The integral membrane protein, ponticulin, acts as a monomer in nucleating actin assembly

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Abstract. Ponticulin, an F-actin binding transmembrane glycoprotein in *Dictyostelium* plasma membranes, was isolated by detergent extraction from cytoskeletons and purified to homogeneity. Ponticulin is an abundant membrane protein, averaging $\sim 10^6$ copies/cell, with an estimated surface density of $\sim 300$ per $\mu m^2$. Ponticulin solubilized in octylglucoside exhibited hydrodynamic properties consistent with a ponticulin monomer in a spherical or slightly ellipsoidal detergent micelle with a total molecular mass of $56 \pm 6$ kD.

Purified ponticulin nucleated actin polymerization when reconstituted into *Dictyostelium* lipid vesicles, but not when a number of commercially available lipids and lipid mixtures were substituted for the endogenous lipid. The specific activity was consistent with that expected for a protein comprising $0.7 \pm 0.4\%$, by mass, of the plasma membrane protein. Ponticulin in octylglucoside micelles bound F-actin but did not nucleate actin assembly. Thus, ponticulin-mediated nucleation activity was sensitive to the lipid environment, a result frequently observed with transmembrane proteins. At most concentrations of *Dictyostelium* lipid, nucleation activity increased linearly with increasing amounts of ponticulin, suggesting that the nucleating species is a ponticulin monomer. Consistent with previous observations of lateral interactions between actin filaments and *Dictyostelium* plasma membranes, both ends of ponticulin-nucleated actin filaments appeared to be free for monomer assembly and disassembly. Our results indicate that ponticulin is a major membrane protein in *Dictyostelium* and that, in the proper lipid matrix, it is sufficient for lateral nucleation of actin assembly. To date, ponticulin is the only integral membrane protein known to directly nucleate actin polymerization.

Membrane-associated actin assembly has been proposed as the driving force behind many motile processes, including pseudopod extension (1, 22, 94, 96), microvillus elongation (64), translocation of cell surface components (35, 43), and the intracellular spread of invasive bacteria (26, 95). Newly assembled actin filaments become associated with the cell periphery within 2 to 6 s after chemotactic stimulation of neutrophils and *Dictyostelium* amebas (22, 29, 68), and actin assembly at the cytoplasmic membrane surface has been observed in living cells (34, 67, 89, 90, 94, 99).

Membrane-mediated actin assembly has been characterized in vitro using purified plasma membranes from the cellular slime mold, *Dictyostelium discoideum* (39, 59). These membranes both bind F-actin and nucleate actin polymerization at the membrane surface (57, 77, 78, 80). Binding is specific, saturable, rapid, and of high avidity, with estimated $K_d$'s in the submicromolar range (47, 77, 88). Actin assembly appears to involve the generation of membrane-associated actin trimers (78) with both the barbed and pointed ends free to elongate (80). A 17-kD glycoprotein called ponticulin has been implicated in both actin binding and nucleation although the nature of the nucleating species is unknown (58, 80, 103).

Ponticulin apparently serves as a transmembrane link between the cell surface and the cytoskeleton. Extracellular sites have been identified by surface labeling of intact cells and Con A binding (103). A cytoplasmic domain is indicated by the continued recognition of ponticulin by antibody that has been exhaustively adsorbed against intact cells (103). Ponticulin appears to be responsible for most of the basal actin-binding activity of *Dictyostelium* plasma membranes because 96% of the actin-membrane binding in sedimentation assays is inhibited by univalent antibody fragments against the cytoplasmic domain (103). The plasma membrane localization and cytoskeletal association of ponticulin in *Dictyostelium* amebas and human neutrophils also have been demonstrated using immunofluorescence microscopy (104).

Direct binding between ponticulin and F-actin has been shown by F-actin affinity chromatography (103) and on 125I-
other synthetic phospholipids were purchased from Sigma Chem. Co. (St. Louis, MO). TX-114 (TX-114), deuterium oxide (D2O), DTT, N-ethyl-3-(3-dimethylaminopropyl) carbodiimide, dimyristoyl L-alpha-phosphatidylcholine (DMPC) 1, and lipoproteins were from Pierce Chem. Co. (Rockford, IL). Bio-Beads were prepared by the method of Holloway et al. (28, 29). The cytoskeletal pellet was resuspended in cytoskeletal buffer (2x CSK; 20 mM sodium phosphate, pH 6.1, and washed once with 20 mM sodium phosphate, pH 6.8 (PB). Cells were concentrated in PB to 2 × 10^6 cells/ml and extracted with an equal volume of 2% (vol/vol) TX-114 in 2x cytoskeletal buffer (2x CSK; 20 mM KC1, 20 mM EGTA, 20 mM imidazole, 4 mM MgC12, pH 7.0) (62). The mixture was incubated for 10 min on ice and then for 10 min at room temperature. Cytoskeletal pellets were recovered as a pellet after centrifugation in a swinging bucket rotor for 4 min at 11,000 g.

**Purification of Ponticulin**

**Preparation of Cytoskeletons.** D. discoideum amebas (strain K-A3X from Dr. Richard Kein, Columbia University) were grown in HL-5 medium (19) to >10^7 cells/ml. A total of 4 × 10^9 cells were used for analytical analyses. 1.5 × 10^9 cells were used in preparative experiments. All centrifugation steps were carried out at 4°C. Cells were harvested by pelleting for 2 min at 400 g, washed twice with 14.6 mM KH2PO4, 2.0 mM Na2HPO4, pH 6.1, and washed once with 20 mM sodium phosphate, pH 6.8 (PB). Cells were concentrated in PB to 2 × 10^6 cells/ml and extracted with an equal volume of 2% (vol/vol) TX-114 in 2x cytoskeletal buffer (2x CSK; 20 mM KC1, 20 mM EGTA, 20 mM imidazole, 4 mM MgC12, pH 7.0) (62). The mixture was incubated for 10 min on ice and then for 10 min at room temperature. Cytoskeletal pellets were recovered as a pellet after centrifugation in a swinging bucket rotor for 4 min at 11,000 g.

**Isolation of Ponticulin from Cytoskeletons.** The cytoskeletal pellet was resuspended to the initial cell suspension volume by homogenization into 2 M NaCl, 1x CSK, 1% TX-114, 1 μM phallolidin. After 30 min of slow rotary mixing at room temperature, the suspension was centrifuged in an SW41 rotor (Beckman Instruments, Inc., Fullerton, CA) at 4°C for 45 min at 28,000 rpm (100,000 x g). The mixture separated into a buoyant detergent phase, a denser high salt aqueous layer, and a pellet (extracted cytoskeletons). This pellet was reextracted as described above. The two detergent layers were pooled before TX-114 removal.

**Removal of TX-114.** Bio-Beads were prepared by the method of Holloway et al. (45) and stored in PB. The concentration of TX-114 was determined by reading the OD275 of the sample and using the extinction coefficient for the structurally similar TX-100 (ε = 21) (45). 1 g of Bio-Beads was sufficient to remove 70 mg of TX-114. The detergent layer was mixed gently with Bio-Beads at 4°C for 30 min. This lowered the TX-114 concentration to ~1%. Immediately after incubation, enough 30% (vol/vol) OG in column buffer (CB; 20 mM KC1, 1 mM MgC12, 20 mM Tris-acetate, pH 7.0) was added to make the detergent layer 3% OG. This solution was mixed gently at 4°C overnight.

