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## F-actin binds to the cytoplasmic surface of ponticulin, a 17-kD integral glycoprotein from Dictyostelium discoideum plasma membranes

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*Et al.*

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# F-Actin Binds to the Cytoplasmic Surface of Ponticulin, a 17-kD Integral Glycoprotein from *Dictyostelium discoideum* Plasma Membranes

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**Abstract.** F-actin affinity chromatography and immunological techniques are used to identify actin-binding proteins in purified *Dictyostelium discoideum* plasma membranes. A 17-kD integral glycoprotein (gp17) consistently elutes from F-actin columns as the major actin-binding protein under a variety of experimental conditions. The actin-binding activity of gp17 is identical to that of intact plasma membranes: it resists extraction with 0.1 N NaOH, 1 mM dithiothreitol (DTT); it is sensitive to ionic conditions; it is stable over a wide range of pH; and it is eliminated by proteolysis, denaturation with heat, or treatment with DTT and *N*-ethylmaleimide. gp17 may be responsible for much of the actin-binding activity of plasma membranes since monovalent antibody fragments (Fab) directed primarily against gp17 inhibit actin-membrane binding by 96% in sedimentation assays. In contrast, Fab directed against cell surface determinants inhibit binding by only 0–10%. The actin-binding site of gp17

appears to be located on the cytoplasmic surface of the membrane since Fab against this protein continue to inhibit 96% of actin-membrane binding even after extensive adsorption against cell surfaces.

gp17 is abundant in the plasma membrane, constituting 0.4–1.0% of the total membrane protein. A transmembrane orientation of gp17 is suggested since, in addition to the cytoplasmic localization of the actin-binding site, extracellular determinants of gp17 are identified. gp17 is surface-labeled by sulfo-*N*-hydroxysuccinimido-biotin, a reagent that cannot penetrate the cell membrane. Also, gp17 is glycosylated since it is specifically bound by the lectin, concanavalin A. We propose that gp17 is a major actin-binding protein that is important for connecting the plasma membrane to the underlying microfilament network. Therefore, we have named this protein “ponticulin” from the Latin word, *ponticulus*, which means small bridge.

**B**ASIC cell activities such as adhesion, motility, chemotaxis, endocytosis, and the modulation of cell surface receptors are thought to involve interactions between the plasma membrane and the underlying microfilament network. Actin, one of the most abundant and highly conserved cytoskeletal proteins (reviewed in Korn, 1982; DeRosier and Tilney, 1984), is a major component of this submembranous network. Although the association of actin with the plasma membrane has been extensively investigated (reviewed in Geiger, 1983; Jacobson, 1983), the molecular details of actin-membrane linkages remain obscure. The cellular slime mold *Dictyostelium discoideum* offers many advantages for the study of actin-membrane interactions. This eukaryotic amoeba displays a number of cellular activities, such as adhesion, motility, chemotaxis, endocytosis, and cell surface receptor modulation (see reviews in Loomis, 1982). In addition, *D. discoideum* is amenable to biochemical analysis since large quantities of amoebae can be grown easily in axenic culture (Loomis, 1971).

Information about the cytoplasmic surface of the *D. discoideum* plasma membrane is extensive. Actin filaments

have been visualized underlying the membrane (Clarke et al., 1975; Eckert and Lazarides, 1978) and actin has been isolated with plasma membrane preparations (Parish and Müller, 1976; Giffard et al., 1983). Many cytoskeletal proteins have been purified, including actin (Uyemura et al., 1978), myosin (Clarke and Spudich, 1974), calmodulin (Clarke et al., 1980), and a number of actin-binding proteins that cross-link, cap, or otherwise modify actin filaments (reviewed in Taylor and Fehheimer, 1982; Stossel et al., 1985).

Several assays have shown that plasma membranes isolated from *D. discoideum* specifically bind F-actin (Luna et al., 1981, 1984; Stratford and Brown, 1985; Schwartz and Luna, 1986). This binding appears to be predominantly to the sides, rather than to the ends, of actin filaments (Bennett and Condeelis, 1984; Goodloe-Holland and Luna, 1984). Further, *D. discoideum* plasma membranes can organize actin into filaments at actin concentrations below the critical concentration required for polymerization in solution (Schwartz and Luna, 1986). Integral membrane proteins appear to mediate both the actin-binding and the actin assembly activity of the membrane since these activities persist after extraction of

the membranes with chaotropic agents such as 0.1 N NaOH and 1 mM dithiothreitol (DTT), but are destroyed when membranes are proteolyzed, heat-treated, or alkylated with *N*-ethylmaleimide (NEM)<sup>1</sup> after reduction with DTT (Luna et al., 1981, 1984; Schwartz and Luna, 1986). Although initial studies have identified potential candidates for integral membrane actin-binding proteins (Luna et al., 1984; Stratford and Brown, 1985; Brown and Petzold, 1987), the exact molecules involved are not yet known.

To investigate the molecular basis for the binding of F-actin to purified *D. discoideum* plasma membranes, we have used two very different but complementary approaches: a biochemical approach and an immunological approach. In the biochemical approach, low-speed sedimentation assays are used to define conditions for actin-membrane binding with respect to salt concentration and pH. F-actin-binding proteins then are isolated by fractionating detergent-solubilized membrane proteins on F-actin affinity columns. In the immunological approach, polyclonal monovalent antigen-binding fragments of IgG (Fab) directed against integral membrane proteins are used to inhibit actin-membrane binding and the proteins recognized by these antibodies are identified by immunoprecipitation.

In this paper, we report that a 17-kD integral membrane glycoprotein (gp17) is responsible for much of the actin-binding activity of plasma membranes. The actin-binding activity of gp17 is identical to that observed for intact plasma membranes: it resists extraction with 0.1 N NaOH, 1 mM DTT; it is sensitive to ionic conditions; it is stable over a wide range of pH; and it is eliminated by proteolysis, denaturation with heat, or treatment with DTT and NEM. gp17 is relatively abundant in the plasma membrane, constituting ~0.4–1.0% of the total membrane protein. Further, gp17 appears to be a transmembrane protein with its actin-binding site located on the cytoplasmic surface of the membrane. Therefore, we propose that gp17 is a major actin-binding plasma membrane protein that is important for linking the plasma membrane to the microfilament network during cellular activities such as adhesion, motility, chemotaxis, endocytosis, and the modulation of cell surface receptors.

This work was presented in a preliminary form at the 26th Annual Meeting of the American Society for Cell Biology, Washington, DC, December 7–11, 1986 (Wuesthube and Luna, 1986).

## Materials and Methods

### Chemicals

Octyl glucoside (OG), ATP, and phalloidin were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). DTT, NEM, diisopropylfluorophosphate, phenylmethylsulfonyl fluoride (PMSF), Triton X-100 (TX-100), and BSA were supplied by Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase-conjugated goat anti-rabbit IgG was acquired from Cooper Biomedical, Inc. (Malvern, PA). Acrylamide and SDS were purchased from Bio-Rad Laboratories (Richmond, CA). Twice crystallized ovalbumin was obtained from Millipore Corp. (Bedford, MA) and con-

1. *Abbreviations used in this paper:* anti-NaIO<sub>4</sub>-PM, antiserum raised against NaIO<sub>4</sub>-treated, NaOH-extracted plasma membranes; anti-NaOH-PM, antiserum raised against NaOH-extracted plasma membranes; Fab, monovalent antigen-binding fragments of IgG; gp17, 17-kD integral glycoprotein; NEM, *N*-ethylmaleimide; OG, octyl glucoside; SNHS-biotin, sulfo-*N*-hydroxysuccinimidobiotin; TX-100, Triton X-100.

canavalin A (Con A) was from Miles-Yeda (Rehovot, Israel). Sephacryl S-1000 was procured from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ). All other chemicals were reagent grade.

### Proteins

Actin was isolated from rabbit skeletal muscle according to the method of Spudich and Watt (1971). For actin-membrane binding assays, actin was further purified by gel filtration (MacLean-Fletcher and Pollard, 1980).

Rabbit plasma gelsolin was prepared following a protocol graciously provided by Dr. Carl Frieden (Frieden, C., unpublished observations). Protein concentrations were determined in the presence of 1% SDS by the procedure of Lowry et al. (1951), using BSA as a standard.

