

A Membrane Cytoskeleton from *Dictyostelium discoideum*.

II. Integral Proteins Mediate the Binding of Plasma Membranes to F-Actin Affinity Beads

ELIZABETH J. LUNA, CATHERINE M. GOODLOE-HOLLAND, and
HILARY M. INGALLS

Department of Biology, Princeton University, Princeton, New Jersey 08544

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 S-1000 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 $\sim 0.6 \mu\text{g}$ of membrane protein per microgram of bead-bound F-actin, (c) rapid with a $t_{1/2}$ 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, proteolyzed 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 pre-

sumed 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 endocytotic, 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 addi-

tion, 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 inherently poor and background binding tends to be high. Low-shear viscometry, recently introduced as a technique for monitoring F-actin interactions with membranes (22, 23, 43), is more sensitive and semiquantitative but is only useful for measuring interactions between F-actin and bi- or multivalent F-actin-binding moieties, such as intact membrane vesicles.

In this and the accompanying paper (27), we introduce sedimentation analysis using F-actin affinity beads (45) as 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 univalent F-actin-binding moieties, such as detergent-solubilized integral membrane proteins.

Using sedimentation binding and competition assays, we show that highly purified, sonicated *D. discoideum* plasma membrane fragments bind saturably, 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 indirect or direct 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),¹ BSA, diisopropylfluorophosphate, dithiothreitol (DTT), Triton X-100, *N*-carboxyphenylalanine, and 1,10-phenanthroline monohydrate were supplied

by 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 1×10^7 cells/ml. The cells were collected by centrifugation at 1,500 g for 2 min and were washed twice with 14.6 mM KH_2PO_4 , 2.0 mM Na_2HPO_4 , 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 2×10^7 cells/ml and swirled 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 5×10^7 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*-carboxyphenylalanine, 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-D-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-D-mannoside, 0.02% sodium azide, 100 mM Tris-HCl, pH 8.5, 1 mM EDTA, 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). These 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 4°C, resuspended, and stored at 0°C in this buffer. Any clumps of membranes were disrupted by gentle homogenization.

MEMBRANE EXTRACTIONS: Purified plasma membranes were extracted with 0.1 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. DuPont 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, urea-extracted membranes, proteolytically digested membranes, heat-denatured membranes, and *D. discoideum* lipid vesicles were also prepared from 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 0–0.14 mg/ml of membrane protein were incubated at 28°C for 2 h (43). Residual, active protease was never observed for pronase-digested membranes treated with both diisopropylfluorophosphate and PMSF (43). However, both pronase-digested membranes treated with

¹ Abbreviations used in this paper: AF-actin, F-labeled at Cys-374 with 5-iodoacetamidofluorescein; Con A, concanavalin A; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.

PMSF only or papain-digested membranes treated with 7.4 mM iodoacetic acid cleaved actin in solution. In competition assays (see below), membranes with residual, active protease prevented the co-sedimentation of ^{125}I -labeled membranes 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 [^{125}I]Bolton-Hunter reagent (reference 5; $\sim 2,000$ Ci/mmol, Amersham Corp., Arlington Heights, IL) in 20 mM sodium phosphate, pH 7.8, for 16–20 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 KCl, 1 mM MgCl_2 , 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 nCi/ μg membrane protein; the specific activities of NaOH-extracted membrane fragments and *D. discoideum* lipid vesicles were ~ 125 $\mu\text{Ci}/\mu\text{mol}$ phospholipid phosphorus.

Cell Surface Labeling

Cell surfaces were labeled using a modification of the procedure of Hubbard and Cohn (33). About 4×10^8 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 KI, 20 μl of 0.5 M glucose, 10 μl of 2 mg/ml lactoperoxidase (Sigma Chemical Co.) and 300 μCi of carrier-free Na^{125}I (Amersham Corp.). The reaction was initiated by 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 2×10^7 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-FLUORESCENIN ANTIBODY: Anti-fluorescein IgG was prepared and isolated from late hyperimmune rabbit antisera as described previously (45, 78). The r -value of each antibody preparation, a measure of the mole 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: Sephacryl S-1000 beads (300–400 nm porosity, Pharmacia Inc.) were activated with cyanogen bromide (46) and conjugated to rabbit anti-fluorescein 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 anti-fluorescein IgG-Sephacryl S-1000 beads essentially as described previously (45). However, to increase the final concentration of bead-bound actin, we added a third 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 phalloidin 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. Anti-fluorescein IgG-Sephacryl S-1000 beads containing bound AF-actin (F-actin beads) were stored on ice as a 25% suspension in 0.5 mM ATP, 0.5 mM DTT, 50 mM KCl, 1 mM MgCl_2 , 20 μM phalloidin, 0.02% NaN_3 , 20 mM Tris-acetate, pH 7.0.

The average amount of actin associated with F-actin beads was 2.7 mg/ml 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 anti-fluorescein IgG-Sepharose 4B beads (i.e., the ratio of functional to total actin was at least 0.2 as assayed by the binding of ^{125}I -heavy meromyosin; see reference 45). Similarly, the association between AF-actin and anti-fluorescein IgG-Sephacryl S-1000 beads was as stable as that described previously (45). The concentration of free actin in the bead supernatant averaged only 80 $\mu\text{g}/\text{ml}$, even after 4 wk of repeated resuspensions interspersed with storage at 0°C . Since little or no actin remained in solution after F-actin bead 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 shearing during bead resuspension or by leaching of IgG from the bead matrix (20, 81). The site of F-actin release is probably not the anti-fluorescein IgG link with AF-actin since incubation of F-actin beads with 1% SDS at 20 – 22°C for several hours failed to release all bead-bound fluorescence.

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 KCl, 1 mM MgCl_2 , 2.3 μM phalloidin, 45 μM 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 IgG-Sephacryl S-1000 beads, with or without bound actin, were pipetted into a 1-ml plastic syringe fitted with a plug of porous polyethylene (35- μm porosity, 1.6-mm thick, BoLab Inc., Lake Havasu, AZ). The resulting 100- μl columns were washed with 1 ml of 1.0 mg/ml ovalbumin in column buffer (50 mM KCl, 1 mM MgCl_2 , 0.02% NaN_3 , 0.5 μM phalloidin, 20 mM Tris-acetate, pH 7.0 at 20°C) and then with 0.4 ml of column buffer. About 1 μCi of [^{125}I]Bolton-Hunter-labeled membranes in 10–40 μl of column buffer was slowly loaded onto the column and equilibrated for 20 min. After unbound membranes were eluted with 1.0 ml of column buffer, nonspecifically bound material and solubilized membrane components were eluted with 1.0 ml of 2% Triton X-100 followed by 1.0 ml of 0.1% Triton X-100, both in column buffer. Finally, most of the actin and specifically bound membrane components were eluted with 1.0 ml of 1% SDS. All the affinity columns were run at room temperature (20 – 22°C); two-drop fractions (~ 100 μl) were collected by hand. Fractions were counted in a Beckman Gamma 4000 Counting System (Beckman Instruments, Inc., Irvine, CA). Peak fractions were pooled and solubilized in SDS electrophoresis sample buffer and analyzed on SDS slab gels (see below).

