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

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Et al.
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 \( \sim 10^5 \) 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), microvillar 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 \( 125\)I-
labeled F-actin blot overlays with SDS gel-purified ponticulin (16). Ponticulin–actin binding in both assays resembles actin binding to purified plasma membranes in that all these interactions are sensitive to high salt, heat, and disulfide reducing agents. Ponticulin is involved in membrane-mediated actin nucleation activity because its removal from detergent-solubilized membranes precludes the reconstitution of this activity (80). However, it is not known whether ponticulin nucleates actin assembly alone or in concert with other membrane components.

Because previous purification schemes generated only small amounts of partially purified ponticulin, very little is known about its physical properties or mechanism of action. The major 17-kD band observed on SDS–polyacrylamide gels has been shown to contain six isoforms (pI 4.1 to 5.3) on two-dimensional gels (16, 104). The amino acid composition of ponticulin purified from plasma membranes by F-actin affinity chromatography and preparative SDS-PAGE suggests that this protein is unusually high in serine (20%), glycine (10%), and alanine (10%) (105). The NH2-terminal amino acid sequence also has been determined (105). However, essentially nothing is known about the biochemical properties of the native protein.

To characterize ponticulin and its role in nucleating actin assembly, we have developed a rapid and efficient purification procedure, starting with cytoskeletons instead of plasma membranes. We also have determined the hydrodynamic properties of highly purified ponticulin in octylglucoside micelles and have demonstrated that, when reconstituted into Dictyostelium lipid vesicles, ponticulin is sufficient for the nucleation of actin assembly. Like actin assembled onto Dictyostelium plasma membranes, ponticulin-nucleated actin filaments have both ends free. The hydrodynamic properties and the observed first-order dependence of actin nucleating activity on the protein-to-lipid ratio in reconstituted vesicles indicate that ponticulin behaves as a monomer in both detergent micelles and lipid bilayers. In addition to its previous identification as an actin–membrane anchor, our results suggest that monomeric ponticulin laterally nucleates actin filaments at the plasma membrane during motile processes. These results further suggest that clustering of ponticulin by extracellular factors is not an obligate step in membrane-mediated actin nucleation.

**Materials and Methods**

**Chemicals**

All chemicals used were reagent grade unless otherwise specified. Triton X-114 (TX-114), deuterium oxide (D2O), DTT, 1-ethyl-3-(3-dimethylamino)propryl carbodiimide, dimyristoyl L-a-phosphatidylcholine (DMPC), and other synthetic phospholipids were purchased from Sigma Chem. Co. (St. Louis, MO). Other cross-linking agents were from Pierce Chem. Co. (Rockford, IL). TX-114 was precondensed three times by the method of Border (8). The plasmalogens of bovine brain phosphatidylethanolamine (PE) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Bio-Beads (SM-2) were purchased from Bio-Rad Laboratories (Richmond, CA). Octylglucoside (OG), phalloidin, and ATP were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). N-(5-fluorescein-thiocarbamoyl)dipalmitoyl-1-O-phosphatidylethanolamine triethylammonium salt (fluorescein PE) was from Molecular Probes Inc. (Eugene, OR).

**Proteins**

Actin used for cosedimentation assays and actin affinity columns was isolated from rabbit skeletal muscle according to the method of Spudich and Watt (86). Pyrene actin, used in actin nucleation assays (51), was purified further by gel filtration Sephadex G-150 in 0.2 mM CaCl2, 0.2 mM ATP, 0.5 mM DTT, 2 mM Tris-HCl, pH 8.0 (buffer A, reference 60). Adsorbed rhG, an antibody recognizing ponticulin and two related polypeptides, was prepared as described (16, 103, 104). CapZ was the kind gift of Dr. John A. Cooper, Washington University School of Medicine, St. Louis, MO.