### Cell Culture, Fixation, and Surface-Labeling

**Cell Culture.** *D. discoideum* amoebae (strain A×3, a gift from Dr. Richard Kessin, Columbia University) were grown in HL-5 medium (Cocucci and Sussman, 1970) to ~1 × 10<sup>7</sup> cells/ml for membrane preparations or to ~6 × 10<sup>6</sup> cells/ml for glutaraldehyde fixation. The cells were harvested at 1,500 g for 2 min and were washed twice with 14.6 mM KH<sub>2</sub>PO<sub>4</sub>, 2.0 mM Na<sub>2</sub>PO<sub>4</sub>, pH 6.1 (Sorensen's buffer) at 650 g for 2 min at 20°C.

**Glutaraldehyde Fixation of Cells.** Washed cells were resuspended to 1.0 × 10<sup>8</sup> cells/ml in 2% glutaraldehyde, Sorensen's buffer and incubated for 5 min at room temperature with gentle agitation. Cells were collected and washed twice in Sorensen's buffer. To block reactive sites, cells were incubated for 2 h at 2.0 × 10<sup>8</sup> cells/ml in 100 mM glycine ethyl ester, Sorensen's buffer and washed five times in Sorensen's buffer. Glutaraldehyde-fixed cells appeared intact and no cell fragments were observed under the light microscope.

**Cell Surface Labeling.** Cell surface amino groups were labeled with sulfo-*N*-hydroxysuccinimidobiotin (SNHS-biotin) as described (Ingalls et al., 1986).

### Membrane Preparation and Extraction

**Membrane Preparation.** Unless otherwise noted, highly purified plasma membranes were prepared from log phase *D. discoideum* amoebae using the Con A stabilization, Triton-extraction method described by Luna et al. (1984). All membranes were stored on ice in 0.02% NaN<sub>3</sub>, 20 mM sodium phosphate, pH 6.8.

**NaOH Extraction.** Plasma membranes were extracted with 0.1 N NaOH, 1 mM DTT, pH 12.5, for 30 min at 0°C. The extraction mixture was sonicated with one 5-s burst from a model 200 Branson Sonifier (Branson Sonic Power Co., Danbury, CT) at 20 W. The NaOH-extracted membranes were pelleted at 27,900 g for 20 min at 4°C. The pellet, containing 30% of the initial membrane protein, was immediately resuspended and washed with 20 mM sodium phosphate, pH 6.8.

**Periodate Oxidation.** Highly antigenic carbohydrate residues were altered or removed from membrane proteins by periodate oxidation (Dyer, 1956). NaOH-extracted membranes at ~0.6 mg/ml were incubated in the dark with 50 mM NaIO<sub>4</sub>, 10 mM sodium phosphate, pH 6.0, for 12 h on ice. The reaction was quenched by the addition of ethylene glycol to a final concentration of 1% (vol/vol). After 30 min on ice, membranes were diluted with 4 vol of 20 mM sodium phosphate, pH 6.8, collected at 25,000 g for 40 min, and washed in 20 mM sodium phosphate, pH 6.8. Control membranes were treated identically except that 1% (vol/vol) ethylene glycol was added before incubation with NaIO<sub>4</sub>. At least 83% of the protein from both periodate-treated and control membranes was recovered in the final pellet.

**Protease Treatment.** Proteolytically digested membranes were prepared essentially as described (Luna et al., 1981). Briefly, membranes were sonicated (Luna et al., 1984) and then proteolyzed for 2 h at 37°C in a 2.2-ml reaction mixture containing 1.5 mg membrane protein, 1.2 mg pronase (protease type XIV, 5.3 U/mg solid; Sigma Chemical Co.), 20 mM sodium phosphate, pH 6.8. After 30 min, 200 μl of 100 mM EDTA were added. Proteolysis was inhibited by adding 3.0 ml of 0.4 mg/ml PMSF, 1.4% ethanol, 9 mM diisopropylfluorophosphate, 20 mM sodium phosphate, pH 6.8 (inhibition buffer). After 30 min at 37°C, proteolyzed membranes were collected and washed with inhibition buffer at 134,000 g for 40 min. The pellets, containing ~31% of the initial membrane protein, were resuspended and stored in 20 mM sodium phosphate, pH 6.8.

**DTT-NEM Treatment.** Plasma membranes were incubated with DTT at 37°C for 30 min in a 1.4-ml reaction mixture containing ~2.5 mg membrane protein, 50 mM DTT, 100 mM Tris, pH 7.5. Membranes were pelleted

at 25,300 g for 20 min at 4°C and washed twice in 200 mM Tris, pH 7.5. The pellet was resuspended with an equal volume of 100 mM NEM, 200 mM Tris, pH 7.5, using gentle homogenization or vortexing. After 30 min at 37°C, membranes were centrifuged as described above and washed three times in 40 ml of 20 mM sodium phosphate, pH 6.8. The final pellet, containing 71–79% of the initial membrane protein, was resuspended to 700 µl with 20 mM sodium phosphate, pH 6.8.

**Heat Denaturation.** Plasma membranes in a 1.5-ml polypropylene tube were immersed in a water bath at 90°C for 1 h.

**Glutaraldehyde Fixation of Membranes.** Plasma membranes at 2.0 mg/ml were incubated in 2% glutaraldehyde, 20 mM sodium phosphate, pH 6.8, for 5 min at room temperature. Membranes were collected at 25,000 g for 10 min and washed three times in 20 mM sodium phosphate, pH 6.8. Reactive sites were blocked by incubating membranes at 1.0 mg/ml in 100 mM glycine ethyl ester, 20 mM sodium phosphate, pH 6.8, for 2 h. Fixed membranes were centrifuged as described above and washed five times in 17.5 mM potassium phosphate, pH 8.0.

## Radioiodination

**Radioiodination of Membranes and Molecular Mass Standards.** Sonicated membranes at 2.0 mg/ml were radiolabeled with 125 µCi <sup>125</sup>I-Bolton Hunter reagent (Bolton and Hunter, 1973) in 200 µl 0.2 M sodium phosphate, pH 8.0, for 2 h at 0°C. Labeled membranes were separated from unreacted reagent by chromatography on a column of Sephadex G-25 (Sigma Chemical Co.) equilibrated with 20 mM sodium phosphate, pH 6.8. Pre-stained molecular mass standards (Bethesda Research Laboratories, Bethesda, MD) were labeled with <sup>125</sup>I-Bolton Hunter reagent using the same procedure.

**Radioiodination of Actin.** Gel-filtered actin was labeled, polymerized, depolymerized, and stored as described by Schwartz and Luna (1986) with two exceptions. First, <sup>125</sup>I-Bolton Hunter reagent in benzene was purchased from New England Nuclear (Boston, MA) and evaporated to dryness before addition of G-actin. Second, radiolabeled F-actin was collected at 150,000 g for 2.5 h at 4°C and radiolabeled G-actin was clarified at 150,000 g for 30 min at 2°C.

**Radioiodination of Con A.** Con A was radiolabeled using carrier-free Na<sup>125</sup>I (Amersham Corp., Arlington Heights, IL) and chloramine T (Eastman Kodak Co., Rochester, NY) as described by Chang and Cuatrecasas (1976) with the modifications described in Luna et al. (1984).

## F-Actin Affinity Chromatography

**Bead Preparation.** F-actin beads were prepared as described (Luna et al., 1982) with the exception that F-actin was labeled either with 5-iodoacetamidofluorescein (Molecular Probes Inc., Junction City, OR) by the method of Wang and Taylor (1980), as modified by Luna et al. (1982), or with fluorescein-5-maleimide (Molecular Probes, Inc.) as described by Wang and Taylor (1981).

**Affinity Columns.** Columns were constructed of a 1-ml plastic syringe fitted with a plug of porous polyethylene and contained 100–500 µl of packed antifluorescein IgG-Sepharacyl S-1000 beads with bound actin. Control columns contained IgG-Sepharacyl S-1000 beads without bound actin. Nonspecific protein binding sites were blocked with 4% (wt/vol) BSA in column buffer (50 mM KCl, 1 mM MgCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, 0.5 µM phalloidin, 20 mM Tris-acetate, pH 7.0 at 20°C).