**F-Actin Affinity Chromatography.** The F-actin affinity matrix was prepared as described (105). A 1-ml column of actin beads was prepared in a 5-ml syringe. The beads were preeluted with 5 ml of 2 M NaCl, 2 mM EGTA, 1% OG, 0.5 μM phallolidin, and equilibrated with 10 ml of 1% OG, 1 μM phallolidin. After concentration to >10 ml with a Centriprep-10 (Amicon, Beverly, MA), the detergent solution containing ponticulin and OG was dialyzed overnight at 4°C against 1% OG, CB. After centrifugation at 100,000 g for 30 min, the clarified detergent solution was slowly loaded onto an F-actin column at room temperature or slowly rotated end-over-end with the actin beads in a plastic tube for 1 h at 4°C. Beads and sample were returned to the column and the unbound fraction (run-through) was collected. The beads were washed with 10 ml of 1% OG, 0.5 μM phallolidin, CB, and the ponticulin-containing fraction was eluted with 5 ml of 2 M NaCl, 2 mM EGTA, 1% OG, 0.5 μM phallolidin, CB. The beads were resuspended, reincubated with the run-through fraction, and eluted as above once or twice more. (With freshly prepared F-actin beads, two incubations and NaCl elutions were sufficient to remove all ponticulin from the run-through; older beads sometimes required a third round.) The NaCl eluates were pooled and concentrated to ~2 ml in a Centricon-10 (Amicon). The concentrated NaCl eluates were dialyzed against 1% OG, CB at 4°C for at least 24 h.

**High Pressure Liquid Chromatography Gel Filtration.** Ponticulin was gel filtered using two Bio-Sil TSK-125 columns, each 300 × 7.5 mm (Bio-Rad Laboratories), mounted in series on an Ultro Chrom GTI Bioseparation System (Pharmacia LKB Biotechnology, Piscataway, NJ). The system was calibrated with Bio-Rad gel filtration standards. A ponticulin-enriched frac-

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1. Abbreviations used in this paper: CB, column buffer (50 mM KCl, 1 mM MgCl2, 20 mM Tris-acetate, pH 7.0); DMPC, dimyristoyl L-alpha-phosphatidylcholine; HIC, hydrophobic interaction chromatography; OG, octylglycoside; PB, 20 mM sodium phosphate buffer, pH 6.8; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TX-114, Triton X-114.
tion (typically 30 μg), obtained by concentration and dialysis of the NaCl-eluted fraction from the actin-affinity matrix, was chromatographed in 1% OG in CB, pH 7.0, at a flow rate of 0.5 ml/min. The volumes of collected fractions were 1.5 ml (fractions 1-5); 0.2 ml (fractions 6-60); and 0.5 ml (fractions 61-75). The total elution volume was 22 ml. The void volume was 10.2 ml.

Hydrophobic Interaction Chromatography (HIC). High pressure HIC was performed at ambient temperature using a TSK Phenyl-SPW column (8 x 75 mm; Pharmacia LKB Biotechnology) equilibrated with 10 column volumes (38 ml) of 0.7% OG, 20 mM Pipes, pH 7.0. A ponticulin-enriched fraction (65 μg in 100 μl), obtained by concentration of the NaCl-eluted fraction from the actin-affinity matrix, was dialyzed extensively at 0-4°C against 1.5% OG, CB. The dialyzed ponticulin was purified with an equal volume of 20 mM Pipes, pH 7.0, centrifuged through a Spin-X® filter unit (Costar Corp., Cambridge, MA), and immediately loaded onto the HIC column at a flow rate of 0.1 ml/min. The column was washed with 3.8 ml of 0.7% OG, 20 mM Pipes, pH 7.0, and bound protein was eluted at 0.2 ml/min with 7.6 ml of a gradient of 0.7-1% OG in 20 mM Pipes, pH 7.0. Approximately 60 fractions of 200 μl each were collected. Portions of HIC-purified ponticulin were iodinated with 125I-3'-Bolton-Hunter reagent (New England Nuclear, Boston, MA) in 100 mM sodium phosphate, pH 8.0, to a specific activity of ~3 μCi/μg ponticulin.

SDS-PAGE and Protein Blotting. Samples were denatured at 70°C for 10 min, run on polyacrylamide gradient (10-20%) SDS slab gels using the discontinuous system of Laemmli (53), electrophoretically transferred to nitrocellulose (97), probed with antibody against ponticulin (R67 IgG), and visualized by incubation with 125I-protein A (New England Nuclear) as described previously (16, 104). Prestained molecular mass markers (BRL, Gaithersburg, MD) were lysozyme (15 kD), β-lactoglobulin (18 kD), carbonic anhydrase (29 kD), ovalbumin (46 kD), BSA (69 kD), phosphorylase B (107 kD), and myosin (224 kD). Gels were silver stained according to the method of Merril et al. (63).

Protein Determinations. Protein concentrations were determined in the presence of 1% SDS with the room temperature protocol of the BCA Protein Assay (Pierce Chem. Co.), using BSA as the standard. The amounts of purified ponticulin also were determined by PTC amino acid analyses (31) (see below).

Quantification of Ponticulin

Enrichment during Purification. Various amounts (~0.03 to 90 μg) of whole cells, cytoskeletons, and fractions from the later steps of the purification scheme were analyzed for ponticulin content by immunoblotting as described above. Exposed film was scanned with a densitometer (model 222-020 Ultrascan XL; Pharmacia LKB Biotechnology, Inc., or pdi scanning densitometer, Huntington Station, NY), and the areas under the peaks were determined automatically. In some experiments, the autoradiogram was used as a template to excise the ponticulin signal at 17 kD, and the nitrocellulose pieces were counted in a gamma counter. Known amounts of HIC-purified ponticulin were analyzed simultaneously, generating a standard curve from which the amounts of ponticulin in the different fractions were determined. The results from these different methods generally were in close agreement.

Characterization of Purified Ponticulin

Amino Acid Analysis. HIC-purified ponticulin was absorbed to ProBlott® polyvinylidene difluoride membrane pieces (Applied Biosystems, Inc., Foster City, CA) as described (89). Briefly, 100 μl of ponticulin (5.5 μg) containing a trace amount of 125I-ponticulin were diluted into 1.8 ml of sterile filtered PBS (150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4) in silanized plastic tubes containing pieces of ProBlott® (previously wetted in 100% methanol followed by several rinses in PBS). After gentle agitation for 24 h at room temperature, the pieces of ProBlott® were washed seven times with filter-sterilized water. The washed ProBlott® pieces and recovered PBS supernatant were counted to determine the percentage of adsorbed ponticulin, and the ProBlott® pieces were stored at -20°C until analysis. Amino acid compositions of the adsorbed protein were determined using standard techniques (6, 21, 107). Amino acid compositions also were obtained for ponticulin purified by preparative SDS-PAGE and electrophoresed to polyvinylidene difluoride membranes.

Covalent Actin-Binding Assays. Actin was polymerized at room temperature by adding 1 vol of 5X polymerization buffer (200 mM KCl, 100 mM Pipes, 10 mM MgCl₂, 250 μM CaCl₂, pH 7.0) to 4 vol of actin (1-4 mg/ml) in buffer A and stored on ice. 12-18 h before use, the F-actin was dialyzed at 4-6°C against 300-400 vol of 1X polymerization buffer. 125I-Ponticulin was diluted into 1.5% OG in CB and clarified at room temperature in an airfuge (178,000 g for 20 min) (Beckman Instrs., Inc.). Each assay (~250 μl final volume) contained 50 μg actin, ~65 ng ~125I-ponticulin, 20 μg BSA, ~1% OG, 13 mM Tris, 7 mM Pipes, 50 mM KCl, 2 mM MgCl₂, and 50 mM NaCl, pH 7.0. After 15 min incubations were done on an ice bath. After a 15 min incubation, the assay mixture was centrifuged at room temperature in an airfuge (78,000 g for 20 min; Beckman Instrs., Inc.). The supernatant was collected by aspiration and the pellet was washed carefully with 250 μl CB to remove traces of the supernatant. The supernatant, rinse, and pellet were counted, and the supernatant and pellets were analyzed by SDS-PAGE. The rinses generally contained only a few percent of the total counts and were disregarded. Dried gels were exposed to film at ~85°C in the presence of an intensifying screen or at room temperature.