Radioiodination of Con A

Con A was radiolabeled according to the procedure of Chang and Cuatrecasas (13) with the following modifications. 20 μl of 20 mg/ml Con A in 0.1 M NaPO_4 , pH 7.5, and 0.5–1.0 mCi of carrier free Na^{125}I were added to 100 μl of 0.25 M NaPO_4 , pH 7.5. The iodination reaction was initiated by the addition of chloramine T and was stopped after 50 s by the addition of sodium metabisulfite. After an additional 10 s, 0.3 ml of 0.1 M NaPO_4 , pH 7.5, was added to the reaction mixture. The iodinated Con A was purified by affinity chromatography as described (13) except that a 1-ml Sephadex G-150 (Sigma) column was used and the Con A was selectively eluted with 1 M glucose, 1 M NaCl, 0.3% (wt/vol) BSA, 0.1 M Tris-HCl, pH 8.5.

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, 0.02% Triton X-100, 50 mM Tris-HCl, pH 7.2, containing 200,000 cpm ^{125}I -Con A per ml of staining solution (3–5 μg Con A). Controls for ^{125}I -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 four changes of 150 mM NaCl, 0.02% Triton X-100, 50 mM Tris-HCl, pH 7.2, and for 1 h with four 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 Bligh and Dyer (4). The chloroform layer from the final centrifugation was assayed for total phosphorus by the method of Ames (1) with dipalmitoyl phosphatidylcholine (Sigma) 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 (Mallinckrodt, 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% $\text{K}_4\text{Fe}(\text{CN})_6$ (Fisher Scientific Co., Springfield, NJ) at 0°C, samples were post-fixed in 0.5% OsO_4 (Polysciences Inc., Warrington, PA), 0.8% $\text{K}_4\text{Fe}(\text{CN})_6$, 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 proteolysis 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 tenaciously 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 1), 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 have 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), [^{125}I]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 sedimen-

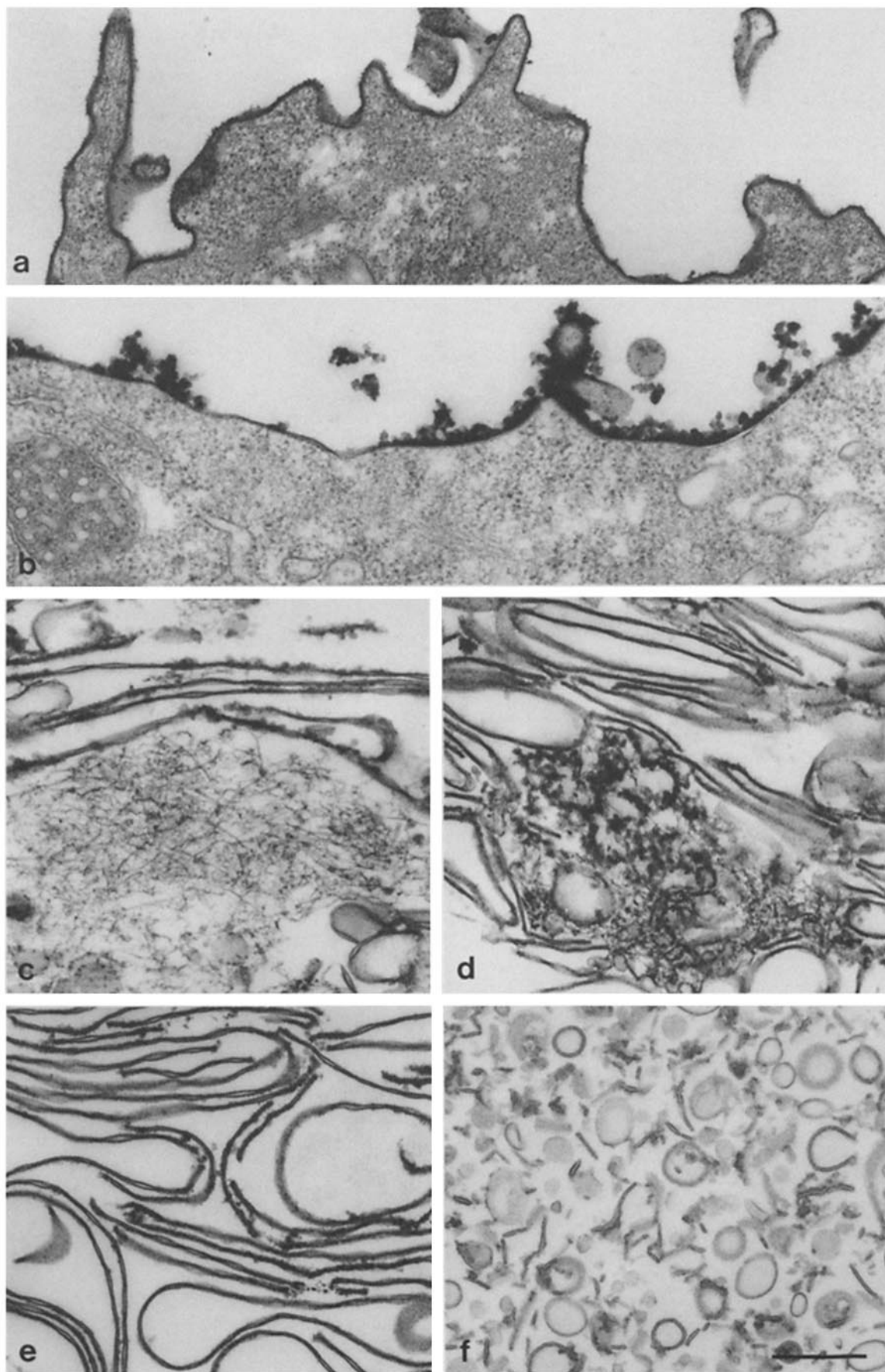


FIGURE 1 Electron micrographs of amebae before (a) or after (b) incubation with Con A. Plasma membrane fragments after cell lysis (c), after removal of Con A, actin, and myosin (d), after purification on a second sucrose gradient (e), and after sonication (f). Bar, 0.4 μ m. $\times \sim 40,000$.