Two F-actin affinity chromatographic procedures were developed to identify F-actin-binding proteins. In the first procedure, sonicated <sup>125</sup>I-labeled membranes were bound at room temperature (20–25°C) to F-actin columns and unbound membranes were washed away with excess column buffer. Membranes were solubilized and unbound proteins were removed by washing with 10 column vol of 3% OG in column buffer. Bound proteins were eluted from the columns either with 1% SDS, which also removes actin filaments from the beads, or with 2 M NaCl, 2 mM EGTA, 1% OG in column buffer followed by 1% SDS. In the second chromatographic procedure, <sup>125</sup>I-labeled membranes were presolubilized with 3% OG in column buffer for ~10 min at 6°C and clarified by centrifugation at 150,000 g for 1 h at 6°C. The supernatant then was incubated at room temperature with F-actin affinity columns, which were washed and eluted as in the first procedure.

## Actin-Membrane Binding Assays

Actin-membrane binding assays were performed essentially as described by Schwartz and Luna (1986). Briefly, <sup>125</sup>I-labeled gel-filtered actin at 60 µg/ml was polymerized for 30 min at 25°C with gelsolin at a mole ratio of actin to gelsolin of 25:1 in polymerization buffer (50 mM KCl, 2 mM MgCl<sub>2</sub>,

50 µM CaCl<sub>2</sub>, 1.0 mM DTT, 0.4 mM ATP, 100 µM PMSF, 20 mM Pipes, pH 7.0) containing 1 µM phalloidin and 1 mg/ml ovalbumin. The pre-assembled short actin filaments then were diluted with 2 vol of polymerization buffer. In actin-membrane binding inhibition studies, membranes were preincubated with Fab in 50 mM KCl, 2 mM MgCl<sub>2</sub>, 20 mM Pipes, pH 7.0, for 1 h at room temperature. For each assay point, 0.1 µg of <sup>125</sup>I-labeled short actin filaments were incubated with 1.25 µg of NaOH-extracted membranes in a total volume of 50 µl polymerization buffer for 1 h at room temperature. Membrane-bound actin was measured after centrifugation through 10% (wt/vol) sucrose as described (Schwartz and Luna, 1986). For binding assays in which NaCl was present, 24% (wt/vol) sucrose was used. Error bars on binding curves represent high and low values for duplicate points when the range is greater than the size of the symbol.

## PAGE and Protein Blotting

Samples were denatured (Luna et al., 1981) and run in 6–16%, 10–20%, or 15–20% polyacrylamide gradient SDS slab gels using the discontinuous system of Laemmli (Laemmli, 1970). Gels were silver stained according to the method of Merrill et al. (1981) using the color development modification of Nielsen and Brown (1984), or dried onto filter paper for autoradiography. Kodak XAR-5 film was exposed to the dried gels at –70°C in the presence of a DuPont Cronex Lightning Plus intensifying screen. Molecular mass determinations were based on the molecular mass (Tsang et al., 1984) of Bethesda Research Laboratories prestained standards.

Blots were prepared by transferring electrophoretically separated proteins onto nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) for 12 h at 30 V or for 5 h at 60 V. To prevent elution of low molecular mass proteins from the nitrocellulose, proteins were fixed onto the nitrocellulose with glutaraldehyde (Van Eldik and Wolchok, 1984). Membrane glycoproteins were visualized by probing blots with <sup>125</sup>I-Con A as described (Luna et al., 1984).

Biotinylated membrane proteins were visualized by staining blots with 0.5 µg/ml avidin-alkaline phosphatase (460 U/mg solid; Sigma Chemical Co.) in 150 mM NaCl, 4% BSA, 0.02% TX-100, 50 mM sodium borate, pH 10.5, for 2 h at room temperature. Blots were washed for a total of 1 h with three changes of 150 mM NaCl, 0.01% TX-100, 50 mM sodium borate, pH 10.5, and three changes of 0.1% TX-100, 50 mM sodium borate, pH 10.5. Blots then were developed in 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 100 mM Tris, pH 9.5, containing 0.33 mg/ml nitro blue tetrazolium (Sigma Chemical Co.) and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co.) as described by Leary et al. (1983).

## Antibody Production, Purification, and Adsorption

**Preparation of Antisera.** Two New Zealand rabbits were injected intradermally with either 600 µg of NaOH-extracted membranes or 100 µg NaIO<sub>4</sub>-treated, NaOH-extracted membranes mixed 1:1 with Freund's complete adjuvant (Miles Laboratories Inc.). 4 wk later, a booster injection was given to each rabbit using the same antigens, i.e., 800 µg of NaOH-extracted membranes or 100 µg of NaIO<sub>4</sub>-treated, NaOH-extracted membranes, in Freund's incomplete adjuvant (Miles Laboratories Inc.). The rabbits were bled from the marginal ear vein every week after the booster injection.

**IgG and Fab Purification.** IgG was isolated by ammonium sulfate precipitation and chromatography through DEAE-cellulose (Hudson and Hay, 1980). Fab were prepared from IgG by papain digestion and were purified by chromatography through carboxymethylcellulose (Hudson and Hay, 1980). The purity of IgG and Fab preparations was verified by SDS-PAGE.

**Adsorption of IgG against Cells.** IgG was adsorbed by five 1-h incubations with glutaraldehyde-fixed cells in 17.5 mM potassium phosphate, pH 8.0, at a ratio of 1 mg IgG per 5 × 10<sup>7</sup> cells. After each incubation, cells were pelleted at 955 g for 2 min and the IgG supernatant was collected. The adsorption was considered complete since no difference in titer was observed between the IgG supernatants after the fourth and fifth incubations, as measured by ELISA (see below). The final IgG supernatant was clarified at 25,000 g for 30 min and the IgG was re-purified by ammonium sulfate precipitation and chromatography through DEAE-cellulose. As a control, non-adsorbed IgG also was re-purified.

**Adsorption of IgG against Membranes.** IgG was adsorbed by five 1-h incubations with glutaraldehyde-fixed membranes in 17.5 mM potassium phosphate, pH 8.0, at a ratio of 1 mg IgG per mg membrane protein. After each incubation, membranes were pelleted at 10,000 g for 5 min and the IgG supernatant was collected. The final IgG supernatant was clarified at 150,000 g for 1 h at 6°C.

## Immunological Methods

**Immune Precipitation.**  $^{125}\text{I}$ -labeled membranes ( $1 \times 10^5$  to  $2 \times 10^5$  cpm/ $\mu\text{g}$ ) were solubilized at  $10 \mu\text{g}/\text{ml}$  in  $0.3 \text{ M NaCl}$ ,  $5 \text{ mM EDTA}$ ,  $25 \text{ mM Tris-HCl}$ ,  $\text{pH } 8.0$ ,  $1\% \text{ TX-100}$ ,  $1 \text{ mg}/\text{ml BSA}$  (solubilization buffer). To minimize background, solubilized membranes were precleared by incubation with either  $2 \mu\text{l}$  preimmune serum or  $10 \mu\text{g}$  preimmune IgG per  $1 \mu\text{g}$  membrane protein for 1 h at  $25^\circ\text{C}$  followed by incubation for 30 min at  $25^\circ\text{C}$  with  $10 \mu\text{l}$  of  $10\%$  prewashed *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring Corp., La Jolla, CA) per  $1 \mu\text{l}$  serum or  $5 \mu\text{g}$  IgG. The solution then was clarified at  $150,000 \text{ g}$  for 1 h at  $6^\circ\text{C}$  and  $10 \mu\text{g}$  immune IgG or  $2 \mu\text{l}$  immune serum was added to each supernatant. After a 2-h incubation at  $25^\circ\text{C}$ , the solution was incubated for an additional 30 min with  $25 \mu\text{l}$  of  $10\%$  Pansorbin. The immune complexes were collected at  $10,000 \text{ g}$  for 4 min at  $25^\circ\text{C}$  and washed three times with solubilization buffer. The immunoprecipitated membrane proteins were prepared for electrophoresis by heating in sample buffer at  $70^\circ\text{C}$  for 10 min after which the Pansorbin was removed by centrifugation at  $10,000 \text{ g}$  for 4 min.