Hydrodynamic Properties

Stokes Radius. The Stokes radius of HIC-purified ponticulin was determined at room temperature using both high pressure and conventional gel filtration chromatography. The Bio-Rad gel filtration standards (molecular mass; Stokes radius, Rs) used in the HFLC experiments were bovine IgG (158 kD; 5.1 μm), ovalbumin (43 kD; 2.8 μm), and myoglobin (17 kD; 1.9 μm). Conventional gel filtration chromatography employed a Sephadex G-100 column (17.5 x 0.75 cm) equilibrated with 2% OG in CB. A trace amount of 125I-ponticulin was added as an aid in determining the elution position of ponticulin relative to the following molecular mass standards (molecular mass; Stokes radius, Rs): cytochrome c (12.3 kD; 1.7 μm), myoglobin (17 kD; 1.9 μm), chymotrypsigen (23.7 kD; 2.2 μm), ovalbumin (43 kD; 2.8 μm), and lactoperoxidase (92.6 kD; 3.6 μm). Column fractions were collected and then analyzed by SDS-PAGE. Most Stokes radii were from Le Maire et al. (54) and Tanford et al. (93); Rs for lactoperoxidase was calculated using a diffusion coefficient (D = 10 x 10⁻⁹ cm² s⁻¹) of 5.95 (84) in the equation, Rs = kD/6πηuD (83).

Sedimentation Coefficient and Partial Specific Volume (v*). The standard proteins used in sedimentation analyses and the accepted values (84) for their Sedwerg coefficients (10 x 10⁻¹⁰) and partial specific volumes (v*) were as follows: cytochrome c (1.71 S; 0.728 cm²/g), myoglobin (2.04 S; 0.741 cm²/g), chymotrypsigen (2.54 S; 0.721 cm²/g), ovalbumin (3.55 S; 0.749 cm²/g), lactoperoxidase (5.37 S; 0.764 cm²/g), and aldolase (7.35 S; 0.742 cm²/g). HIC-purified ponticulin (2 μg) and the standard proteins (10 μg each) were run together on 5-20% sucrose gradients (prepared in 2% OG, CB, and either H2O or D2O) at 240,000 g for 18 h at 4°C and 25 fractions per gradient (200 μl each) were collected using a density gradient fractionator (model 640; ISCO, Lincoln, NE). Aliquots were analyzed by SDS-PAGE and ponticulin-containing fractions were determined by immunoblotting. The refractive index of each fraction was measured with a Bausch & Lomb refractometer (catalogue No. 33.460; Rochester, NY). As the refractive indices appeared to be unaltered by the detergent and buffer used, the percent of sucrose in each fraction and the density of each H2O-containing fraction were determined from standard tables (73, 100). Densities of D2O-containing fractions were obtained by linear interpolation between the density of the 5% sucrose (1.122 g/cm³) and the 20% sucrose (1.168 g/cm³) (18). Values for average distance sedimented, average density, average viscosity, and v* were determined for the standard proteins and ponticulin-OG micelles using the procedure and equations described in Clarke (17) and Clarke and Smigel (18). The molecular weight of the ponticulin-OG micelle was calculated from the equation (83, 91),
(47), and phospholipid content was estimated from the amount of organic solvent-extractable phosphorus (56). Lipids were dissolved in 1% OG, 20 mM Pipes, pH 7.0, to a final concentration of 1 mM phospholipid, sonicated briefly, and equilibrated for 12-16 h on ice. Lipid solutions then were clarified by centrifugation at room temperature in a Beckman Instrs. Inc. ultracentrifuge at 200,000 g for 20 min or at 4°C in a Beckman 42.2 Ti rotor (223,000 g for 30 min), and mixed with HIC-purified ponticulin in the same buffer. The ponticulin- lipid-detergent mixture (usually ~840-fold molar excess phospholipid per protein) was incubated at room temperature for 4-5 h and then diluted 10-15-fold into buffer. These dilutions lowered the OG concentration far below the critical micelle concentration (~0.7% (82)). Unless stated otherwise, vesicles contained 0.5 μg/ml ponticulin and 14 μM phospholipid. Vesicles containing D. discoideum lipid without ponticulin were prepared similarly.

**Vesicle Characterization**

**Electron Microscopy.** Vesicles of ponticulin and D. discoideum lipid were reconstituted as described above and collected by centrifugation at ~183,000 g° for 3.5 h at 5°C in a Beckman 50Ti rotor. The pellet was resuspended into 2% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.4, and compacted in two Beckman airfugè tubes at ~165,000 g° for 1 h at 20°C. The pellets were washed three times with buffer and postfixed with 2% Oso4, 0.1 M cacodylate, pH 7.4. After three more washes with buffer, the pellets were stained en bloc with 1% uranyl acetate, 70% ethanol, dehydrated in ethanol, and embedded in Poly/Bed 812 (Polysciences, Inc., Warrington, PA). Silver sections were cut both parallel and perpendicular to the axis of centrifugation with a Diatome diamond knife on a Reichert Ultracut E ultramicrotome. Grids were poststained with 4% uranyl acetate in methanol; 70% ethanol (1:1) for 30 min and with Reynold's lead citrate for 1 min.

**Sucrose Gradients.** Fluorescein PE (1 μM; 0.1% of the total) and 125I-ponticulin (<25 ng; ~0.2% of the total) were added as tracers to mixtures containing 1% OG, ponticulin, and DMPC or D. discoideum lipid (1 mM phospholipid). The mixtures then were diluted 10-fold with 20 mM Pipes, pH 7.0. After collection by centrifugation at 200,000 g for 3 h, vesicles (100-μl aliquots) were loaded onto 5-20% sucrose gradients containing 50 mM NaCl, 20 mM Pipes, pH 7.0, and centrifuged in a Beckman SW50.1 rotor (18 h, 45,000 rpm, 4°C). Gradient fractions (200 μl each) were collected with an ISCO density gradient fractionator (model 640) and counted in a gamma counter. 100-μl aliquots of each fraction were diluted into 700 μl of 1% SDS, 50 mM borate, pH 9.0, and their relative fluorescence was measured (Xr = 365 nm; λem = 407 nm). Background fluorescence was subtracted and the corrected numbers were normalized between 0 and 100%, with 100% denoting the fluorescence of each sample at the earliest measurable time point.

**Pyrene-Actin Assays**

**Actin Nucleation.** Reconstituted ponticulin vesicles and control vesicles were assayed for actin nucleation activity using the pyrene-actin fluorescence assay (23, 80). Vesicles, without fluorescein PE or radiolabel, were prepared in assay buffer (25 mM imidazole, 100 mM KCl, 2 mM MgCl2, 1% OG, 14 μM phospholipid, 14 μM phosphoponticulin) and sonicated briefly, and equilibrated for 12-16 h on ice. Lipid solutions then were clarified by centrifugation at room temperature in a Beckman Instrs. Inc. ultracentrifuge at 200,000 g for 20 min or at 4°C in a Beckman 42.2 Ti rotor (223,000 g for 30 min), and mixed with HIC-purified ponticulin in the same buffer. The ponticulin-lipid-detergent mixture (usually ~840-fold molar excess phospholipid over protein) was incubated at room temperature for 4-5 h and then diluted 10-15-fold into buffer. These dilutions lowered the OG concentration far below the critical micelle concentration (~0.7% (82)). Unless stated otherwise, vesicles contained 0.5 μg/ml ponticulin and 14 μM phospholipid. Vesicles containing D. discoideum lipid without ponticulin were prepared similarly.

**Results**

**Purification**

Ponticulin, an F-actin binding plasma membrane glycoprotein (16, 103) also is a component of the cytoskeleton (104). When log-phase D. discoideum amebas were extracted with 1% Triton X-100 in CSK buffer, 92.6 ± 4.0% (n = 3) of the ponticulin, as detected on immunoblots, pelleted with the cytoskeletons (data not shown). Trace amounts of 15- and 19-kD polypeptides, proteins that may be structurally related to ponticulin (104), also were observed in these Triton-insoluble residues.

