation with Con A. Plasma membrane fragments after cell lysis (c), after removal of Con A, actin, and myosin (d), after purification on a second sucrose gradient (e), and after sonication (f). Bar, 0.4 μm. × 40,000.
Radiolabeled, sonicated plasma membrane fragments bind with saturation kinetics to F-actin on antifluorescein IgG-Sepharl S-1000 beads (F-actin beads). In the presence of excess F-actin beads, a maximum of 35-45% of the labeled membranes sediment with the beads (Fig. 3A). In the presence of excess labeled membranes, F-actin beads bind, on average, 0.6 µg of membrane protein per microgram of bead-bound actin (Fig. 3B). Half-maximal binding to ~10 µg/ml bead-associated F-actin (~0.2 µM actin monomers) is observed in the presence of only 8 µg/ml of labeled, sonicated membranes (Fig. 3B). These values reflect both the steric constraints on the number of membrane fragments that can fit onto an actin filament and the amount of bead-associated actin that is accessible to membranes. Only 5-10% as much membrane protein sediments with control beads as with the F-actin beads. Much of this background "binding" is observed in the absence of beads (Fig. 3B) and can be reduced by precentrifugation of the membranes immediately before assaying them. Analysis of the binding data in Fig. 3B by the method of Hill (31) shows that the association between sonicated membranes and F-actin beads demonstrates a weak, positive cooperativity with an apparent $n_H$ of 1.1-1.2 (Fig. 3C). A simple explanation for the positive cooperativity is a small amount of membrane-membrane interaction in addition to the predominant binding between sonicated membranes and F-actin.

Assay Conditions

Membrane binding to F-actin beads is essentially complete after 1 h and stable for at least 4 h (Fig. 4). Over the concentration ranges of labeled membranes and bead-bound F-actin used in binding assays, $t_0$ varies between 4 and 20 min. Therefore, all the points on binding curves represent equilibrium values at the end of a 2-h incubation.
Changes in the ionic conditions of the binding assay have had, to date, no dramatic effects on the binding of membranes to F-actin beads. Increasing the free calcium ion concentration in assay buffer from \(-1 \times 10^{-8} \text{ M}\) to \(-3 \times 10^{-6} \text{ M}\) has no effect on binding. Varying the concentration of magnesium ions between 0.1 and 5 mM also has no effect on binding, except that at least 0.1 mM MgCl\(_2\) is required to stabilize the F-actin. Increasing the KCl concentration in assay buffer from 50 mM to 0.5 M diminishes the specific binding of membranes to F-actin beads. However, increasing KCl concentrations also increase background binding to control beads to such an extent that total binding actually increases (data not shown).

**Competition Assays**

The binding of \([^{125}\text{I}]\)Bolton-Hunter-labeled, sonicated membranes to F-actin beads is competed to background levels by unlabeled, sonicated membranes added to the assay mixture either 1 h before or at the same time as the labeled, sonicated membranes (Fig. 5). However, when labeled, sonicated membranes are prebound to F-actin beads for 1 h, even a large excess of unlabeled, sonicated membranes is incapable of displacing the labeled membranes from the beads (Fig. 5). This result suggests that the rate of membrane disassociation from F-actin beads is much slower than the rate of association and, therefore, implies that the association constant of this interaction is significant.

In these competition assays, the abilities of pretreated,
sonicated membranes to compete (Fig. 6) parallel the abilities of similarly pretreated, unsonicated membranes to co-sediment with F-actin and to induce viscosity increases in solutions containing F-actin (43). Sonicated, unlabelled membranes pre-extracted with chaotropes efficiently inhibit the binding of 125I-labeled membranes to F-actin beads (Fig. 6a).

Sonicated, heat-denatured membranes (Fig. 6a) or sonicated, proteolyzed membranes (Fig. 6b) do not compete with the labeled membranes. Thus, integral membrane proteins apparently mediate both the binding of plasma membrane fragments to F-actin on Sephacryl S-1000 beads and the interaction between plasma membranes and F-actin in solution.

While competition assays are a quick and useful first step in the determination of whether or not a membrane fraction interacts with F-actin, any apparent activity in a competition assay should always be verified by a direct binding assay. As expected, 125I-labeled, sonicated, NaOH-extracted membranes do bind directly to F-actin beads (Fig. 7). 125I-labeled, sonicated lipid vesicles do not bind to F-actin beads (Fig. 7), a result that is consistent with the inability of lipid vesicles to increase the viscosity of actin in low-shear viscometric analyses (43).