**ELISA.** The titer<sup>2</sup> of Fab or IgG against membranes and against partially purified gp17 was measured in an ELISA (Engvall and Perlman, 1972). Briefly,  $1 \mu\text{g}$  membrane protein or  $\sim 20 \text{ ng}$  partially purified gp17 in PBS ( $150 \text{ mM NaCl}$ ,  $10 \text{ mM sodium phosphate}$ ,  $\text{pH } 7.2$ ) was allowed to attach to wells in Nunc immunoplates (Vanguard International, Inc., Neptune, NJ) for 2 h at  $25^\circ\text{C}$ . Nonspecific binding sites were blocked with  $2\% \text{ BSA}$ , PBS at  $25^\circ\text{C}$  for 2 h. Serial dilutions of IgG or Fab in  $1\% \text{ BSA}$ , PBS were incubated in each well for 1 h at  $25^\circ\text{C}$ . The binding of primary antibody was visualized by incubating wells with  $100 \mu\text{l}$  of horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:1,000 or 1:100 in  $1\% \text{ BSA}$ , PBS for 1 h at  $25^\circ\text{C}$  followed by a 30-min incubation at  $25^\circ\text{C}$  with  $150 \mu\text{l}$  of  $0.03\% \text{ H}_2\text{O}_2$ ,  $0.5 \text{ mg}/\text{ml } o\text{-phenylenediamine}$  (Sigma Chemical Co.),  $50 \text{ mM sodium tartrate}$ ,  $\text{pH } 4.5$ . Between each incubation, wells were washed extensively with PBS. The absorbance at  $405 \text{ nm}$  of each well was measured using a Bio-Rad model 2550 EIA Reader.

Fab titers against plasma membranes and against gp17 were compared using the ratios of the slopes obtained by plotting OD 405 versus Fab concentration ( $\mu\text{g}/\text{ml}$ ). For example, the percentages of Fab directed against the intracellular surface of the membrane ( $\text{Fab}_i$ ) were determined using the equation  $\text{Fab}_i = 100 \times (\text{OD } 405/\text{adsorbed Fab} \div \text{OD } 405/\text{non-adsorbed Fab})$ . Each calculation was based on 6–10 data points from the linear section of the graph. Correlation coefficients were 0.97–1.00.

## Results

### Biochemical Analysis of Actin–Membrane Binding

**Conditions of Actin–Membrane Binding.** The molecular nature of the interactions involved in the binding of F-actin to membranes was explored by analyzing the sensitivity of actin–membrane binding to changes in salt concentration and pH. A quantitative sedimentation assay (Schwartz and Luna, 1986) in which  $^{125}\text{I}$ -labeled actin filaments, limited in length by the actin capping protein gelsolin, are bound to large membrane sheets was used for this purpose. Fig. 1 shows that the extent of actin–membrane binding changes as a function of NaCl concentration. Maximal binding is observed between 20 and 100 mM NaCl. At concentrations higher than 100 mM NaCl, binding is observed to decrease steadily. In the presence of 2 M NaCl, binding is reduced 82%. In contrast, the extent of actin–membrane binding does not vary significantly over a wide range of pH. The amount of F-actin bound to membranes remains relatively constant from pH 5.5 to 7.0. Binding decreases gradually above pH 7.0 and is reduced  $\sim 25\%$  at pH 9.5 (data not shown).

**Octyl Glucoside Solubilization of Plasma Membranes.** Integral membrane proteins that are anchored in the lipid

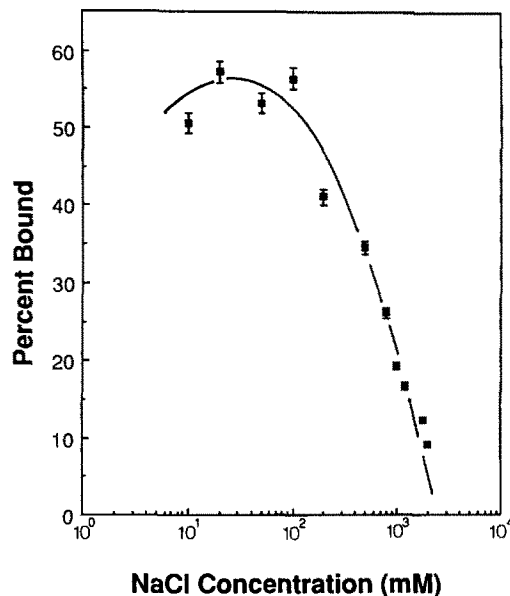


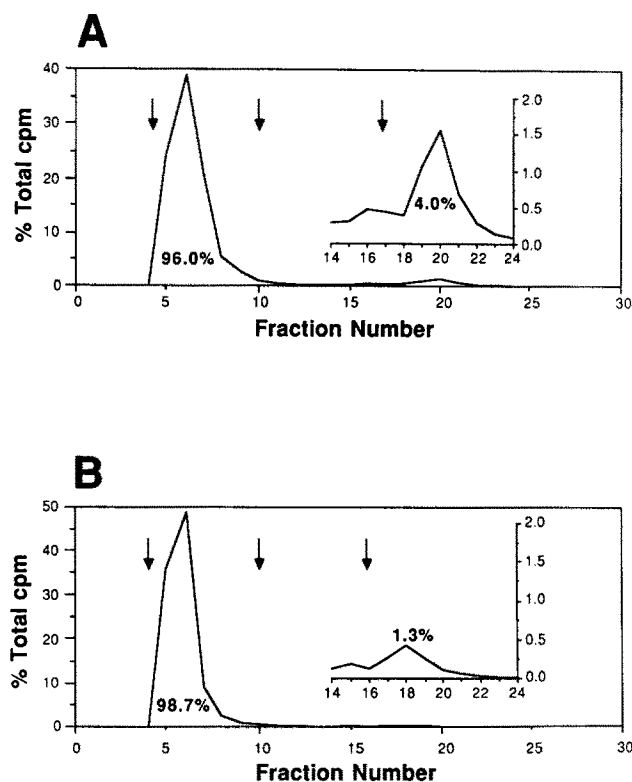
Figure 1. The binding of  $^{125}\text{I}$ -labeled actin to membranes as a function of NaCl concentration.

bilayer by hydrophobic interactions require the use of detergents for solubilization into aqueous solution (Helenius and Simons, 1975). We have used two established criteria to judge the efficiency of OG in solubilizing *D. discoideum* plasma membrane proteins. First, membrane proteins are considered solubilized if retained in the supernatant fraction after centrifugation at  $150,000 \text{ g}$  for 1 h at  $6^\circ\text{C}$  (Hjelmeland and Chrambach, 1984). The concentration of OG required for efficient solubilization of both purified plasma membranes and of plasma membranes that were preextracted with  $0.1 \text{ N NaOH}$ ,  $1 \text{ mM DTT}$  to remove peripheral proteins was examined using a sedimentation assay. In this assay, membranes at  $0.2 \text{ mg}/\text{ml}$  protein are solubilized with concentrations of OG ranging from 0% (wt/vol) to 3.0% (wt/vol) and then pelleted onto sucrose cushions at  $150,000 \text{ g}$  for 1 h at  $6^\circ\text{C}$ . Sedimentation assays of  $^{125}\text{I}$ -labeled membranes reveal that at a final concentration of 3% OG, 90% of the counts associated with both plasma membranes and NaOH-extracted membranes are recovered in the supernatant fraction. SDS-PAGE analysis shows that the overall protein composition of the supernatant does not change appreciably as the concentration of OG increases (data not shown). This result indicates that, at these concentrations, OG is not selectively extracting membrane proteins, but rather is solubilizing membrane proteins by disrupting the lipid bilayer.

The efficiency of OG in solubilizing *D. discoideum* plasma membrane proteins is confirmed by a second criterion in which membrane proteins are considered to be solubilized if they partition in the included volume of a Sepharose 6B column (Hjelmeland and Chrambach, 1984). Since at least 93% of the counts of  $^{125}\text{I}$ -labeled membrane proteins found in the supernatant fraction elute in the included volume of a Sepharose 6B column (data not shown), protein complexes appear to be no larger than  $4 \times 10^6 \text{ D}$ , the exclusion limit of Sepharose 6B.