We developed a protocol for the rapid purification of ponticulin that takes advantage of its cytoskeletal association, hydrophobic nature, and salt-sensitive binding to F-actin (103). The initial steps of this procedure are outlined in Fig. 1 (see also Materials and Methods). First, cytoskeletons are prepared by extracting whole cells with a cytoskeleton-stabilizing buffer containing TX-114. Solutions containing this detergent spontaneously form a TX-114-enriched hydrophobic phase and a TX-114–depleted aqueous phase when heated above 20°C, the cloud point of the detergent (38). The
As expected, ponticulin was greatly enriched in the detergent layers generated by this procedure (Fig. 2). A 43-kD polypeptide, probably actin, was prominent in the whole cell extract (Fig. 2 A, lane 1), relatively depleted in the initial TX-114 supernatant (Fig. 2 A, lane 2) and enriched in the TX-114-insoluble cytoskeletons (Fig. 2 A, lane 3). The TX-114 cytoskeletons also contained ~85% of the total cellular ponticulin (Table I; Fig. 2 B, lane 3). After phase separation, most of the cytoskeletal ponticulin usually was recovered in the first detergent layer (Fig. 2, A and B, lanes 4). However, appreciable amounts (sometimes most) of the ponticulin continued to pellet with the cytoskeletons (Fig. 2, A and B, lanes 6). Therefore, the cytoskeletal pellet was reextracted with 2 M NaCl and 1% TX-114, and additional ponticulin was recovered in the second detergent layer (Fig. 2, A and B, lanes 7). Very little ponticulin was found in either aqueous layer (Fig. 2, A and B, lanes 5 and 8), confirming the hydrophobic nature of this protein. Even with the difficulty of cleanly separating the detergent and aqueous layers, ponticulin was enriched ~58-fold in the combined detergent layers compared with the whole cell extract (Table I). The detergent layers were pooled and most of the TX-114 was exchanged with OG before F-actin affinity chromatography.

As much as one third of the cellular ponticulin always remained tightly bound to the cytoskeletons and was discarded (Fig. 2, A and B, lanes 9). Only two high salt extractions usually were performed because resuspension of the cytoskeletal pellets was difficult after repeated ultracentrifugations. Extractions with 4 M NaCl resulted in higher initial yields of ponticulin but the additional ponticulin aggregated irreversibly and final recovery was lower.

### Table I. Purification of Ponticulin

| Step            | Volume | Protein | Ponticulin | % Ponticulin of total protein | Step-fold purification | Total fold purification | Recovery |
|-----------------|--------|---------|------------|-------------------------------|------------------------|-------------------------|----------|
|                 | ml     | (in 10⁶ cells) | (in 10⁶ cells) | mg | mg | mg | % | % | % | % |
| Cells           | 69     | 505     | 0.33        | 0.065 | 1.0 | 1.0 | 100 |
| Cytoskeletons   | 69     | 48.5    | 0.28        | 0.58 | 8.9 | 8.9 | 85  |
| Pooled detergent layers | 19 | 4.68 | 0.18 | 3.8 | 6.6 | 58 | 54  |
| Concentrated NaCl eluate | 1.2 | 0.12 | 0.090 | 75 | 19.7 | 1150 | 27 |
| NaCl eluate     | 1.2 | 0.084 | 0.084 | >99 | 1.32 | 1523 | 25  |
| Gel Filtration  | 1.5 | 0.066 | 0.066 | >98 | 1.30 | 1508 | 20  |

* Data from a single analytical experiment. In replicate experiments, ponticulin was estimated to comprise 0.040% and 0.047% of the total cell protein, and the fold purification of the final, highly purified ponticulin was proportionately higher.

† Determined by BCA assay and normalized to 10⁶ cells.

‡ Determined by autoradiography and normalized to 10⁶ cells. Values for ponticulin in cells and cytoskeletons may be underestimates because the total signal at 17 kD increased during purification. Other 17-kD protein(s) in these crude fractions apparently interfered with the detection of ponticulin on immunoblots, especially at high protein loads (Fig. 2).
Ponticulin was separated from most other proteins in the pooled detergent layers by F-actin affinity chromatography. As observed with detergent-solubilized D. discoideum plasma membranes (103), a major 17-kD polypeptide in the detergent layers bound F-actin columns under physiological buffer conditions and was eluted with high salt (Fig. 2 C, lanes 4–6). Minor amounts of other proteins, including actin and 15-kD and 19-kD polypeptides, also eluted under these conditions. Immunoblotting with adsorbed R67 antibody identified the 17-kD polypeptide as ponticulin (Fig. 2 D). Essentially all of the ponticulin in the detergent layers was recovered after two or three successive passages through the F-actin column (Fig. 2 D, lanes 1–3). Ponticulin was enriched nearly 20-fold in the salt-eluted fractions relative to the pooled detergent layers (Table I).

Ponticulin was further purified by either gel filtration or hydrophobic interaction chromatography (Fig. 3). Because ponticulin has essentially no absorbance at 280 nm, fractions containing ponticulin were identified using SDS-PAGE. Both gel filtration and HIC yielded fractions containing a major 17-kD protein and minor 15-kD and 19-kD polypeptides (Fig. 3, lanes 1 and 2). The 17-kD polypeptide was identified as ponticulin by immunoblotting with absorbed R67 IgG (data not shown).

HIC was more efficient than gel filtration chromatography as a final purification step. Almost 93% of the initial ponticulin load was recovered after HIC in contrast to an 80% recovery after gel filtration (Table I). Based on the recovery of 84 μg of ponticulin per 10¹⁰ cells, as determined from amino acid analyses, a 1,520-fold purification of ponticulin was achieved. This degree of purification was slightly larger than the 1,508-fold purification calculated for gel-filtered ponticulin load was recovered after HIC in contrast to an 80% recovery after gel filtration (Table I). Based on the recovery of 84 μg of ponticulin per 10¹⁰ cells, as determined from amino acid analyses, a 1,520-fold purification of ponticulin was achieved. This degree of purification was slightly larger than the 1,508-fold purification calculated for gel-filtered ponticulin. Almost 93% of the initial ponticulin load was recovered after HIC in contrast to an 80% recovery after gel filtration (Table I).

The purity of HIC-purified ponticulin (Fig. 3, lane 2) was examined by radiodination followed by SDS-PAGE and autoradiography (Fig. 3, lanes 3 and 4). Although a number of contaminating proteins could be detected by this method, even overexposed films showed no signal at the position of actin (Fig. 3, lane 4).

As another test of purity, we compared the amino acid composition of HIC-purified ponticulin (Fig. 3, lanes 2–4) with the amino acid composition of ponticulin purified by preparative SDS-PAGE followed by electrotransfer to PVDF membranes (Table II; (105). The amino acid compositions obtained for ponticulin purified by these different methods were very similar (Table II). Ponticulin contains relatively large amounts of serine (17–20 mol %), asparagine + aspartate (10–12 mol %), alanine (~10 mol %), threonine (~9 mol %), and glycine (7–9 mol %). It has little or no methionine or histidine. Ponticulin's weak absorbance at 280 nm indicates that it probably does not contain tryptophan, which could not be measured after acid hydrolysis. Based on the amino acid composition (20), the partial specific volume, \( \nu^* \), of ponticulin was predicted to be 0.716 cm³/g (the inverse of a density of 1.40 g/ml), which is close to the average \( \nu^* \) observed for a wide range of proteins (14). This predicted value for \( \nu^* \) should be considered an upper estimate because oligosaccharides, known to be present on ponticulin (16, 103), may reduce \( \nu^* \) to 0.68–0.70 cm³/g (37, 79).