Affinity Columns

Since sonicated plasma membranes specifically bind to F-actin beads as evinced by low-speed sedimentation binding and competition assays, F-actin affinity columns will be useful for the identification and isolation of membrane-associated F-actin binding proteins. In our F-actin affinity chromatographic procedure, (a) sonicated plasma membranes are bound to the F-actin affinity matrix, (b) unbound membranes are washed away, (c) bead-bound membranes are solubilized with detergent, (d) unbound membrane proteins are removed, and (e) the F-actin-associated polypeptides are eluted. 125I-labeled, sonicated, plasma membrane fragments before (Fig. 8a) or after (not shown) extraction with 0.1 N NaOH specifically bind to columns containing F-actin on beads. Although much of the bound radioactivity is removed when the column is eluted with 2% Triton X-100 in buffer, the rest is eluted only when bound F-actin is removed with 1% SDS. In contrast, 125I-labeled, sonicated lipid vesicles bind only nonspecifically to F-actin affinity columns since they readily bind to plastic columns and tubes (not shown) as well as to antifluorescein IgG-Sephacryl S-1000 columns with and without bound F-actin (Fig. 8b).

Membrane-associated, F-Actin-binding Polypeptides

 Autoradiographs of the SDS polyacrylamide gel profiles of detergent solubilized, 125I-labeled plasma membrane proteins eluted with 1% SDS show that certain membrane-associated proteins are preferentially retained by the F-actin affinity column (Fig. 9). Although not all plasma membrane polypep-
tides bind F-actin, at least 17, including residual Con A, bind and are eluted by SDS (Figure 9a, lane 4). Because 125I-labeled Con A does not bind specifically to F-actin beads (not shown), it is clear that at least this polypeptide is bound indirectly to the F-actin column through associations with other proteins. Since many of the plasma membrane polypeptides bind Con A (see below), one or more membrane proteins that bind both Con A and F-actin could cause the binding of other polypeptides to the F-actin column. However, membranes from which Con A has been completely extracted also contain a number of polypeptides that specifically bind to an F-actin column (Fig. 9b). Thus, protein-protein associations mediated by Con A are not solely responsible for the multiplicity of membrane polypeptides bound by the F-actin affinity column. While the molecular basis for this binding is still an open question, it is interesting to note that none of the proteolipids that coextract and reconstitute with lipids bind to F-actin affinity columns (Fig. 9c).

A comparison of the SDS polyacrylamide gel profiles of plasma membrane fragments, proteolyzed plasma membrane fragments, and plasma membrane fragments sonicated during NaOH extraction (Fig. 10) suggests several candidate polypeptides that may directly bind F-actin. Coomassie Blue-stained SDS polyacrylamide gels show that a number of membrane polypeptides are resistant to extraction with 0.1 N NaOH but are cleaved during proteolysis (Fig. 10a). Any or all of these polypeptides may be F-actin binding proteins since F-actin binding activity is observed for NaOH-extracted membranes but not for proteolyzed membranes (Fig. 6). Six polypeptides that are present in NaOH-extracted membranes, but depleted in proteolyzed membranes, bind 125I-Con A (Fig. 10b).

At least four of these six Con A-binding polypeptides—those with apparent molecular weights of 180,000, 130,000, 86,000, and 77,000—probably span the membrane (7). First, we note that much of the residual Con A associated with plasma membrane fragments (Fig. 10a, lane 1) remains associated with these fragments after pronase digestion and subsequent washing (Fig. 10a, lane 2). Con A in solution or bound to leaky erythrocyte ghosts is completely digested under similar conditions (not shown). Because Con A remains bound to proteolyzed D. discoideum membranes (Fig. 10a, lane 2), the Con A must be sheltered within inside-out vesicles that are sealed to protease. Therefore, formerly extracellular Con A-binding portions of membrane proteins, now inside the inside-out vesicles, will also be sheltered from proteolytic digestion. If essentially all of a given Con A-binding polypeptide in these membranes is cleaved (Fig. 10b), either the polypeptide is a transmembrane protein that has been cleaved at its cytoplasmic face or it is selectively excluded from the sealed inside-out vesicles. Selective exclusion from inside-out vesicles is unlikely since preliminary fractionation of membrane fragments by Con A-affinity chromatography (40, 74, 76, 83) and by equilibrium density centrifugation (69) have thus far resulted in fractions with essentially identical SDS polyacrylamide gel profiles (G. Q. Daley, C. M. Goodloe-Holland, and E. J. Luna, unpublished observations).

Assuming that each band in SDS polyacrylamide gels represents a single polypeptide, we note that polypeptides with apparent molecular weights of 180,000, 130,000, 86,000 and 77,000 are eluted by SDS from F-actin affinity columns (Fig. 7).
9, a and b), are present in NaOH-extracted membranes but undetectable in proteolyzed membranes (Fig. 10, a and b), bind ^125^I-Con A (Fig. 10 b), and are apparently labeled at the cell surface by lactoperoxidase-catalyzed iodination of intact cells (Fig. 10 c). Therefore, we suggest that these bands represent excellent candidate polypeptides for transmembrane, F-actin-binding proteins. Obviously, however, additional experimentation is required to confirm such a function for one or more of these polypeptides.