**F-Actin Affinity Chromatography.** F-actin affinity chromatography has been used to identify potential actin-binding proteins in detergent extracts of plasma membranes. Fig. 2

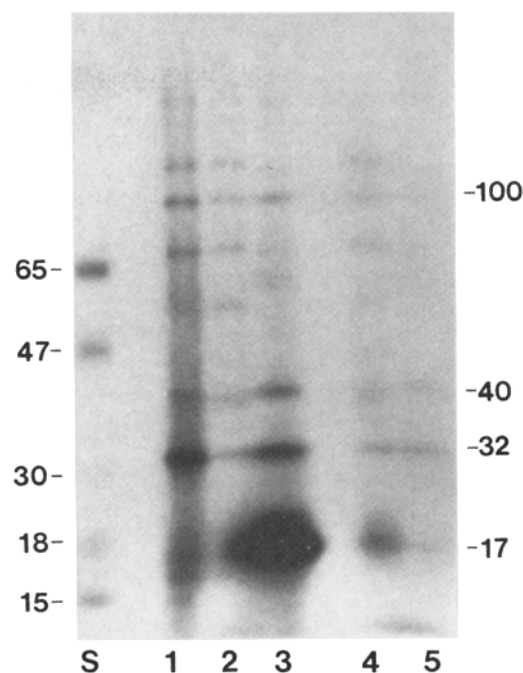
2. In this paper, the term "titer" refers to the concentration of Fab or IgG required to generate a given absorbance reading at  $405 \text{ nm}$  in an ELISA, as described in Materials and Methods.



**Figure 2.** Chromatography of  $^{125}\text{I}$ -labeled plasma membranes on F-actin affinity (**A**) and control (**B**) columns. Membranes were solubilized in 3% octyl glucoside and clarified at 150,000 g immediately before loading onto the columns. The arrows indicate when the columns were loaded and successively eluted with 1% octyl glucoside and with 1% SDS. The run-through peak containing unbound membrane proteins was collected in fractions 5 and 6 for both columns. SDS-elution of the actin column released specifically bound proteins which were collected in fractions 19, 20, and 21. Background radioactivity eluted with SDS from the control column was collected in fractions 17, 18, and 19.

shows typical elution profiles of OG presolubilized  $^{125}\text{I}$ -labeled membranes fractionated on F-actin affinity columns and on control columns which lack actin filaments. Whereas most of the counts associated with  $^{125}\text{I}$ -labeled membranes are eluted from the columns with OG,  $\sim 4\%$  of the radioactivity remains bound to the actin column and is eluted with SDS. By comparison, the SDS-eluted fraction from the control column contains only 1.3% of the total radioactivity. Therefore, we estimate that 2.5–3.0% of the radioactivity associated with  $^{125}\text{I}$ -labeled membranes specifically binds to F-actin on affinity columns.

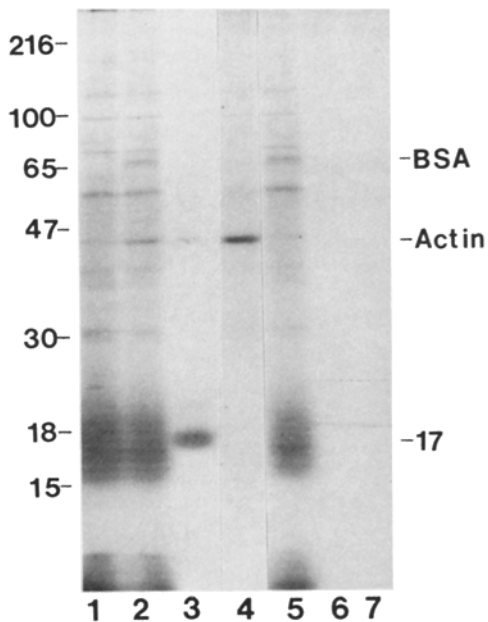
SDS-PAGE analysis of the peak fractions from F-actin and control columns shows that only a subset of OG pre-solubilized membrane proteins specifically bind to F-actin on columns (Fig. 3). The major actin-binding protein that is eluted from the F-actin column with SDS has a molecular mass of 17 kD. Smaller amounts of membrane proteins with molecular masses of 32, 40, and 100 kD also bind preferentially to F-actin columns. The 17-kD protein is also the major actin-binding protein in silver-stained gels of SDS-eluted fractions after actin affinity chromatography of unlabeled membranes. This observation indicates that the intensity of the 17-kD protein in autoradiographs is not due to selective labeling with Bolton-Hunter reagent. The appearance of the



**Figure 3.** Autoradiograph of a 6–16% polyacrylamide gradient SDS gel profile of  $^{125}\text{I}$ -labeled plasma membrane fractions from F-actin affinity and control columns such as those shown in Fig. 2. Membranes before affinity chromatography are shown in lane 1. Peak fractions from the F-actin column include the run-through (lane 2) and the SDS-eluted fraction (lane 3). The run-through and SDS-eluted fractions from the control column are shown in lanes 4 and 5, respectively.  $^{125}\text{I}$ -labeled molecular mass standards are shown in lane S. Numbers on the left refer to the molecular mass of the standards in kilodaltons. Numbers on the right refer to the molecular mass of the major proteins in lane 3. Lanes 1, 2, and 4 contained 7,500 cpm. Lane 3 contained 7,500 cpm in 125  $\mu\text{l}$  and lane 5 contained 2,500 cpm in 125  $\mu\text{l}$ . The total cpm loaded into each lane may not reflect the cpm incorporated into membrane proteins since many cpm are bound to phospholipids that are electrophoresed off the bottom of the gel. Film was exposed to the dried gel for 2 d.

17-kD protein in Con A-stabilized, Triton-extracted membranes is not qualitatively dependent on the membrane preparation technique because this protein also is isolated upon F-actin affinity chromatography of membranes prepared without Con A (Das and Henderson, 1983; Spudich, 1974). These less pure plasma membrane preparations contain several membrane proteins, in addition to the 17-kD protein, that bind specifically to F-actin columns. These proteins have molecular masses of 16, 19, 28, 32, 40, 68, and 100 kD (data not shown). It is not clear, however, whether these other actin-binding membrane proteins are localized to the plasma membrane and perhaps are excluded from Con A-stabilized regions of the membrane, or whether these proteins are localized to other membranous organelles that are contaminants in the plasma membrane preparation.

F-actin affinity chromatography was used further to explore the actin-binding activity of the 17-kD protein. Columns were eluted with a step gradient of NaCl to determine the sensitivity of the actin-binding activity of the 17-kD protein to ionic conditions. Although some 17-kD protein elutes from actin columns at NaCl concentrations as low as 200 mM, more efficient elution of the 17-kD protein is obtained with 2 M NaCl (data not shown). The 17-kD protein is virtu-

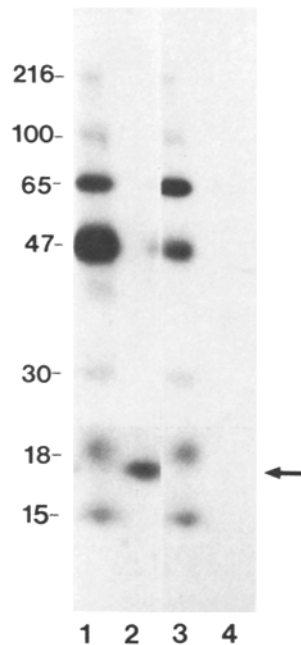


**Figure 4.** A silver-stained 10–20% polyacrylamide SDS gel profile of plasma membrane fractions from F-actin affinity and control columns. Lane 1 shows membranes before affinity chromatography. Peak fractions from the F-actin column include the run-through (lane 2), the fraction eluted with 2 M NaCl (lane 3), and the SDS-eluted fraction (lane 4). The run-through, NaCl-eluted fraction, and SDS-eluted fraction from the control column are shown in lanes 5–7, respectively. Each lane was loaded with 10  $\mu$ l. Numbers on the left refer to the molecular mass of Bethesda Research Laboratories prestained molecular mass standards.

ally the only membrane protein that is eluted from the actin column with 2 M NaCl (Fig. 4). Except for occasional traces of a 19-kD protein, the other membrane proteins remain bound to the actin column unless eluted with SDS. High NaCl concentrations do not irreversibly denature the actin-binding activity of the 17-kD protein since this protein will bind again to F-actin columns when the NaCl concentration is lowered (data not shown). Salt elution of F-actin columns allows partial purification of the 17-kD protein under non-denaturing conditions. We estimate that the 17-kD protein constitutes  $\sim$ 0.4–1.0% of the total membrane protein, based on the relative amount of radioactivity eluting with 2 M NaCl after F-actin affinity chromatography of  $^{125}$ I-labeled membranes.