Amino acid analyses also were used to assess the accuracy of the BCA protein assay for ponticulin. Trace amounts of \(^{125}\)I-ponticulin were used to monitor the adsorption of the protein to the polyvinylidene difluoride membrane. Because 93.8 ± 1.8% (n = 4) of the radiolabeled protein was adsorbed and retained on the membranes through multiple washes, we assumed that ~94% of the unlabeled protein mass also was retained. A comparison of the amino acid analyses with the BCA assay results indicated that the BCA assay overestimated by a factor of two the protein concentration of highly purified ponticulin. Calculations for the concentrated NaCl eluate, HIC, and gel-filtered ponticulin take into account this twofold overestimate (Table I).

**Characterization of Purified Ponticulin**

HIC-purified ponticulin retained the ability to bind F-actin (Fig. 4). In sedimentation assays, at least 74% of freshly
Figure 4. HIC-purified ponticulin binds F-actin. Supernatants (S) and pellets (P) from sedimentation assays containing \(~15\) nM \(^{125}\)I-ponticulin with (lanes 1 and 2) and without (lanes 3 and 4) \(4.6\) 
\(\mu\)M F-actin. Samples were analyzed by SDS-PAGE and autoradiography. Arrow indicates the position of ponticulin; arrowheads indicate the positions of the 15- and 19-kD polypeptides. Film was exposed for 24 h.

purified, OG-solubilized \(^{125}\)I-ponticulin pelleted with F-actin (Fig. 4, lane 2). By contrast, only \(18 \pm 6\%\) \((n = 4)\) of the counts sedimented in the absence of F-actin (Fig. 4, lane 4). Therefore, at least half of the ponticulin appeared to specifically associate with actin. Unbound ponticulin may be in equilibrium with ponticulin bound to actin because OG-solubilized ponticulin in initial run-through fractions from F-actin columns bound actin in subsequent passages through this column (Fig. 2 D, lanes 1-3). If this is true, the dissociation constant for the ponticulin-actin interaction is \(~5\) \(\mu\)M. However, the stochastic aggregation of the \(^{125}\)I-ponticulin during these assays (Fig. 4, lane 4) confounded a precise determination of this value.

To determine the likely size and shape of ponticulin-detergent complexes, estimates of the partial specific volume \(\left(\nu^*\right)\) and the sedimentation coefficient \(\left(S_{20,w}\right)\) were derived from the sedimentation behavior of these complexes in sucrose density gradients prepared with \(H_2O\) and \(D_2O\) (17, 18) (Table III). Like other detergent-solubilized integral membrane proteins (17, 42, 70), ponticulin in OG micelles exhibited a lower apparent sedimentation coefficient in \(D_2O\) than in \(H_2O\) (Fig. 5 A). HIC-purified ponticulin exhibited an apparent sedimentation coefficient of \(2.5 \pm 0.2\) \(S\) \((n = 4)\) in \(H_2O\) gradients, while the apparent sedimentation coefficient was only \(1.4 \pm 0.3\) \(S\) \((n = 2)\) in \(D_2O\) gradients. This difference in apparent sedimentation rate indicates that the ponticulin-OG micelle has a larger \(\nu^*\) than those of the soluble protein standards which average \(0.74 \pm 0.02\) \(cm^3/g\) (see Materials and Methods). Specifically, \(\nu^*\) for the ponticulin-OG micelle was calculated to be \(0.825 \pm 0.015\) \(cm^3/g\) (theoretically equivalent to a density of \(~1.21\) g/cm\(^3\)), a value larger than that \(~0.716\) \(cm^3/g\) predicted from amino acid analyses of the protein alone, but comparable to values \(0.82-0.84\) \(cm^3/g\) obtained for other integral membrane proteins in detergent micelles (14, 24, 30, 39, 67). Using the calculated value for \(\nu^*\) to convert the apparent sedimentation coefficients to standard conditions \((H_2O\) at \(20^\circ C\) (17, 18), we obtained a value of \(2.42 \pm 0.05\) \(S\) for the ponticulin-OG micelle (Table III).

The Stokes radius of ponticulin in OG micelles was determined by gel filtration chromatography under both high pressure and normal atmospheric conditions. From the HPLC gel filtration experiments (not shown), a Stokes ra-

Table III. Hydrodynamic Properties of the Ponticulin-OG Micelle

| Property                          | Value       |
|----------------------------------|-------------|
| Sedimentation coefficient, \(S_{20,w} \times 10^{13} s\) | \(2.42 \pm 0.05\) |
| Partial specific volume, \(\nu^* \left(cm^3/g\right)\) | \(0.825 \pm 0.015\) |
| Stokes radius, \(R_s \left(nm\right)\) | \(3.6 \pm 0.3\) \((n = 5)\) |
| Molecular mass \((kd)\) | \(56 \pm 6\) |
| Detergent bound (g OG/g protein) | \(2.3 \pm 0.4\) |

\* \(2\%\) OG.

\dagger Determined by the method of Clarke and Smigel (18).

\ddagger Determined by HPLC gel filtration.

\† Determined by conventional gel filtration chromatography.

\‡ Calculated using \(R_s = 3.6\) nm, \(S_{20,w} = 2.42\) \(S\), and \(\nu^* = 0.825\) \(cm^3/g\).

\| Calculated using \(R_s = 3.6\) nm, \(M = 56\) kd, and \(\nu^* = 0.825\) \(cm^3/g\).

\(\text{Frictional ratio, } f/f_0\)\ (--

\(0.2\) g \(H_2O/\)g (minimum estimate) | \(1.26\)

\(1.4\) g \(H_2O/\)g (maximum estimate) | \(0.98\)

\(A\)

Figure 5. Physical properties of the ponticulin-octylglucoside micelle. (A) Sedimentation in sucrose density gradients \((5-20\%)\). The apparent sedimentation coefficient of HIC-purified ponticulin in \(2\%\) OG, \(H_2O\) \((\bigcirc)\) is \(2.5 \pm 0.2\) \(S\) \((n = 4)\). In gradients prepared in \(D_2O\) \((\bullet)\), the apparent sedimentation coefficient is \(1.4 \pm 0.3\) \(S\) \((n = 2)\) in \(D_2O\) gradients. Arrows indicate position of ponticulin. (B) Chromatography on Sephadex G-100. The Stokes radius \((R_s)\) of HIC-purified ponticulin in 2 % OG is \(~3.6\) nm (see text). Arrow indicates elution position of ponticulin.
diameter, \( R_s \), of 3.6 \( \pm \) 0.3 nm \((n = 5)\) was calculated using the
method of Nalecz et al. (66). This value was confirmed by
conventional chromatography on a Sephadex G-100 column,
which yielded a Stokes radius of 3.5 \( \pm \) 0.4 nm \((n = 2)\) (Fig.
5B).

Using the values \( R_s = 3.6 \text{ nm}, s = 2.42 S\), and \( v^* =
0.825 \text{ cm}^3/\text{g} \), we calculate a molecular mass of 56 \( \pm \) 6 kDa
for the ponticulin–OG micelle. This size and \( v^* \) are those
expected for a ponticulin monomer of 13–17 kDa in an OG
micelle of 30–40 kDa, the approximate size reported for pure
OG micelles in sedimentation velocity experiments (55).

The relatively large \( v^* \) for the complex, which is not far from
the reported \( v^* \) for OG \((0.859 \text{ cm}^3/\text{g})\) (75), is inconsistent
with the presence of ponticulin dimers or oligomers in OG
micelles. For instance, a 26-kDa ponticulin dimer in a micelle
with 30-kDa of OG should exhibit a \( v^* \) of \([26/56(0.716) +
30/56(0.859)] = 0.792\), a number well outside the ex-
perimental range. Thus, the micellar detergent-to-protein
mass ratio appears to be at least 2.3 mg OG per mg ponticu-
lin. The presence of residual TX-114 is contraindicated by
the absence of a detectable absorbance at 280 nm, but the
presence of tightly associated endogenous lipid cannot be ex-
cluded.