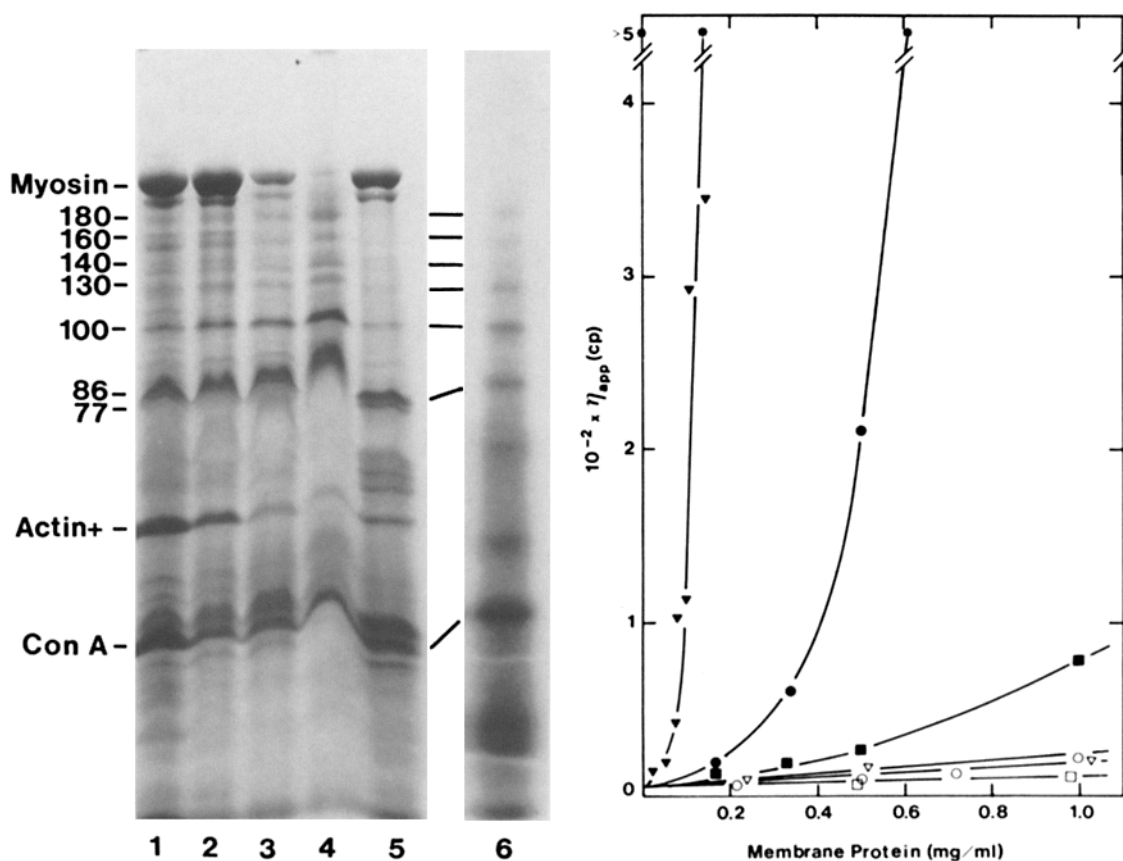


FIGURE 2 (left) Coomassie Blue-stained SDS polyacrylamide gels (SDS PAGE) of plasma membrane fragments after cell lysis (lane 1), removal of Con A (lane 2), subsequent removal of actin and myosin (lane 3), and purification on a second sucrose gradient (lane 4); SDS PAGE of dense membrane band from second sucrose gradient that contains contaminants and residual actin and myosin (lane 5). 60 μ g of protein was loaded onto each of lanes 1–5. (lane 6) Autoradiograph after a 2-d exposure of sonicated plasma membrane fragments labeled after isolation with [125 I]Bolton-Hunter reagent. About 100,000 cpm were loaded onto this lane. Numbers to the left of the figure designate the apparent molecular weights ($\times 10^{-3}$) of the membrane polypeptides. Since peptide maps of the 43,000-dalton polypeptide in lane 4 are distinct from peptide maps of *D. discoideum* actin, polypeptides with apparent molecular weights of 43,000 are designated “actin +”. (right) Apparent viscosity (centipoise) vs. concentration of membrane protein (mg/ml) for membrane preparations mixed with 0.8 mg/ml G-actin and incubated at 28°C for 60 min under actin-polymerizing conditions. Plasma membrane fragments after cell lysis and centrifugation on the first sucrose gradient (∇), removal of Con A (\circ), subsequent removal of actin and myosin (\blacksquare), purification on a second sucrose gradient (\bullet), and sonication (\blacktriangledown). Apparent viscosity vs. concentration curve for the dense membrane band from the second sucrose gradient (\square).

tation assays nor compete with 125 I-labeled, sonicated membranes in competition assays (see below). Since the diameters of unsonicated membrane vesicles average 646 nm, about twice the exclusion limit of Sephacryl S-1000 beads, we suggest that essentially all binding between membranes and F-actin occurs within the pores of the bead matrix (see also reference 27).

Radiolabeled, sonicated plasma membrane fragments bind with saturation kinetics to F-actin on anti fluorescein IgG-Sephacryl 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 μ g of membrane protein per microgram of bead-bound actin (Fig. 3B). Half-maximal binding to ~ 10 μ g/ml bead-associated F-actin (~ 0.2 μ M actin monomers) is observed in the presence of only 8 μ g/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, $t_{1/2}$ varies between 4 and 20 min. Therefore, all the points on binding curves represent equilibrium values at the end of a 2-h incubation.

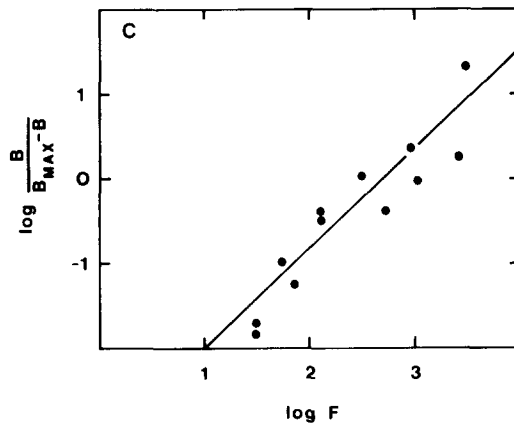
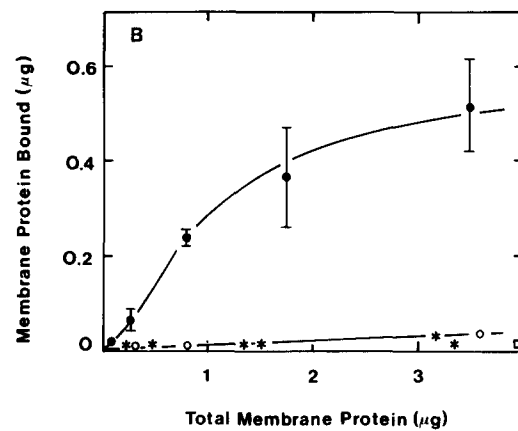
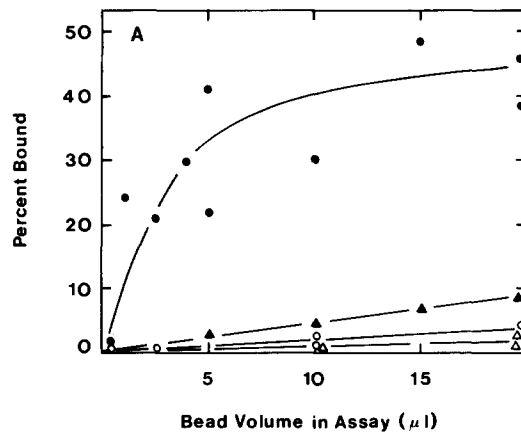


FIGURE 3. Binding curves of ^{125}I -labeled, sonicated plasma membranes to AF-actin on anti fluorescein beads. (A) 0.8- μg membranes incubated with increased amounts of anti fluorescein beads with (●, ▲) or without (○, Δ) 0.6 μg AF-actin/ μl beads. Anti fluorescein on Sephacryl S-1000 beads (●, ○). Anti fluorescein on Sepharose 2B beads (▲, Δ). (B) Increased amounts of membrane protein incubated with 0.5 μl of Sephacryl S-1000 beads (packed volume) with 0.8 μg of bound AF-actin (●), with 0.1 μg of bound fluorescein-conjugated ovalbumin (asterisk), or with no bound fluorescent protein (○). Background observed in a sample lacking beads (□). Points shown are the averages from duplicate assays. (C) Individual data points from the binding curve (panel B) plotted according to the Hill equation (31). B , membrane

protein bound to beads (ng); B_{max} , binding capacity of beads (800 ng); F , free (unbound) membrane protein (ng) in the 90- μl assay volume. B and B_{max} were corrected for background binding to control beads; F was corrected for membrane protein incapable of binding (panel A).