The binding of the 17-kD protein to F-actin columns appears to be stable over a wide range of pH. The 17-kD protein does not elute from actin columns between pH 4.0 and 8.0. Trace amounts of the 17-kD protein are eluted from F-actin columns at pH 9.0. At pH 10.0, significant amounts of the 17-kD protein are eluted from F-actin columns (data not shown).

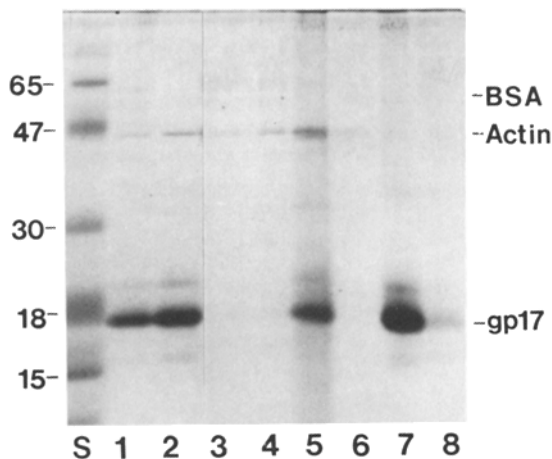
The orientation in the membrane of the 17-kD protein was explored using F-actin affinity chromatography of membranes isolated from cells that had been surface-labeled with SNHS-biotin. On protein blots of the NaCl-eluted fraction, avidin specifically binds to 17-kD protein isolated from surface-biotinylated membranes but not to 17-kD protein isolated from nonlabeled membranes (data not shown). Since the SNHS-biotin label cannot penetrate the cell membrane



**Figure 5.**  $^{125}$ I-Con A-stained nitrocellulose blots of a 10–20% polyacrylamide SDS gel containing 25  $\mu$ l of the NaCl-eluted fraction from F-actin affinity columns.  $^{125}$ I-labeled molecular mass standards are shown in lanes 1 and 3. Con A-binding proteins were visualized by incubation of blots with  $^{125}$ I-Con A in the absence (lane 2) and presence (lane 4) of  $\alpha$ -methyl-D-mannoside. Film was exposed to the nitrocellulose blots for 2 d. The arrow denotes the position of gp17.

(Ingalls et al., 1986; Goodloe-Holland and Luna, 1987), this result indicates that a domain of the 17-kD protein is exposed to the extracellular surface of the membrane. A second line of evidence supporting the conclusion that a domain of the 17-kD protein is exposed extracellularly comes from protein blots of the NaCl-eluted fraction probed with Con A (Fig. 5). The 17-kD protein binds Con A in the absence (Fig. 5, lane 2), but not in the presence (Fig. 5, lane 4), of the competing sugar  $\alpha$ -methyl-D-mannoside. We interpret this result to mean that the 17-kD protein is glycosylated and henceforth will refer to this protein as gp17. Fig. 5 also shows that no other prominent Con A-binding glycoproteins are found in the NaCl-eluted fraction, a further indication of the relative purity of this fraction.

The binding of gp17 to F-actin was characterized using F-actin affinity chromatography of membranes subjected to various pretreatments (Fig. 6). gp17 can be isolated on F-actin columns from NaOH-extracted membranes (Fig. 6, lane 2) and therefore is considered to be an integral component of the membrane. As in the case of actin binding to intact plasma membranes, the actin-binding activity of gp17 is sensitive to denaturation. gp17 is not isolated on F-actin columns from pre-solubilized membranes that have been denatured either with heat (Fig. 6, lane 3) or by treatment with DTT and NEM (Fig. 6, lane 4). Since gp17 also is not recovered from denatured membrane vesicles solubilized after incubation with F-actin beads (data not shown), the absence of gp17 from the NaCl-eluted fraction must be due to loss of its actin-binding activity and not due to changes in its solubility properties upon denaturation. The actin-binding activity of gp17 also is sensitive to protease treatment of the membranes. In contrast to F-actin affinity chromatography of column-solubilized control membranes (Fig. 6, lane 5), chromatography of proteolyzed membrane vesicles yields no detectable gp17 when proteolyzed membranes are solubilized after incubation with F-actin beads (Fig. 6, lane 6). However, when compared with presolubilized control membranes (Fig. 6, lane 7), trace amounts of gp17 are isolated on F-actin columns



**Figure 6.** The binding of gpl7 from pre-treated membranes to F-actin affinity columns. gpl7 was visualized on a silver-stained 15–20% polyacrylamide SDS gel containing 40  $\mu$ l of the NaCl-eluted fraction from F-actin affinity chromatography of: untreated plasma membranes (lane 1), NaOH-extracted membranes (lane 2), presolubilized heat-denatured membranes (lane 3), membranes treated with DTT and NEM and presolubilized (lane 4), column-solubilized proteolysis control (lane 5), column-solubilized proteolyzed membranes (lane 6), presolubilized proteolysis control (lane 7), and presolubilized proteolyzed membranes (lane 8). Bethesda Research Laboratories prestained molecular mass standards are shown in lane S. Numbers on the left refer to the molecular mass of the standards in kilodaltons.

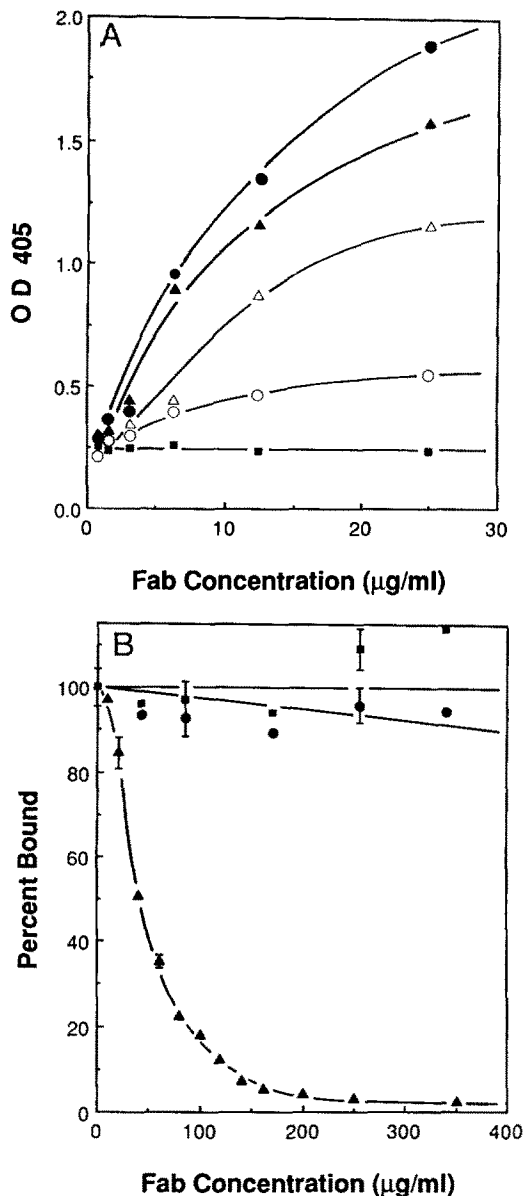
from protease-treated membranes that are solubilized before incubation with F-actin beads (Fig. 6, lane 8). This is most likely due to the release of nonproteolyzed gpl7 from sealed membrane vesicles upon detergent solubilization.

### Immunological Analysis of Actin–Membrane Binding

**Characterization of Antigens.** Two antigens were used to raise polyclonal antibodies against integral membrane actin-binding proteins: NaOH-extracted membranes and NaOH-extracted membranes that were further treated with NaIO<sub>4</sub> to alter or remove highly antigenic carbohydrate residues.

NaOH-extracted membranes bind F-actin and contain relatively few major membrane proteins (Luna et al., 1984). However, since many of these membrane proteins are glycosylated, we anticipated that the majority of the immune response might be directed against the highly immunogenic carbohydrate residues (Knecht et al., 1984). Therefore, periodate oxidation of NaOH-extracted membranes was explored as a means to deglycosylate membrane proteins. Since these antibodies were to be used to inhibit actin–membrane binding, it was important to verify that NaIO<sub>4</sub>-treated membranes retained actin-binding activity.