Although tightly bound lipid also would increase \( v^* \), its
possible presence should not affect the conclusion that pon-
ticulin is monomeric in OG micelles. To account for our ex-
perimental values, \(~66\%\) of the OG in a dimer-containing
micelle would have to be replaced by endogenous phospho-
lipid (average \( v^* \sim 0.95 \text{ cm}^3/\text{g} \)) (61). This eventuality
is highly unlikely because OG–phospholipid mixed micelles
are stable only when OG is present in a 3.2-fold molar ex-
cess (2).

The calculated frictional ratio, \( f/f_0 \), for a 56-kDa ponticu-
lin–OG micelle is 1.26, assuming a hydration factor \((\delta)\) of
0.2 g H\(_2\)O/g micelle, a minimum estimate often used for
soluble proteins (14, 27, 91). A particle with this frictional
coefficient is ellipsoidal with an axial ratio of about 5 (12,
24). However, \( \delta \) for a protein–detergent micelle may be much
larger (18). For instance, a maximum hydration factor of
1.4 g H\(_2\)O/g has been reported for Triton X-100 micelles
(106). If this higher estimate is used, \( f/f_0 \) = 0.98, indicating
an essentially spherical protein–detergent micelle.

**Reconstitution of Bilayer Structure**

Ponticulin was readily incorporated into lipid bilayers made
either with lipids extracted from Dictyostelium plasma mem-
branes or with the synthetic lipid DMPC (Fig. 6). However,
the efficiency of incorporation was higher with Dictyostelium
lipid. About 66% of \(^{125}\)I-labeled ponticulin cosedimented
with Dictyostelium lipid (Fig. 6A, fractions 4–10), com-
pared to \(~28\%\) of ponticulin reconstituted with DMPC
(Fig. 6B, fractions 14–19). This behavior contrasted with
that of the lipid tracer, fluorescein-PE, which incorporated
more efficiently into DMPC vesicles. When reconstituted
with Dictyostelium lipid (Fig. 6A), \(^{125}\)I-labeled ponticulin
sedimented a shorter distance into the sucrose gradient than
after reconstitution into DMPC (Fig. 6B), but further than
ponticulin diluted in the absence of lipid (P, Fig. 6A), indi-
cating that the protein’s final position in the gradient de-
dependent upon association with lipid. The lipid-dependent
differences in sedimentability might be due to intrinsic
differences in vesicle density (apparent density of 1.031
g/cm\(^3\) for ponticulin/Dictyostelium lipid, compared to 1.050
g/cm\(^3\) for ponticulin/DMPC), but also may reflect a greater
degree of resealing for vesicles containing Dictyostelium
lipid (39, 87).

Reconstituted mixtures of ponticulin and Dictyostelium
lipid formed vesicles and membrane sheets with \(~6\)-nm
wide trilaminar bilayers (Fig. 7). Apparent vesicle diameters
varied from \(~80\) nm to \(~4\) \( \mu \)m, with most between \(~0.2\) and
\(~0.5\) \( \mu \)m. No amorphous material indicative of denatured
protein was seen. The electron densities of the outer leaflets
of the trilaminar bilayers were variable (Fig. 7, inset), per-
haps reflecting local differences in lipid or amounts of incor-
porated protein, possibly induced by glutaraldehyde treat-
ment.

**Actin Nucleation Activity**

HIC-purified ponticulin reconstituted into Dictyostelium
lipid vesicles promoted the assembly of pyrene-labeled actin in nucleation assays (Fig. 8). The shapes of the polymerization time courses were similar to those reported previously for Dictyostelium plasma membranes (80), with the maximal polymerization rate observed after a short lag. In agreement with previous observations (16, 80), ponticulin-mediated actin nucleation activity required both the presence of ponticulin and its incorporation into a lipid bilayer (Fig. 8 A). Neither Dictyostelium lipid vesicles without ponticulin nor ponticulin diluted in the absence of lipid promoted actin filament assembly.

Ponticulin-mediated actin nucleation activity depended critically on the composition of the lipid bilayer. Ponticulin reconstituted into vesicles composed of either DMPC (Fig. 8 A) or several other commercially available lipids and lipid mixtures (not shown) exhibited <11% of the nucleation activity observed for ponticulin in Dictyostelium lipid vesicles. Even after correcting for the lower incorporation efficiency (Fig. 6), the nucleation activity of ponticulin/DMPC vesicles is < 26% of that observed for ponticulin in Dictyostelium lipid. The critical factor appeared not to be bilayer fluidity per se because neither phosphatidylcholines (PCs) with low gel-fluid chain melting temperatures (Tm's), such as dioleoyl-L-α-PC (Tm = -21°C) and egg PC (Tm = -15 to -5°C), nor relatively high-melting PCs, such as DMPC (Tm = 23.5°C) and dipalmityl-L-α-PC (Tm = 41.5°C), supported ponticulin-mediated actin nucleation. Also, neither the bilayer curvature nor the elastic stress (40, 41) attributed to hexagonal-phase phospholipids (25) was the sole critical factor since ponticulin-mediated actin nucleation activity was not supported by PEs with low lamellar-to-hexagonal phase transitions (Tn's), such as dioleoyl-L-α-PE (Tn = -16°C, Tn = 12°C) and the plasmalogen form of bovine brain PE (Tn = 3°C, Tn = 18°C). (Tn and Tn values are taken from references 7, 52, 61.) In addition, no activity was observed with an equimolar mixture of dioleoyl-L-α-PC, dioleoyl-L-α-PE, and bovine brain plasmalogen PE, a composition that approximates that of the three most prevalent phospholipids, ~84% of the total (101), in the Dictyostelium plasma membrane. Finally, 1,2-dioctanoyl-sn-glycerol (1.0 μM), a lipid recently shown to increase the actin nucleation activity of isolated Dictyostelium plasma membranes (81), did not affect the nucleation activity of ponticulin in DMPC bilayers. This result is consistent with the previous conclusion that diacylglycerols act through a tightly bound peripheral protein, rather than directly with ponticulin (81).

Essentially all of the basal actin nucleation activity of Dictyostelium plasma membranes appears to be attributable to ponticulin. First, the maximal rate of actin polymerization as a function of the concentration of ponticulin in Dictyostelium lipid vesicles is 1.45 ± 0.48 min⁻¹ (μg/ml)⁻¹.
A) and HIC (Fig. 9 B). In both chromatographic steps, frac-
tein) -1 (n = 5) (Fig. 8 B). The comparable rate for
olysed Dictyostelium plasma membrane lipids (7
reconstituted into vesicles by an 11-fold dilution into assay buffer. (B) 30 
reconstituted into vesicles by a 13-fold dilution. Peaks of
reconstituted into vesicles. (B) 30 µM of each fraction was premixed
of OG-solubilized plasma membrane lipids (7
for actin nucleation activity. Activity is shown above the 12-22-kD re-
frac-
ation activities were observed for vesicles reconstituted from phospholipid-to-protein mole ratios varying from \( \sim 1:10 \) (1.4 \( \mu \)M phospholipid:0.5 \( \mu \)g/ml ponticulin) to \( \sim 6000:1 \) (70 \( \mu \)M phospholipid:0.2 \( \mu \)g/ml ponticulin). Only for mole ratios exceeding \( \sim 10:1 \) (140 \( \mu \)M phospholipid and 0.2 or 0.3 \( \mu \)g/ml ponticulin) was there perhaps a deviation from linearity (not shown). These observations strongly suggest that ponticulin functions as a monomer. If dimeric ponticulin is the nucleating species, it is not in equilibrium with monomer in the range of lipid and protein concentrations used in these experiments.