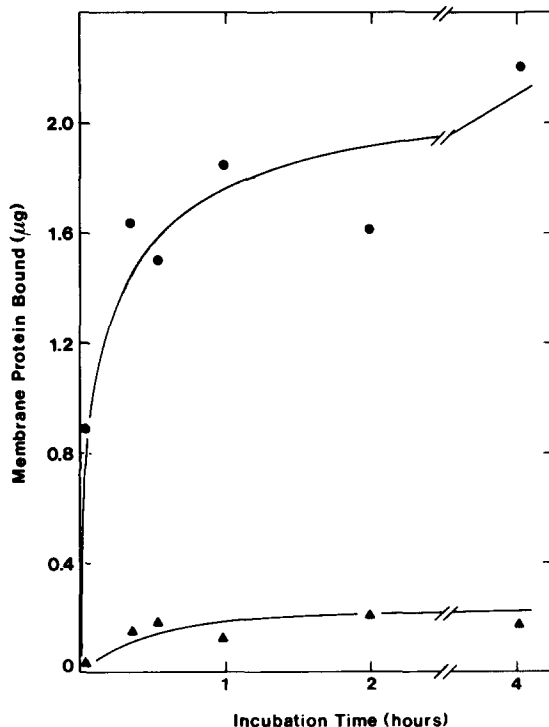


FIGURE 4. Time courses of ^{125}I -labeled, sonicated plasma membrane binding to F-actin Sephacryl S-1000 beads. 5.7 μg membranes, 0.7 μg actin (●); 0.8 μg membranes, 0.2 μg actin (▲). Nonspecific binding was subtracted.

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 $\sim 1 \times 10^{-8}$ M to $\sim 3 \times 10^{-6}$ 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}I -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, unlabeled membranes pre-extracted with chaotropes efficiently inhibit the binding of ^{125}I -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, ^{125}I -labeled, sonicated, NaOH-extracted membranes do bind directly to F-actin beads (Fig. 7). ^{125}I -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. ^{125}I -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, ^{125}I -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-Sepharose columns with and without bound F-actin (Fig. 8b). Essentially all of this nonspecifically bound radioactivity is eluted with 2% Triton X-100; almost none elutes with 1% SDS.

Membrane-associated, F-Actin-binding Polypeptides

Autoradiographs of the SDS polyacrylamide gel profiles of detergent solubilized, ^{125}I -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-

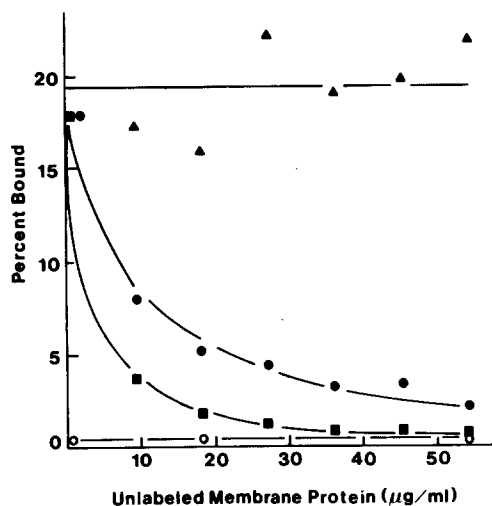


FIGURE 5 Competitive displacement of ^{125}I -labeled, sonicated membrane binding to F-actin beads with unlabeled, sonicated membranes added 1 h before (■), at the same time as (●), or 1 h after (▲) 6 $\mu\text{g}/\text{ml}$ of ^{125}I -labeled membranes were added to 0.5 μg of bead-bound F-actin. All mixtures were incubated for 1½ h after the final addition. ^{125}I -membrane binding to control beads (○) was independent of the order of addition.

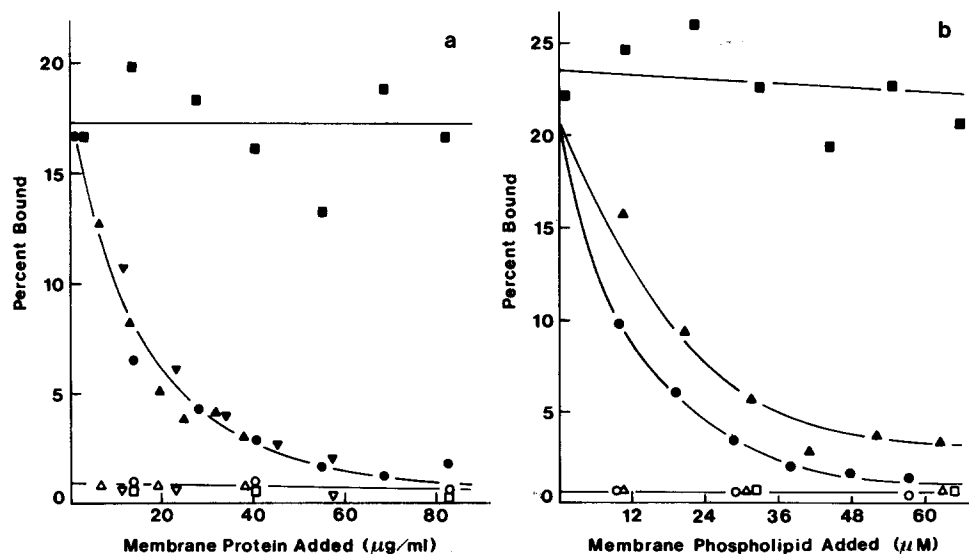


Figure 6 Competitive displacement of ^{125}I -labeled, sonicated membrane binding to F-actin beads with pretreated, unlabeled, sonicated membranes. (a) Purified sonicated membranes before pretreatment (●), membranes heated with excess crystalline DTT at 90°C for 15 min (■), KCl, urea-extracted membranes (▼), and NaOH-extracted membranes (▲). (b) Purified membranes before pretreatment (●), protease-pretreated membranes (■), and membranes incubated and washed as a control for the protease-pretreated membranes (▲). The same preparation of purified, sonicated membranes before pretreatment was used to generate competition curves (●) in

both a and b. Open symbols correspond to the closed symbols but represent binding to control beads rather than to F-actin beads. All assay mixtures included 1.7 μg of ^{125}I -labeled membrane protein and 0.7 μg of bead-bound F-actin.

tides bind F-actin, at least 17, including residual Con A, bind and are eluted by SDS (Figure 9a, lane 4). Because ^{125}I -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 columns. While the molecular basis for this binding is still an open question, it is interesting to note that none of the proteolipids that co-extract 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 ^{125}I -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. We justify this statement with an argument analogous to that first propounded for band 3 of the human erythrocyte (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.