The successful removal of carbohydrate residues from membrane proteins by periodate oxidation was assayed by SDS-PAGE and by <sup>125</sup>I-Con A staining of nitrocellulose blots (data not shown). Treatment with NaIO<sub>4</sub> eliminates the binding of <sup>125</sup>I-Con A to membrane proteins, indicating that the carbohydrate residues of these proteins have been cleaved. Periodate oxidation also results in a lowering of the molecular mass of many membrane proteins. This is due, at least in part, to the removal of carbohydrate residues from



**Figure 7.** (A) ELISA analysis of the titer against plasma membranes of Fab prepared from the following: anti-NaOH-PM (●), anti-NaIO<sub>4</sub>-PM (▲), anti-NaIO<sub>4</sub>-PM after adsorption against glutaraldehyde-fixed cells (△), anti-NaOH-PM after adsorption against glutaraldehyde-fixed cells (○), and preimmune IgG (■). (B) Inhibition of binding of <sup>125</sup>I-labeled actin to membranes by anti-NaOH-PM (●), anti-NaIO<sub>4</sub>-PM (▲), and preimmune (■) Fab.

membrane proteins, but also may be caused by cleavage of the peptide backbone of these proteins at the amino acid residues, serine and threonine (Dyer, 1956). However, periodate oxidation does not destroy the actin-binding activity of membranes since NaIO<sub>4</sub>-treated membranes competitively inhibit the binding of <sup>125</sup>I-labeled membrane vesicles to F-actin beads in low-speed sedimentation assays (data not shown).

**Characterization of Antisera.** Adsorption experiments suggest that the antiserum raised against NaOH-extracted plasma membranes (anti-NaOH-PM) is directed predominantly against cell surface determinants, whereas the antiserum raised against plasma membranes treated with both

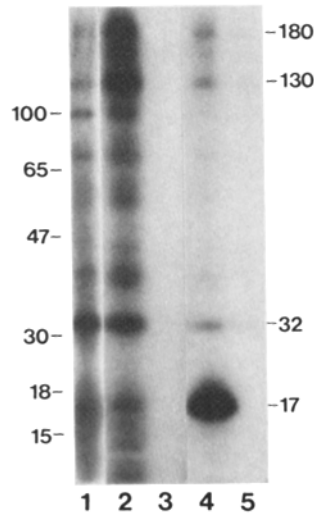


NaOH and NaIO<sub>4</sub> (anti-NaIO<sub>4</sub>-PM) recognizes determinants on both the extracellular and the cytoplasmic surfaces of the membrane (Fig. 7 A). After adsorption against glutaraldehyde-fixed cells, the titer against plasma membranes of anti-NaOH-PM is reduced by ~92%. From this result, we estimate that only ~8% of the plasma membrane-specific anti-NaOH-PM antibodies recognize determinants on the cytoplasmic surface of the membrane. In contrast, the titer against plasma membranes of anti-NaIO<sub>4</sub>-PM is reduced by ~33% after adsorption against glutaraldehyde-fixed cells. Therefore, we estimate that as much as 67% of the plasma membrane-specific anti-NaIO<sub>4</sub>-PM antibodies recognize determinants on the cytoplasmic surface of the membrane. The alternative explanation, that the antisera recognize glutaraldehyde-sensitive sites, is unlikely since adsorption against glutaraldehyde-fixed membranes reduces the titers against plasma membranes of both anti-NaOH-PM and anti-NaIO<sub>4</sub>-PM to preimmune levels (data not shown). These results indicate that the determinants recognized by anti-NaIO<sub>4</sub>-PM and anti-NaOH-PM are not destroyed by glutaraldehyde fixation.

**Actin-Membrane Binding Inhibition by Fab.** Fab from anti-NaOH-PM and anti-NaIO<sub>4</sub>-PM antisera were used to inhibit actin-membrane binding in sedimentation assays. Fig. 7 B shows that anti-NaIO<sub>4</sub>-PM Fab inhibit actin-membrane binding by 96%, whereas anti-NaOH-PM Fab inhibit binding by only 0-10% at comparable Fab concentrations. This difference is more striking in light of the observation that anti-NaOH-PM has a higher titer against plasma membranes than does anti-NaIO<sub>4</sub>-PM, as measured in an ELISA (Fig. 7 A). These results indicate that it is not the titer of the antisera against plasma membranes, but rather the specificity of the antisera that is important for the observed inhibition of actin-membrane binding.

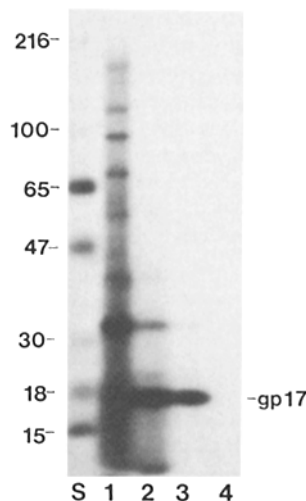
**Specificity of Anti-NaOH-PM and Anti-NaIO<sub>4</sub>-PM.** The specificity of anti-NaOH-PM and anti-NaIO<sub>4</sub>-PM was determined by immunoprecipitating <sup>125</sup>I-labeled plasma membrane proteins (Fig. 8). Anti-NaOH-PM IgG immunoprecipitates many membrane proteins, possibly due to cross-reactive carbohydrate determinants. In contrast, anti-NaIO<sub>4</sub>-PM IgG immunoprecipitates predominantly one protein with a molecular mass of 17 kD. This 17-kD protein is almost certainly the same protein first identified using F-actin affinity chromatography since anti-NaIO<sub>4</sub>-PM IgG selectively immunoprecipitates gp17 from a mixture of all the membrane proteins eluting from F-actin columns with SDS (Fig. 9).

**Characterization of the Actin-binding Site of gp17.** The titers of anti-NaOH-PM and anti-NaIO<sub>4</sub>-PM Fab against partially purified gp17 were measured by ELISA (Fig. 10 A). Although both anti-NaOH-PM and anti-NaIO<sub>4</sub>-PM Fab exhibit a substantial titer against partially purified gp17, the titer of anti-NaIO<sub>4</sub>-PM Fab is approximately sixfold higher than that of anti-NaOH-PM Fab. After exhaustive adsorption against glutaraldehyde-fixed cells, anti-NaIO<sub>4</sub>-PM Fab maintains a high titer against partially purified gp17 while the titer of anti-NaOH-PM Fab drops to preimmune levels. In addition, anti-NaIO<sub>4</sub>-PM IgG still immunoprecipitates gp17 after extensive adsorption against glutaraldehyde-fixed cells (data not shown). These results suggest that anti-NaOH-PM recognizes mostly extracellular determinants of gp17, whereas anti-NaIO<sub>4</sub>-PM also recognizes cytoplasmic determinants of gp17.

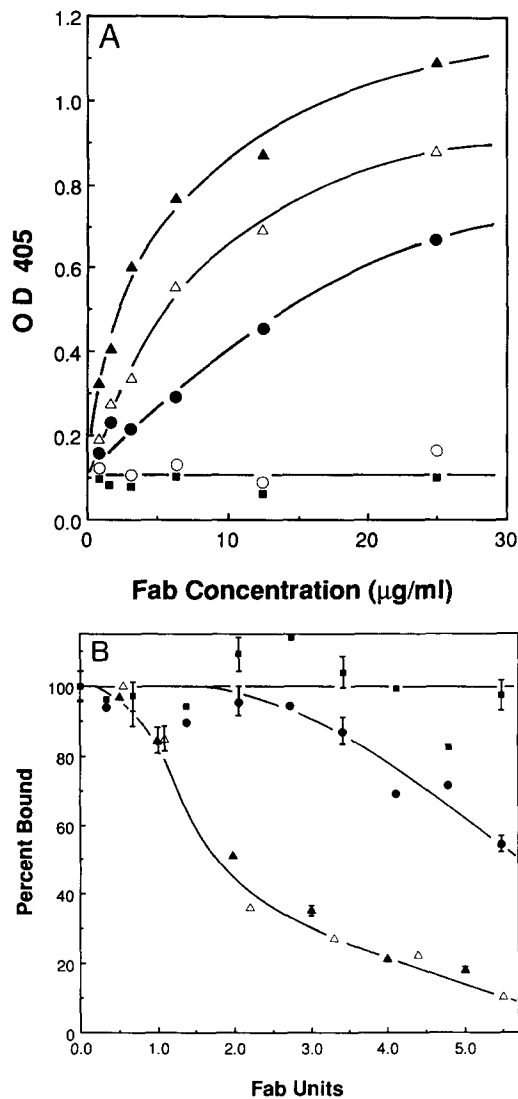


**Figure 8.** Autoradiograph of a 6-16% polyacrylamide SDS gel profile of <sup>125</sup>I-labeled membrane proteins before immunoprecipitation (lane 1) and after immunoprecipitation with IgG prepared from the following: anti-NaOH-PM (lane 2), preimmune anti-NaOH-PM (lane 3), anti-NaIO<sub>4</sub>-PM (lane 4), and preimmune anti-NaIO<sub>4</sub>-PM (lane 5). Numbers on the left refer to the molecular masses of <sup>125</sup>I-labeled standards in kilodaltons. Numbers on the right refer to the molecular mass of the major membrane proteins in lane 4. Each lane was loaded with 3,000 cpm or 100  $\mu$ l, whichever was the lesser amount. Film was exposed to the dried gel for 6 d.