The quaternary structure of ponticulin reconstituted with Dictyostelium lipid also was probed using water-soluble cross-linking agents. No reproducible cross-linked dimers or multimers were observed on silver-stained gels or immunoblots after treating reconstituted ponticulin with up to 20 \( \mu \)M 1-ethyl-3(3-dimethylaminopropyl)carbodiimide, m-maleimidobenzoyl-N-hydroxysulfo-succinimid ester, ethylene glycolbis(sulfosuccinimidyl)sulfonate, or bis[2(sulfosuccinimido)oxycarbonyloxy]ethyl]sulfone (data not shown). These results indicate that, if ponticulin dimers exist, the reactive surface-accessible amino and/or sulfhydryl groups are oriented inappropriately for cross-linking by these reagents. In fact, inefficient cross-linking of ponticulin is possible because, at the highest reagent concentrations used, some cross-linking is expected even for monomers, due to random collisions in the plane of the bilayer (36).

### Discussion

The ponticulin isolation procedure described here is much more efficient than the previous method in which 75–90% pure ponticulin was obtained from purified plasma membranes (103, 105). With the present procedure, essentially homogeneous ponticulin is isolated by F-actin affinity chromatography and HIC (Figs. 2 and 3, Table II) after extraction from cytoskeletons and enrichment by TX-114 phase partitioning (Fig. 1). Because the considerable time and material loss associated with plasma membrane purification (39, 47, 57) are avoided, \( \times 8 \)-fold more ponticulin is recovered per cell in about one-third the time.

Quantitative purification tables indicate that ponticulin is a relatively major component of Dictyostelium amebas, averaging 0.05 \( \pm 0.01 \% \) of the total cell protein (Table I). Given that 10\(^{9}\) amebas contain \( \sim 0.5 \) g total protein (Table I), there is \( \sim 0.26 \) mg ponticulin per 10\(^{9}\) cells, or \( \sim 9.1 \times 10^{6} \) copies/cell, with an upper estimate of 1.2 \( \times 10^{7} \) copies/cell (Table I). Thus, on a per cell basis, ponticulin is about as abundant as Band III (1.2 \( \times 10^{6} \)/erythrocyte) and Thy-I glycoprotein (1 \( \times 10^{6} \)/thymocyte or neuronal cell), both of which are considered major plasma membrane proteins (10, 102).

The surface density of ponticulin also is consistent with its proposed role as a major site of cytoskeleton–membrane attachment. Assuming that 43% (\( \sim 4 \times 10^{5} \) molecules) of the ponticulin is at the cell surface at a given time (28) and that the surface area of the Dictyostelium plasma membrane is \( \sim 1,400 \) \( \mu \)m\(^2\)/cell (Swaisgood, M., and T. L. Steck, University of Chicago, personal communication), there are \( \sim 300 \) ponticulin monomers per \( \mu \)m\(^2\) of plasma membrane. This surface density compares favorably with the 250–400 actin oligomers and \( \sim 700 \) ankyrins found per \( \mu \)m\(^2\) in the erythrocyte membrane skeleton, although it is much less than the \( \sim 2,000 \) Band III tetramers/\( \mu \)m\(^2\) (potential ankyrin attachment sites) (5, 10–12, 98). The surface density of ponticulin also is less than the \( \sim 1,200 \) molecules/\( \mu \)m\(^2\) calculated for GPIb, the major membrane attachment site for actin-binding protein in unactivated platelets (3). (The calculation for GPIb is based on 25,000 copies/platelet and the assumption that platelets are smooth cylinders \( \sim 0.75-\mu \)m high with diameters of \( \sim 3 \) \( \mu \)m [65]). Thus, while ponticulin appears to be a major point of actin–membrane attachment in Dictyostelium, there is ample space on the membrane for additional cytoskeleton binding sites.

The estimated number of ponticulin monomers in the membrane is about the same as the number of short actin filaments in the cell. Based on deoxyribonuclease I inhibition assays, Podolski and Steck have reported that Dictyostelium amoebas contain \( \sim 3.6 \times 10^{7} \) actin filaments with unblocked pointed ends; \( \sim 96\% \) of these filaments average 0.22 \( \mu \)m in length and are proposed to populate the submembrane cortical mesh (71). This filament number is intriguingly close to the \( 4 \times 10^{7} \) ponticulin monomers per cell estimated above.

Although limitations of the experimental approach used here have been noted (92), our hydrodynamic data (Fig. 5; Table III) indicate that ponticulin is monomeric in OG micelles. The large \( \times 4 \)-fold increase in the number of OG micelles containing 4–8-fold more ponticulin with OG micelles appears to be attributable to the presence of the (low density) detergent and, possibly, some tightly associated lipid. These results are consistent with those reported for other integral membrane proteins, which tend to be monomers or dimers in detergent micelles containing 0.28–1.12 mg of detergent per mg protein (17, 42, 44, 76). By contrast, typical watersoluble proteins bind <0.03 mg of detergent per mg of protein (17, 44).

Detergent-solubilized ponticulin binds F-actin (Fig. 4), but does not promote actin filament assembly (Fig. 8). However, when reconstituted into Dictyostelium lipid vesicles (Figs. 6 and 7), highly purified ponticulin nucleates actin assembly (Fig. 8) with the activity expected if ponticulin is responsible for all the basal activity in Dictyostelium plasma membranes (80). Like Dictyostelium plasma membranes, reconstituted ponticulin nucleates actin filaments with both ends free for interactions with other proteins, including actin monomers. Activity profiles of ponticulin purified by two different methods (Fig. 9) show clearly that ponticulin is both necessary and sufficient for actin nucleation.

The dependence of nucleation activity on the presence of Dictyostelium lipid (Fig. 8) suggests either that ponticulin function requires a particular lipid environment or that some lipid (or proteolipid) is a cofactor. Although a need for a specific lipid is rare, the activities of many integral membrane proteins depend upon lipid composition (46, 49, 74). For instance, reconstitution of the mitochondrial proton pump is optimal in a 5:5:0.3 mix of PE/PC/cardiolipin (74), and the acetylcholine receptor does not integrate properly into bilayers lacking cholesterol (49). Clearly, the nature of the lipid(s) required for ponticulin-mediated actin nucleation is an interesting area for further investigation.

The first-order dependence of actin nucleation on ponticulin concentration over a range of protein-to-lipid ratios (Fig. 10) indicates that the actin-nucleating moiety behaves like a monomer. This result is consistent with the observation that...
ponticulin in OG micelles appears to be monomeric and with the resistance of ponticulin in vesicles to chemical cross-linking. In conjunction with the previous finding that an actin trimer is the minimum assembly state required for tight binding to Dictyostelium plasma membranes (78), our results suggest that, like talin (50), each ponticulin molecule may promote actin nucleation by lateral stabilization of a trimeric actin nucleus. However, we cannot completely exclude the possibility that a very stable ponticulin dimer (or oligomer), resistant to chemical cross-linking, forms in the presence of Dictyostelium lipid.

Even if the actin-nucleating species is a ponticulin multimer with a high self-association constant in lipid bilayers, our results indicate that ponticulin-mediated actin nucleation is probably not dependent on clustering induced by extracellular factors. This conclusion is consistent with the observation that the number of actin nucleation sites is unchanged during lectin-induced capping in B-lymphocytes (48). However, the localization of nucleation sites in the plane of the membrane could be controlled by clustering ponticulin. Ponticulin function also may be regulated by upregulation from internal stores (59, 104), by oxidation and reduction of disulfide bonds (16), and/or by changes in the local lipid environment (this work). The purification and reconstitution procedures described here should potentiate the further dissection of the molecular basis for ponticulin-mediated actin nucleation which, in turn, may shed light on actin polymerization at the plasma membrane during motile processes.

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Clarke, S. 1975. The size and detergent binding of membrane proteins. J. Biol. Chem. 250:5459–5469.

Clarke, S., and M. Smigiel. 1989. Size and shape of membrane protein detergent complexes: hydrodynamic studies. Methods Enzymol. 172: 696–709.

Cocucci, S. M., and M. Sussman. 1970. RNA in cytoplasmic and nuclear fractions of cellular slime mold amoebae. J. Biol. Chem. 45:399–407.