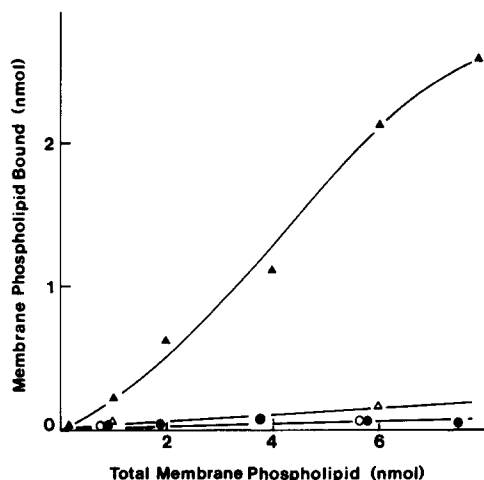


FIGURE 7 Binding curves of ^{125}I -labeled, sonicated, NaOH-extracted membranes (\blacktriangle , \triangle) and ^{125}I -labeled, sonicated *D. discoideum* lipid vesicles (\bullet , \circ) to beads with (\blacktriangle , \bullet) or without (\triangle , \circ) bound F-actin. Assay mixtures included $0.7\ \mu\text{g}$ of bead-bound F-actin.

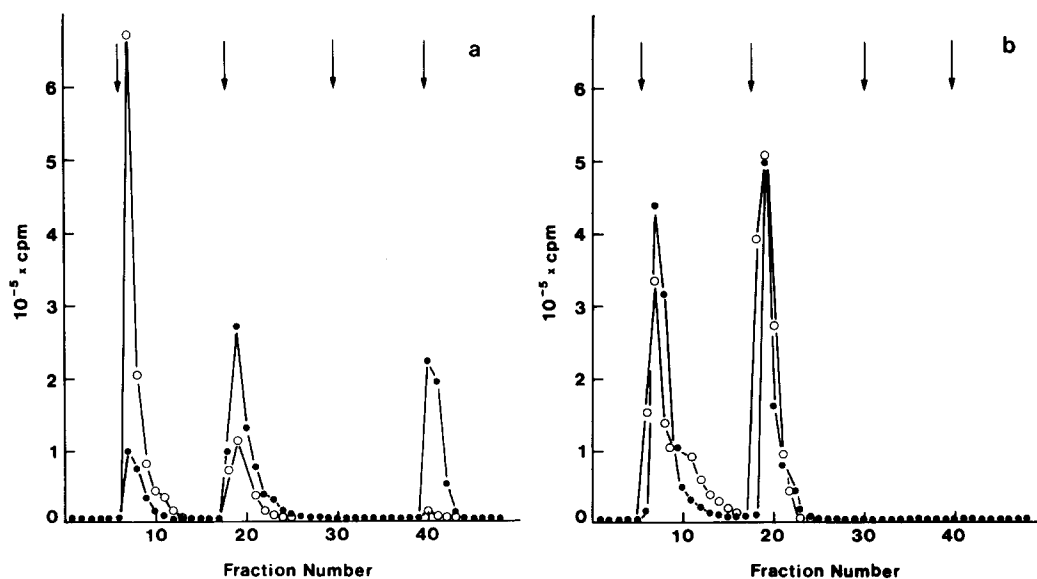


FIGURE 8. Chromatography of (a) ^{125}I -labeled, sonicated plasma membranes and (b) ^{125}I -labeled, sonicated *D. discoideum* lipid vesicles on antifluorescein IgG-Sepharose S-1000 columns with (\bullet) or without (\circ) bound F-actin. The arrows indicate where the columns were loaded and successively eluted with 2% Triton X-100, 0.1% Triton X-100, and 1% SDS.