When compared on the basis of equivalent titer against partially purified gp17, anti-NaIO<sub>4</sub>-PM Fab and anti-NaOH-PM Fab show significant differences in the ability to inhibit actin-membrane binding (Fig. 10 B). Although anti-NaOH-PM Fab will inhibit actin-membrane binding, much higher amounts of anti-NaOH-PM Fab are required to achieve levels of inhibition comparable to those observed with anti-NaIO<sub>4</sub>-PM Fab. Even after extensive adsorption against glutaraldehyde-fixed cells, relatively low concentrations of anti-NaIO<sub>4</sub>-PM Fab can inhibit 96% of actin-membrane binding. These results demonstrate that Fab directed against the cytoplasmic surface of gp17 efficiently inhibit actin-membrane binding and are consistent with the conclusion that the high-affinity actin-binding site of gp17 is cytoplasmically located.



**Figure 9.** Autoradiograph of a 6-16% polyacrylamide SDS gel of immunoprecipitated <sup>125</sup>I-labeled membrane proteins recovered from an F-actin column. Membrane proteins before F-actin affinity chromatography are shown in lane 1. Actin-binding proteins in the SDS-eluted peak fraction from an F-actin column before precipitation (lane 2) and after precipitation with IgG prepared from the following: anti-NaIO<sub>4</sub>-PM (lane 3) and preimmune anti-NaIO<sub>4</sub>-PM (lane 4). <sup>125</sup>I-labeled molecular mass standards are shown in lane 5. Lane 1 contains 15,000 cpm; lane 2 contains 7,000 cpm; lane 3 contains 1,500 cpm in 125  $\mu$ l; and lane 4 contains 125  $\mu$ l. Film was exposed to the dried gel for 1 d.



**Figure 10.** ELISA analysis of the titer against partially purified gp17 of Fab prepared from the following: anti-NaIO<sub>4</sub>-PM before (▲) and after (△) adsorption against glutaraldehyde-fixed cells, anti-NaOH-PM before (●) and after (○) adsorption against glutaraldehyde-fixed cells, and preimmune IgG (■). (B) Inhibition of actin-membrane binding by anti-NaOH-PM (●), anti-NaIO<sub>4</sub>-PM before (▲) and after (△) adsorption against glutaraldehyde-fixed cells, and preimmune (■) Fab. Fab units are based on the concentration of Fab required to give equivalent titer against partially purified gp17. One Fab unit is equivalent to: 100 µg/ml anti-NaIO<sub>4</sub>-PM Fab; 220 µg/ml anti-NaIO<sub>4</sub>-PM Fab after adsorption against cells; 620 µg/ml anti-NaOH-PM Fab. The values of Fab units were obtained by comparing the slopes from the linear sections of the curves shown in Fig. 11 A. As a control, preimmune Fab were included at a concentration of 620 µg/ml per Fab unit. Anti-NaOH-PM Fab that had been adsorbed against cells were not included in this assay since, after adsorption, no titer against gp17 was detected.

## Discussion

In this report, we present evidence that a 17-kD integral glycoprotein (gp17) is responsible for much of the F-actin binding activity of purified *D. discoideum* plasma membranes. Our results show that gp17 is the major F-actin binding protein isolated from OG-solubilized membranes on F-actin

affinity columns (Figs. 3 and 4). The actin-binding activity of gp17 has the same characteristics observed for purified plasma membranes: it resists extraction with 0.1 N NaOH, 1 mM DTT; it is sensitive to ionic conditions; it is stable over a wide range of pH; and it is eliminated by proteolysis, heat-denaturation, or treatment with DTT and NEM (Figs. 1 and 6). Since the actin-binding activity of gp17 persists in the presence of high concentrations of detergent, the actin-binding site of the protein is not hydrophobic. Rather, the salt-sensitivity of the actin-gp17 binding strongly suggests an electrostatic interaction.

gp17 may be the primary link between actin filaments and the plasma membrane since Fab directed predominantly against gp17 inhibit ~96% of actin-membrane binding. In contrast, Fab directed primarily against cell surface determinants inhibit binding by only 0-10% (Fig. 7 B). The high-affinity actin-binding site of gp17 appears to be located on the cytoplasmic surface of the membrane since Fab directed against this protein continue to inhibit 96% of actin-membrane binding even after extensive adsorption against glutaraldehyde-fixed cells (Fig. 10 B). A transmembrane orientation of gp17 is suggested since, in addition to the cytoplasmic localization of the actin-binding site, extracellular determinants of gp17 are identified. gp17 is glycosylated since it is specifically bound by Con A (Fig. 5), a lectin that binds to  $\alpha$ -mannopyranosides and glucopyranosides (Goldstein et al., 1974). Also, gp17 is surface-labeled by SNHS-biotin, a reagent that is restricted to the extracellular surface of the membrane. These results demonstrate that a domain of gp17 is extracellularly exposed. Since gp17 appears to be an important connection between the plasma membrane and the underlying microfilament network, we have named it "ponticulin," from the Latin word, *ponticulus*, which means small bridge.

Although the literature describing cytoplasmic and high molecular mass membrane proteins associated with actin is extensive (reviewed by Pollard and Cooper, 1986), only recently have low molecular mass actin-binding membrane proteins been recognized. While ponticulin is the first integral actin-binding protein to be isolated from plasma membranes, other low molecular mass actin-binding proteins in *D. discoideum* membranes have been identified. Schleicher et al. (1984) have identified a 17-kD protein that binds <sup>125</sup>I-G-actin in gel overlays. However, unlike ponticulin, their protein appears to be peripherally associated with the membrane since it is extracted with 1.5 M KCl. Stratford and Brown (1985) have described a 24-kD integral protein that binds glutaraldehyde-cross-linked F-actin on gel overlays. When purified, this protein also binds radiolabeled F- and G-actin in a two-phase binding assay which partitions proteins based on their hydrophobicity. It is not clear, however, whether this protein is localized to the plasma membrane since these investigators were working with a crude membrane preparation that also contains other membranous organelles.

Recently, Brown and Petzold (1987) have used polyclonal antibodies raised against crude *D. discoideum* membranes to inhibit actin-membrane interactions in viscosity and sedimentation assays. By adsorbing these antibodies against electrophoretically separated membrane proteins transferred to nitrocellulose, these investigators have shown that ~25% of the activity is adsorbed out by proteins with molecular masses between 15.5 and 18.5 kD and that ~40% of the activity is

adsorbed out by proteins with molecular masses between 18.5 and 22.5 kD. Although these investigators have attributed this inhibitory activity to a single protein with a mass of 20 kD, their results do not preclude the existence of several actin-binding proteins in the molecular mass region between 15.5 and 22.5 kD. Supporting the presence of multiple low molecular mass actin-binding proteins in crude membranes, we find that a crude membrane preparation similar to that used by Brown and Petzold contains at least three actin-binding proteins in this molecular mass region. Further investigation is necessary to explore any structural relationship between ponticulin and the protein(s) in the molecular mass region defined by Brown and Petzold.

We do not yet know how ponticulin interacts with other plasma membrane proteins or how the actin-binding activity of ponticulin is controlled. Considering its small size, we feel that ponticulin probably does not act as a complete transmembrane signaling unit. We propose that ponticulin may function as an intermediary protein such as the integral membrane protein, *x*, postulated by Bourguignon and Singer (1977). By binding to other membrane proteins and by providing a membrane attachment site for actin, ponticulin may act to transduce signals from one or more membrane receptors to the contractile elements of the cytoskeleton. Future studies will focus on further characterizing the actin-binding activity of ponticulin in vitro and in vivo.

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