Cohen, E. J., and J. T. Edsall. 1943. Density and Apparent Specific Volumes of Proteins. In Proteins, Amino Acids, and Peptides. Hafner Publishing Co., Inc., New York. 370:381.

Cohen, S. A., B. A. Bidlingmeyer, and T. L. Tarvin. 1986. PTIC derivatives in amino acid analysis. Nature (Land.). 320:769–770.

Condeelis, J., A. Bresnick, M. Demma, S. Dharmawardhanie, R. Eddy, A. L. Hall, B. Sauterer, and V. M. Warren. 1990. Mechanisms of amoeboid motility: an evaluation of the cortical expansion model. Dev. Genet. 11:333–340.

Cooper, J. A., S. B. Walker, and T. D. Pollard. 1983. Pyrene actin: documentation of the validity of a sensitive assay for actin polymerization. J. Muscle Res. Cell Motil. 4:253–262.

Costello, M. J., J. Escaig, K. Matsuoh, P. V. Viitanen, D. R. Menick, and H. R. Kaback. 1987. Purified lac permease and cytochrome o oxidase are functional as monomers. J. Biol. Chem. 262:17072–17082.

Cullis, P. R., and M. J. Hope. 1985. Physical properties and functional roles of lipids in membranes. In Biochemistry of Lipids and Membranes. D. E. Vance and J. E. Vance, editors. The Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA. 25–72.

Dabiri, G. A., M. J. Sanger, D. A. Portnoy, and F. S. Southwick. 1990. Listeria monocytogenes moves rapidly through the host-cell cytoplasm by inducing directional actin assembly. Proc. Natl. Acad. Sci. USA, 87:6068–6072.

Davis, A. 1984. Determination of the hydrodynamic properties of detergent-solubilized proteins. In Molecular and Chemical Characterization of Membrane Receptors. J. C. Venter and L. C. Harrison, editors. Alan R. Liss, Inc., New York. 161–178.

de Chastellier, C., A. Ryter, and L. Thilo. 1983. Membrane shuttle between plasma membrane, phosphogamides, and pinosomes in Dictyostelium discoideum amoeboid cells. Eur. J. Cell Biol. 30:233–243.

Decorrevet, P. N., and S. H. Zigmund. 1988. Chemoattraction in eucaryotic cells: a focus on leukocytes and Dictyostelium. Annu. Rev. Cell Biol. 4:649–686.

Doren, A., and J. Goldfarb. 1970. Electrolyte effects on micellar solution of nonionic detergents. J. Colloid Interface Sci. 32:67–72.

Ebert, R. F. 1986. Amino acid analysis by HPLC: optimized conditions for chromatography of phenylthiocarbamyl derivatives. Anal Biochem. 154:431–435.

Edelman, G. M. 1976. Surface modulation in cell recognition and cell growth. Science (Wash. DC). 192:218–226.

Fleischer, B., and M. Smigiel. 1978. Solubilization and properties of galactosytransferase and sulfotransferase activities of Golgi membranes in Triton X-100. J. Biol. Chem. 253:1633–1642.

Forscher, P., and S. J. Smith. 1988. Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. J. Cell Biol. 107:1505–1516.

Forscher, P., C. H. Lin, and C. Thompson. 1992. Novel form of growth cone motility involving site-directed actin filament assembly. J. Cell Biol. 822:289–327.

Gaffey, B. J. 1985. Chemical and biochemical croslinking of membrane components. Biochin. Biophys. Acta. 822:289–317.

Gibbons, R. A. 1972. Physico-chemical methods for the determination of the purity, molecular size and shape of glycoproteins. In Glycoproteins. A. Gottschalk, ed. Elsevier, Amsterdam. 78–160.

Goldfarb, J., and L. Sepulveda. 1969. Application of a theory of polymer solutions to the cloud points of nonionic detergents. J. Colloid Interface Sci. 31:454–459.

Goodridge-Holland, C. M., and E. J. Luna. 1987. Purification and characterization of Dictyostelium discoideum plasma membranes. Methods Cell Biol. 28:103–128.

References

1. Abercrombie, M. 1980. The crawling movement of metazoan cells. Proc. R. Soc. Lond. Ser. B Biol. Sci. 207:129–147.

2. Almol, S., B. J. Litman, W. Wimley, J. Cohen, E. J. Wachtel, Y. Barenholz, A. Ben-Shaul, and D. Lichtenberg. 1990. States of aggregation and phase transformations in mixtures of phosphatidylcholine and octyl glucoside. Biochemistry. 29:4582–4592.

3. Andrews, R. K., and J. E. B. Fox. 1991. Interaction of purified actin-binding protein with the platelet membrane glycoprotein Ib-IX complex. J. Biol. Chem. 262:67072–17082.

4. Casella, L. F., D. J. Maack, and S. Lin. 1986. Purification and initial characterization of a protein from skeletal muscle that caps the barbed ends of filamentous actin. J. Biol. Chem. 261:10915–10921.

5. Caldwell, J. E., S. H. Heiss, V. Mermall, and J. A. Cooper. 1989. Effects of CapZ, an actin capping protein of muscle, on the polymerization of actin. Biochemistry. 28:8506–8514.

6. Byers, T. J., and D. Branton. 1985. Visualization of the protein association in the erythrocyte membrane skeleton. Proc. Natl. Acad. Sci. USA. 82:6153–6157.

7. Boggs, J. M., D. Stump, D. W. Hughes, and C. M. Deber. 1981. Reduction and oxidation of disulfide bonds (16), and/or by changes in the local lipid environment (this work). The purification and reconstitution procedures described here should potentiate the further dissection of the molecular basis for ponticulin-mediated actin nucleation which, in turn, may shed light on actin polymerization at the plasma membrane during motile processes.

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97. Towbin, H., T. Stahelin, 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.

98. Ursitti, J. A., D. W. Pumplin, J. B. Wade, and R. J. Bloch. 1991. Ultrastructure of the human erythrocyte cytoskeleton and its attachment to the membrane. Cell Motil. Cytoskeleton. 19:227-243.

99. Wang, Y.-L. 1985. Exchange of actin subunits at the leading edge of living fibroblasts: possible role of treadmilling. J. Cell Biol. 101:597-602.

100. Weast, R. C., editor. 1969. Handbook of Chemistry and Physics. 50th ed. The Chemical Rubber Co., Cleveland, OH.

101. Weeks, G., and F. C. Herring. 1980. The lipid composition and membrane fluidity of Dictyostelium discoideum plasma membranes at various stages during differentiation. J. Lipid Res. 21:681-686.

102. Williams, A. F., and J. Gagnon. 1982. Neuronal cell Thy-1 glycoprotein: homology with immunoglobulin. Science (Wash. DC). 216:696-703.

103. Wuestehube, L. J., and E. J. Luna. 1987. F-actin binds to the cytoplasmic surface of pontoculin, a 17-kD integral glycoprotein from Dictyostelium discoideum. J. Cell Biol. 105:1741-1751.

104. Wuestehube, L. J., C. P. Chia, and E. J. Luna. 1989. Immunofluorescence localization of pontoculin in motile cells. Cell Motil. Cytoskeleton. 13:245-263.

105. Wuestehube, L. J., D. W. Speicher, A. Shariff, and E. J. Luna. 1991. F-actin affinity chromatography of detergent-solubilized plasma membranes: purification and initial characterization of pontoculin from Dictyostelium discoideum plasma membranes. Methods Enzymol. 196:47-65.

106. Yedgar, S., Y. Barenholz, and V. G. Cooper. 1974. Molecular weight, shape and structure of mixed micelles of Triton X-100 and sphingomyelin. Biochim. Biophys. Acta. 363:98-111.

107. Yuen, S. W., A. H. Chui, K. J. Wilson, and P. M. Yuan. 1988. Microanalysis of SDS-PAGE electroblotted proteins. Applied Biosystems Inc. User Bulletin. 36:8-9.