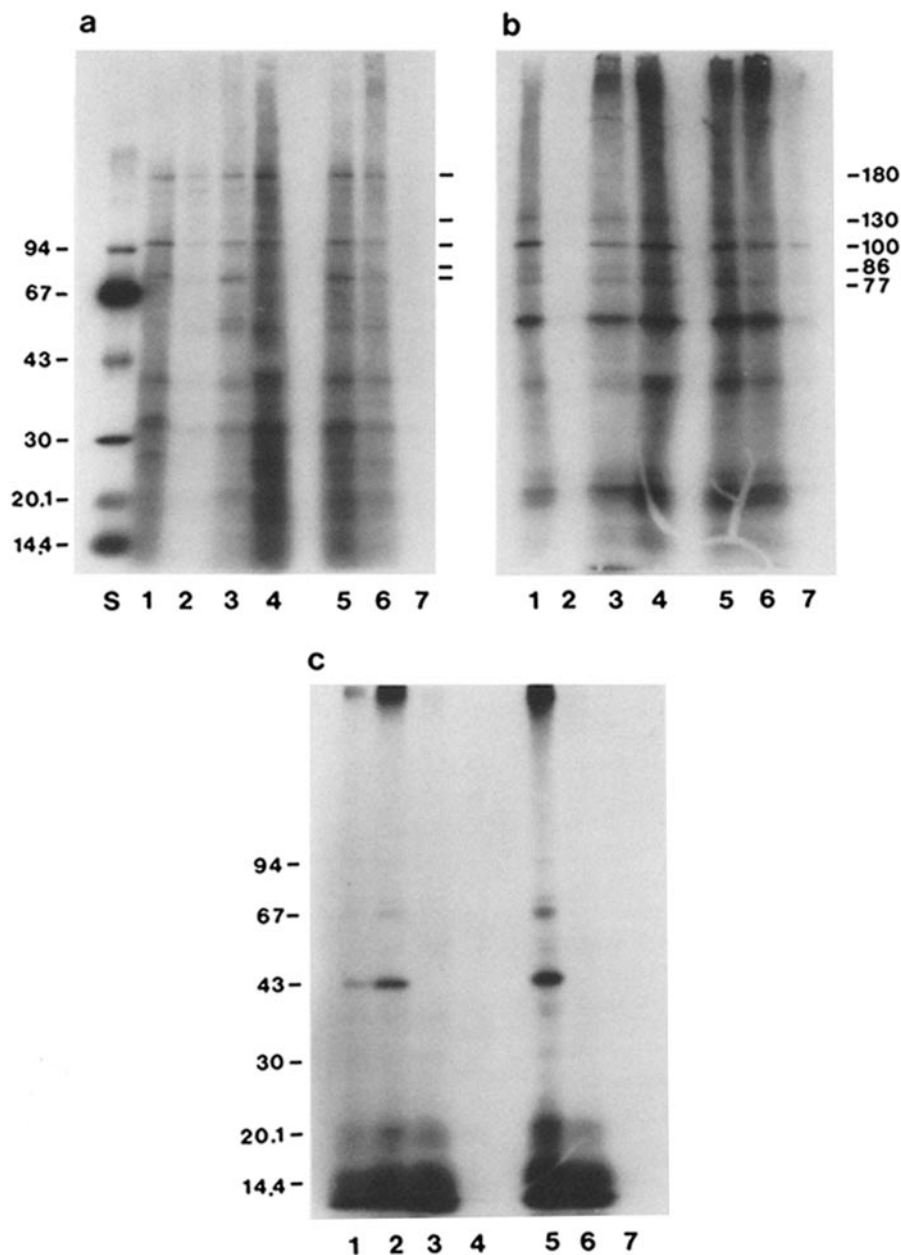


FIGURE 9 Autoradiographs of SDS PAGE profiles of (a) ^{125}I -labeled, sonicated plasma membranes; (b) ^{125}I -labeled, sonicated, NaOH-extracted plasma membranes; and (c) ^{125}I -labeled, sonicated, *D. discoideum* lipid vesicles. Lane 1 in each panel shows membranes before affinity chromatography. The remaining lanes are profiles of fractions from affinity columns such as those shown in Figure 8: (lane 2) run-through peak from an F-actin column; (lane 3) 2% Triton X-100 peak from an F-actin column; (lane 4) 1% SDS peak from an F-actin column; (lane 5) run-through peak from a control bead column; (lane 6) 2% Triton X-100 peak from a control bead column; and (lane 7) 1% SDS peak from a control bead column. Lane S in a shows [^{125}I]Bolton-Hunter-labeled molecular weight standards (Pharmacia Inc.). The numbers to the left of the figures designate the molecular weights ($\times 10^{-3}$) of these standards. Numbers to the right of the figures refer to the apparent molecular weights of the major membrane polypeptides. Each lane was loaded with 100 μl or 150,000 cpm, whichever was the lesser amount. Kodak XAR-2 film was exposed to the dried gels for 4 d (a and b) or for 3 wk (c) with a Cronex Lightening-Plus intensifying screen (E. I. DuPont de Nemours & Co.).

Downloaded from www.jcb.org on October 18, 2007

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. 10b), and are apparently labeled at the cell surface by a lactoperoxidase-catalyzed iodination of intact cells (Fig. 10c). 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-

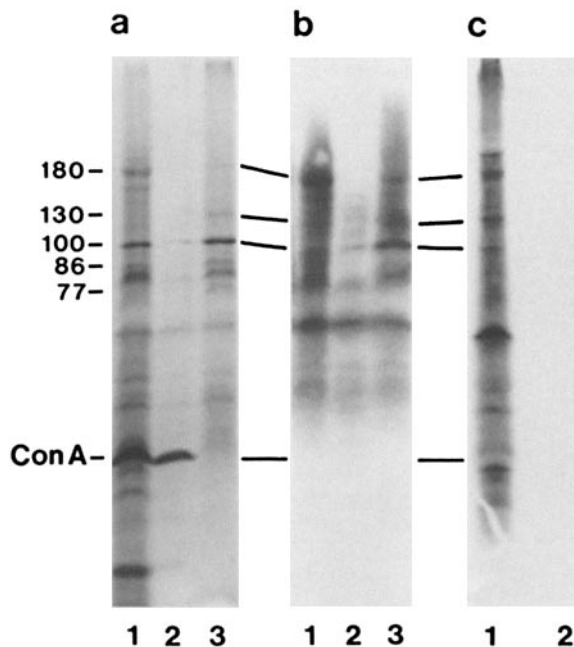


FIGURE 10 SDS PAGE of plasma membrane polypeptides visualized with Coomassie Blue (a), by incubation of nitrocellulose blots with ^{125}I -Con A (b), and by cell surface labeling (c). Both a and b show gels of plasma membrane fragments before pretreatment (lanes 1), after proteolysis with 0.5 mg/ml pronase at 37°C for 2 h (lanes 2), and after extraction with 0.1 N NaOH, 1 mM DTT, pH 12.5 (lanes 3). Although not apparent in lane 3 of b, a faint band at 77,000 daltons was observed on the original autoradiograph. No binding was observed on the α -methyl-D-mannoside control blot. In c we show a gel of surface-labeled polypeptides observed with all labeling components present (lane 1) and with glucose omitted (lane 2). Each lane was loaded with 60 μg of protein. Kodak XAR-2 film was exposed to the gels for 7 d. Although they did not reproduce well, faint bands were observed in lane 2 of c on the original autoradiograph. No detectable labeling was observed when lactoperoxidase was omitted from the labeling solution (not shown). Numbers to the left of the figures designate the apparent molecular weights ($\times 10^{-3}$) of the membrane polypeptides.

sistent with physiologically relevant binding.

Using low-speed sedimentation binding and competition assays, 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 $\sim 10 \mu\text{g}$ of F-actin on beads and no more than 10–15 μg of ^{125}I -labeled *D. discoideum* membrane protein. A single binding determination can be made with $< 1 \mu\text{g}$ 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 twofold. 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.

We thank Karin O'Hara for her excellent secretarial assistance and acknowledge the assistance of S. Proshan and R. Winkelstein in the production of anti-fluorescein IgG and acetamidofluorescein-actin. We appreciate the use of equipment located in the laboratories of Drs. E. C. Cox, A. Newton, and M. S. Steinberg and the critical comments of Drs. A. Ross and M. Holland.

This work was supported by American Cancer Society grant CD-134, by National Institutes of Health grant GM33048, and by Basil O'Connor Starter Research grant No. 5-375 from the March of Dimes

Birth Defects Foundation. H. M. Ingalls was supported by National Cancer Institute Training grant T32CA09167. This work also benefited from the availability of the Whitehall Foundation Center Facilities in the Princeton Biology Department.

Received for publication 21 December 1983, and in revised form 20 March 1984.