**DISCUSSION**

Our results show that highly purified, sonicated plasma membrane fragments, isolated from Con A-stabilized *D. discoideum* amebae by a new modification of published procedures (18, 58), bind to F-actin on Sephacryl S-1000 beads. This binding is probably physiologically important since it is specific (Fig. 3), saturable (Fig. 3), and rapid (Fig. 4). In addition, it is apparently of high affinity. A significant association constant for the membrane-actin interactions is inferred from the low membrane concentrations and low actin concentrations that support binding (Figs. 3 and 4) and from the observation that the on rate is much faster than the off rate (Figs. 4 and 5). However, the measurement of a meaningful association constant in this system is confounded by the non-uniform distribution of F-actin owing to its localization in the bead pores (27), the presumed heterogeneity of the membrane-associated F-actin binding sites, and the likelihood that a given membrane fragment is bound to a given actin filament at more than one site. Apparent binding constants of membranes for F-actin are expected to be complicated functions of the intrinsic binding constants of individual membrane components and the number of sites on a membrane that can bind simultaneously to a given actin filament (49, 68). The apparently tight binding of membranes to F-actin that we observe may be due to a few, high-affinity interactions or to multiple, low-affinity interactions. Either case would be con-
adhesion system known as contact sites B (3, 11). Since any receptors for folic acid and pterin (56, 73), and the cell-cell independent phagocytic receptors (75), separate chemotactic reports that log phase D. discoideum cells contain at least two plasma membrane polypeptides especially in light of the surface. Twelve is a reasonable number of actin-binding transmembrane links between the cytoskeleton and the cell appear to span the membrane and are thus possible direct that at least 12 integral membranepolypeptides bind, directly and F-actin insolution, as monitoredby low-shear viscometry and F-actin beads. This result agrees with the previous observation that integral membrane proteins are responsible for interactions between D. discoideum plasma membranes and F-actin in solution, as monitored by low-shear viscometry (43). Agreement between results from such different tech-
iques suggests that the two approaches monitor the same phenomenon.

Using the F-actin beads as an affinity column, we show that integral membrane proteins mediate an association between highly purified plasma membrane domains and F-actin beads. This result agrees with the previous observation that integral membrane proteins are responsible for interactions between D. discoideum plasma membranes and F-actin in solution, as monitored by low-shear viscometry (43). Agreement between results from such different techniques suggests that the two approaches monitor the same phenomenon.

Using the F-actin beads as an affinity column, we show that at least 12 integral membrane polypeptides bind, directly or indirectly, to F-actin. At least four of these polypeptides appear to span the membrane and are thus possible direct transmembrane links between the cytoskeleton and the cell surface. Twelve is a reasonable number of actin-binding plasma membrane polypeptides especially in light of the reports that log phase D. discoideum cells contain at least two independent phagocytic receptors (75), separate chemotactic receptors for folic acid and pterin (56, 73), and the cell-cell adhesion system known as contact sites B (3, 11). Since any or all of these systems may be comprised of multisubunit proteins and may be associated with cytoplasmic actin, 12 appears to be a reasonable number for, and possibly an underestimate of, the integral, F-actin binding proteins in our membranes.

**Comparison of Low-speed Sedimentation Analysis and Low-shear Viscometry**

Low-speed sedimentation analysis using F-actin beads has three major advantages over low-shear viscometry as a biochemical technique for studying F-actin–membrane interactions. First, low-speed sedimentation assays generate data in a form that is relatively amenable to quantitative analysis. Second, binding assays can be readily scaled up and analyzed with F-actin affinity chromatography, a biochemical technique with both analytic and preparative potential. Third, binding and competition assays can be performed with very small amounts of membranes and beads. A typical saturation binding curve requires only ~10 μg of F-actin on beads and no more than 10–15 μg of 125I-labeled D. discoideum membrane protein. A single binding determination can be made with <1 μg each of membranes and bead-bound F-actin. The ultimate sensitivity of these binding assays is limited only by the extent to which membranes can be radiolabeled while retaining actin-binding activity. The precision of binding assays is limited by the accuracy with which beads, membranes, and buffers can be measured. For instance, each of our data points represents the binding of membranes to about 700 beads, a number small enough to be subject to significant measurement errors. Increasing the number of beads per data point increases precision at the expense of sensitivity since more membranes are then required to generate complete saturation binding curves.

The limitations of low-speed sedimentation assays are two-fold. First, the diameters of the membrane vesicles must be smaller than the exclusion limit of the bead matrix. Therefore, membranes from most cells must be sonicated before analysis and the effects of this sonication on F-actin–membrane interactions must be monitored by independent procedures such as low-shear viscometry. Second, the preparation of F-actin beads is time consuming. However, when F-actin beads are on hand, low-speed sedimentation binding assays are at least as fast as low-shear viscometric experiments. Both low-speed sedimentation and low-shear viscosity assays are more sensitive, more reproducible, and more interpretable than are binding assays based on the co-sedimentation of unsonicated membranes and F-actin (9, 17, 35, 43, 70). Low-speed sedimentation assays, coupled with F-actin affinity chromatography, should be valuable for the identification of membrane polypeptides directly responsible for F-actin binding to plasma membranes. Further work will focus on the correlation between these polypeptides and membrane structures responsible for endocytosis, chemotaxis, and cell-cell and cell–substrate adhesion.

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