REFERENCES

- Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol.* 8:115-118.
- Begg, D. A., R. Rodewald, and L. I. Rebhun. 1978. The visualization of actin filament polarity in thin sections: evidence for the uniform polarity of membrane-associated filaments. *J. Cell Biol.* 79:846-852.
- Beug, H., F. E. Katz, A. Stein, and G. Gerisch. 1973. Quantitation of membrane sites in aggregating *Dictyostelium* cells by use of tritiated univalent antibody. *Proc. Natl. Acad. Sci. USA.* 70:3150-3154.
- Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extractions and purification. *Can. J. Biochem. Physiol.* 37:911-917.
- Bolton, A. E., and W. M. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I -containing acylating agent. *Biochem. J.* 133:529-539.
- Branton, D., C. M. Cohen, and J. Tyler. 1981. Interaction of cytoskeletal proteins on the human erythrocyte membrane. *Cell.* 24:24-32.
- Bretscher, M. S. 1971. A major protein which spans the human erythrocyte membrane. *J. Mol. Biol.* 59:351-357.
- Brown, S. S., H. L. Malinoff, and M. S. Wicha. 1983. Connexin: cell surface protein that binds both laminin and actin. *Proc. Natl. Acad. Sci. USA.* 80:5927-5930.
- Burridge, K., and J. H. Phillips. 1975. Association of actin and myosin with secretory granule membranes. *Nature (Lond.)* 254:526-529.
- Carraway, C. A. C., G. Jung, and K. L. Carraway. 1983. Isolation of actin-containing transmembrane complexes from ascites adenocarcinoma sublines having mobile and immobile receptors. *Proc. Natl. Acad. Sci. USA.* 80:430-434.
- Chadwick, C. M., and D. R. Garrod. 1983. Identification of the cohesion molecule, contact sites B, of *Dictyostelium discoideum*. *J. Cell Biol.* 60:251-266.
- Chaney, L. K., and B. S. Jacobson. 1983. Coating cells with colloidal silica for high yield isolation of plasma membrane sheets and identification of transmembrane proteins. *J. Biol. Chem.* 258:10062-10072.
- Chang, K.-J., and P. Cuatrecasas. 1976. ^{125}I -labelled Concanavalin A of high specific activity. In Con A as a Tool. H. Bittiger and H. P. Schnebli, editors. John Wiley and Sons, London. 187-189.
- Clarke, M., G. Schatten, D. Mazia, and J. A. Spudich. 1975. Visualization of actin fibers associated with the cell membrane in amoebae of *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. USA.* 72:1758-1762.
- Clarke, M., and J. A. Spudich. 1977. Nonmuscle contractile proteins: the role of actin and myosin in cell motility and shape determination. *Annu. Rev. Biochem.* 46:797-822.
- Cocucci, S. M., and M. Sussman. 1970. RNA in cytoplasmic and nuclear fractions of cellular slime mold amoebas. *J. Cell Biol.* 45:399-407.
- Cohen, C. M., and S. F. Foley. 1980. Spectrin-dependent and -independent association of F-actin with the erythrocyte membrane. *J. Cell Biol.* 86:694-698.
- Condeelis, J. 1979. Isolation of concanavalin A caps during various stages of formation and their association with actin and myosin. *J. Cell Biol.* 80:751-758.
- Coudrier, E., H. Reggio, and D. Louvard. 1983. Characterization of an integral membrane glycoprotein associated with the microfilaments of pig intestinal microvilli. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:469-475.
- Cuatrecasas, P., and I. Parikh. 1972. Adsorbents for affinity chromatography. Use of N-hydroxysuccinimide esters of agarose. *Biochemistry.* 11:2291-2299.
- Flanagan, J., and G. L. E. Koch. 1978. Cross-linked surface IgG attaches to actin. *Nature (Lond.)* 273:278-281.
- Fowler, V. M., E. J. Luna, W. R. Hargreaves, D. L. Taylor, and D. Branton. 1981. Spectrin promotes the association of F-actin with the cytoplasmic surface of the human erythrocyte membrane. *J. Cell Biol.* 88:388-395.
- Fowler, V. M., and H. B. Pollard. 1982. Chromaffin granule membrane-F-actin interactions are calcium sensitive. *Nature (Lond.)* 295:336-339.
- Geiger, B. 1983. Membrane-cytoskeleton interaction. *Biochim. Biophys. Acta.* 737:305-341.
- Giffard, R. G., J. A. Spudich, and A. Spudich. 1983. Ca^{2+} -sensitive isolation of a cortical actin matrix from *Dictyostelium* amoebae. *J. Muscle Res. Cell Motil.* 4:115-131.
- Glenney, J. R., Jr., M. Osborn, and K. Weber. 1982. The intracellular localization of the microvillus 110k protein, a component considered to be involved in side-on membrane attachment of F-actin. *Exp. Cell Res.* 138:199-205.
- Goodloe-Holland, C. M., and E. J. Luna. 1984. A membrane cytoskeleton from *Dictyostelium discoideum*. III. Plasma membrane fragments bind predominantly to the sides of actin filaments. *J. Cell Biol.* 99:71-78.
- Gratzer, W. B. 1983. The cytoskeleton of the red blood cell. In *Muscle and Nonmuscle Motility*. A. Stracher, editor. Academic Press, Inc., New York. 2:37-124.
- Griffith, L. M., and T. D. Pollard. 1978. Evidence for actin filament-microtubule interaction mediated by microtubule-associated proteins. *J. Cell Biol.* 78:958-965.
- Haest, C. W. M. 1982. Interactions between membrane skeleton proteins and the intrinsic domain of the erythrocyte membrane. *Biochim. Biophys. Acta.* 694:331-352.
- Hill, A. V. 1910. The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. *J. Physiol. (Lond.)* 40:iv-vii.
- Howe, C. L., and M. S. Mooseker. 1983. Characterization of the 110-kdalton actin-calmodulin- and membrane-binding protein from microvilli of intestinal epithelial cells. *J. Cell Biol.* 97:974-985.
- Hubbard, A. L., and A. Z. Cohn. 1975. Externally disposed plasma membrane proteins. I. Enzymatic iodination of mouse L cells. *J. Cell Biol.* 64:438-460.
- Hynes, R. O. 1981. Relationships between fibronectin and the cytoskeleton. In *Cytoskeletal Elements and Plasma Membrane Organization*. G. Poste and G. L. Nicolson, editors. Elsevier/North Holland Biomedical Press, New York. 99-137.
- Jacobson, B. S. 1980. Actin binding to the cytoplasmic surface of the plasma membrane isolated from *Dictyostelium discoideum*. *Biochem. Biophys. Res. Commun.* 97:1493-1498.
- Koch, G. L. E., and M. J. Smith. 1978. An association between actin and the major histocompatibility antigen H-2. *Nature (Lond.)* 273:274-278.
- Kranz, D. M., and E. W. Voss. 1981. Partial elucidation of an anti-hapten repertoire in BALB/c mice: comparative characterization of several monoclonal anti-fluoresceyl antibodies. *Mol. Immunol.* 18:889-898.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Lehto, V.-P. 1983. 140,000 dalton surface glycoprotein. A plasma membrane component of the detergent-resistant cytoskeletal preparations of cultured human fibroblasts. *Exp. Cell Res.* 143:271-286.
- Lindsay, J. G., G. P. Reid, and P. D'Sousa. 1981. Lectins as biochemical agents for the isolation of sealed membrane vesicles of defined polarity. *Biochim. Biophys. Acta.* 640:791-801.
- Loomis, W. F. 1982. The development of *Dictyostelium discoideum*. Academic Press, Inc., New York. 551 pp.
- Lowry, O. H., N. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Luna, E. J., V. M. Fowler, J. Swanson, D. Branton, and D. L. Taylor. 1981. A membrane cytoskeleton from *Dictyostelium discoideum*. I. Identification and partial characterization of an actin-binding activity. *J. Cell Biol.* 88:396-409.
- Luna, E. J., and C. G. Holland. 1982. Interactions between an F-actin affinity matrix and a plasma membrane fraction from *Dictyostelium discoideum*. *J. Cell Biol.* 95(2, Pt. 2):296a. (Abstr.)
- Luna, E. J., Y.-L. Wang, E. W. Voss, Jr., D. Branton, and D. L. Taylor. 1982. A stable, high capacity, F-actin affinity column. *J. Biol. Chem.* 257:13095-13100.
- March, S. C., I. Parikh, and P. Cuatrecasas. 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. *Anal. Biochem.* 60:149-152.
- Matsuda, P. T., and D. R. Burgess. 1979. Identification and organization of the components in the isolated microvillus cytoskeleton. *J. Cell Biol.* 83:667-673.
- McDonald, K. L. 1982. Membrane and filament distributions in the mitotic spindles of two mammalian tissue culture lines. *J. Cell Biol.* 95(2, Pt. 2):310a. (Abstr.)
- McGhee, J. D., and P. H. von Hippel. 1974. Theoretical aspects of DNA-protein interactions: co-operative and non-co-operative binding of large ligands to a one-dimensional homogeneous lattice. *J. Mol. Biol.* 86:469-489.
- Moss, D. J. 1983. Cytoskeleton-associated glycoproteins from chicken sympathetic neurons and chicken embryo brain. *Eur. J. Biochem.* 135:291-297.
- Murray, B. A. 1982. Membranes. In *The Development of Dictyostelium discoideum*. W. F. Loomis, editor. Academic Press, Inc., New York. 71-116.
- Neff, N. T., C. Lowrey, C. Decker, A. Tovar, C. Damsky, C. Buck, and A. F. Horowitz. 1982. A monoclonal antibody detaches embryonic skeletal muscle from extracellular matrices. *J. Cell Biol.* 95:654-666.
- Oliver, J. M., and R. D. Berlin. 1982. Mechanisms that regulate the structural and functional architecture of cell surfaces. *Int. Rev. Cytol.* 74:55-94.
- Ostlund, R. E., J. T. Leung, and D. M. Kipnis. 1977. Muscle actin filaments bind pituitary secretory granules in vitro. *J. Cell Biol.* 73:78-87.
- Painter, R. G., and M. Ginsberg. 1982. Concanavalin A induces interactions between surface glycoproteins and the platelet cytoskeleton. *J. Cell Biol.* 92:565-573.
- Pan, P., E. M. Hall, and J. T. Bonner. 1972. Folic acid as second chemotactic substance in the cellular slime moulds. *Nature New Biology.* 237:181-182.
- Parish, R. W. 1983. Plasma membrane proteins in *Dictyostelium*. *Mol. Cell. Biochem.* 50:75-95.
- Parish, R. W., and U. Müller. 1976. The isolation of plasma membranes from the cellular slime mold *Dictyostelium discoideum* using concanavalin A and Triton X-100. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 63:40-44.
- Parish, R. W., S. Schmidlin, and U. Müller. 1978. The effects of proteases on proteins and glycoproteins of *Dictyostelium discoideum* plasma membranes. *Exp. Cell Res.* 110:267-276.
- Petrini, M., D. L. Emerson, and R. M. Galbraith. 1983. Linkage between surface immunoglobulin and cytoskeleton of B lymphocytes may involve Gc protein. *Nature (Lond.)* 306:73-74.
- Prem Das, O., and E. J. Henderson. 1983. A novel technique for gentle lysis of eukaryotic cells: isolation of plasma membranes from *Dictyostelium discoideum*. *Biochim. Biophys. Acta.* 736:45-56.
- Rapraeger, A. C., and M. Bernfield. 1982. An integral membrane proteoglycan is capable of binding components of the cytoskeleton and the extracellular matrix. In *Extracellular Matrix*. S. Hawkes and J. L. Wang, editors. Academic Press, Inc., New York. 265-269.
- Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17:208-212.
- Siliciano, J. D., and S. W. Craig. 1982. Metavinculin—a vinculin-related protein with solubility properties of a membrane protein. *Nature (Lond.)* 300:533-535.
- Spudich, J. A. 1974. Biochemical and structural studies of actomyosin-like proteins from non-muscle cells. II. Purification, properties, and membrane association of actin from amoebae of *Dictyostelium discoideum*. *J. Biol. Chem.* 249:6013-6020.
- Spudich, J. A., and A. Spudich. 1982. Cell motility. In *The Development of Dictyostelium discoideum*. W. F. Loomis, editor. Academic Press, Inc., New York. 169-194.
- Spurr, A. R. 1969. A low viscosity epoxy embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26:31-43.
- Stankowski, S. 1983. Large-ligand adsorption to membranes. I. Linear ligands as a limiting case. *Biochim. Biophys. Acta.* 735:341-351.
- Steck, T. L., R. S. Weinstein, J. H. Straus, and D. F. H. Wallach. 1970. Inside-out red cell membrane vesicles: Preparation and purification. *Science (Wash. DC)* 168:255-257.
- Taylor, D. G., V. M. Williams, and N. Crawford. 1976. Platelet membrane actin: solubility and binding studies with ^{125}I -labelled actin. *Biochem. Soc. Trans.* 4:156-160.
- Taylor, D. L., and J. S. Condeelis. 1979. Cytoplasmic structure and contractility in amoeboid cells. *Int. Rev. Cytol.* 56:57-144.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
- Van Haastert, P. J. M., R. J. W. DeWit, and T. M. Konijn. 1982. Antagonists of chemoattractants reveal separate receptors for cAMP, folic acid and pterin in *Dictyostelium*. *Exp. Cell Res.* 140:453-456.
- Virtanen, I., V. P. Lehto, and P. Aula. 1981. Lectins as probes for membrane asymmetry and compartmentalization of saccharide moieties in cells. In *Lectins: Biology, Biochemistry, Clinical Biochemistry*, Vol. I. T. C. Bög-Hansen, editor. Walter deGruyter, New York. 214-220.
- Vogel, G., L. Thilo, H. Schwarz, and R. Steinhart. 1980. Mechanisms of phagocytosis in *Dictyostelium discoideum*: phagocytosis is mediated by different recognition sites as

- disclosed by mutants with altered phagocytic properties. *J. Cell Biol.* 86:456-465.
76. Walsh, F. S., B. H. Barber, and M. J. Crumpton. 1976. Preparation of inside-out vesicles of pig lymphocyte plasma membrane. *Biochemistry*. 15:3557-3563.
 77. Wang, Y.-L., and D. L. Taylor. 1980. Preparation and characterization of a new molecular cytochemical probe: 5-iodoacetamidofluorescein-labeled actin. *J. Histochem. Cytochem.* 28:1198-1206.
 78. Watt, R. M., J. N. Herron, and E. W. Voss, Jr. 1980. First order dissociation rates between a subpopulation of high affinity rabbit anti-fluorescein IgG antibody and homologous ligand. *Mol. Immunol.* 17:1237-1243.
 79. Weatherbee, J. A. 1981. Membranes and cell movement: interactions of membranes with the proteins of the cytoskeleton. *Int. Rev. Cytol.* 12(Suppl):113-176.
 80. Weihing, R. R. 1979. The cytoskeleton and plasma membrane. *Methods Achiev. Exp. Pathol.* 8:42-109.
 81. Wilchek, M., T. Oka, and Y. J. Topper. 1975. Structure of a soluble superactive insulin is revealed by the nature of the complex between cyanogen-bromide-activated Sepharose and amines. *Proc. Natl. Acad. Sci. USA.* 72:1055-1058.
 82. Yahara, I. 1982. Transmembrane control of the motility of surface receptors by cytoskeletal structures. In *Structure, Dynamics, and Biogenesis of Biomembranes*. R. Sato and S.-I. Ohnishi, editors. Plenum Press, New York. 79-95.
 83. Zachowski, A., D. Migliore-Samour, A. Paraf, and P. Jolles. 1975. Non-specific effector-induced enzyme modulation in isolated plasma membranes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 52:57-61.