**Purification of Ponticulin**

**Preparation of Cytoskeletons.** *Dictyostelium* amebas (strain K-A3) from Dr. Richard Kessin, Columbia University) were grown in HL-5 medium (19) to >1010 cells/ml. A total of 4 × 106 cells was used for analytical analyses. 1–1.5 × 1010 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 K2HPO4, 2.0 mM NaH2PO4, pH 6.1, and washed once with 20 mM sodium phosphate, pH 6.8 (PB). Cells were resuspended in PB to 2 × 106 cells/ml and extracted with an equal volume of 2% (vol/vol) TX-114 in 20 mM KC1, 20 mM EGTA, 20 mM imidazole, 4 mM MgCl2, pH 7.0 (62). The mixture was incubated for 10 min on ice and then for 10 min at room temperature. Cytoskeletons 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, 1× CSK, 1% TX-114, 1 μM phalloidin. 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 g). The mixture separated into a buoyant detergent phase, a denser high salt aqueous layer, and a pellet (extracted cytoskeletons). This pellet was resuspended 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 (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 (ε8 = 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% (wt/vol) OG in column buffer (CB; 50 mM KC1, 1 mM MgCl2, 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 prewetted with 5 ml of 2 M NaCl, 2 mM EGTA, 1% OG, 0.5 μM phalloidin, and equilibrated with 10 ml of 1% OG, 1 μM phalloidin, CB. After equilibration, the column was washed with 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 phalloidin, CB, and the ponticulin-containing fraction was eluted with 5 ml of 2 M NaCl, 2 mM EGTA, 1% OG, 0.5 μM phalloidin, CB. The beads were reequilibrated, 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 Ultra Chrom GTI Bioseparation System (Pharmacia LKB Biotechnology, Piscataway, NJ). The system was calibrated with Bio-Rad gel filtration standards. A ponticulin-enriched frac-
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-5PW column (8 x 75 mm; Pharmacia LKB Biotechnology) equilibrated with 10 column volumes 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 poured 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-Bolton-Hunter reagent (New England Nuclear, Boston, MA) in 100 mM sodium phosphate, pH 8.0, to a specific activity of ~ 5 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) in 100 mM sodium phosphate, pH 8.0, to a specific activity of ~ 5 μCi/μg ponticulin.

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 (85). Briefly, 100 μl of ponticulin (5.5 μg) containing a trace amount of 125I-ponticulin were dialyzed into 1.8 ml of sterile filtered PBS (150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4) in slinized 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 supernatants were used 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 electrotransferred to polyvinylidene difluoride membranes.

Affinity matrix Assays. Actin was polymerized at room temperature by adding 1 vol of 5× 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 1× 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 Instrums., 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 a 1-h incubation at room temperature with occasional mixing, the assay mixture was centrifuged at room temperature in an airfuge (178,000 g for 20 min; Beckman Instrums., Inc.). The supernatant was collected by aspiration and the pellet was rinsed 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 results generally contained no significant amount 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, R0) used in the HPLC experiments were bovine IgG (158 kD; 51 nm), ovalbumin (43 kD; 2.8 nm), and myoglobin (17 kD; 1.9 nm). Conventional gel filtration chromatography employed a Sephadex G-100 column (17.5 x 0.7 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, R0): cytochrome c (12.3 kD; 1.7 nm), myoglobin (17 kD; 1.9 nm), chymotrypsigen (23.7 kD; 2.2 nm), ovalbumin (43 kD; 2.8 nm), and lactoperoxidase (92.6 kD; 3.6 nm). Column fractions were counted and then analyzed by SDS-PAGE. Most Stokes radii were from Le Maire et al. (54) and Tanford et al. (93); R0 for lactoperoxidase was calculated using a diffusion coefficient (D x 10-14 cm2 s-1) of 5.95 (84) in the equation, R0 = kD/6πρv* (83).

Sedimentation Coefficient and Partial Specific Volume (v*). The standard proteins used in sedimentation analyses and the accepted values (84) for their Svedberg coefficients (× 10-14) and partial specific volumes (v*) are 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 (3.87 S; 0.784 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. 33.46.10; 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). Values for average distance sedimented, average density, average viscosity, and ρ20,w were determined for the standard proteins and ponticulin-OG micelles using the 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. 33.46.10; 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). The viscosity and ρ20,w 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),

where M is the molecular weight, R0 = Stokes radius, ρ20,w = sedimentation coefficient in pure H2O at 20°C, N = Avogadro's number, ρ20,HO = the viscosity of water at 20°C (1.002 cp), v* = partial specific volume, and ρ20,HO = the density of water at 20°C (0.998 g/cm3). To estimate the asymmetry of the complex, the frictional ratio (f0) for the ponticulin-OG micelle was calculated using the equation (18, 83, 91),

where δ is amount of water in g bound per gram of complex (14).

Reconstitution. Ponticulin was reconstituted into vesicles made from D. discoideum lipid or various commercially available lipids. D. discoideum lipid was extracted from suroce-Renografin purified plasma membranes.
Actin Nucleation.

Half the CapZ was inactivated by binding to were assayed for actin nucleation activity using the pyrene-actin fluorescence assay (23, 80). Vesicles, without fluorescein PE or radiolabel, were concentration of OG in the assays was <0.1%. In some experiments, CapZ (60 nM) was premixed with ponticulin (30 nM) in D. discoideum lipid vesicles (14.4 μM phospholipid) before addition of actin. Controls showed that about half the CapZ was inactivated by binding to Dictyostelium lipid under these conditions. Actin (10% pyrene-labeled; 3 μM final concentration) was added to samples in assay buffer, and the initial changes in pyrene-actin fluorescence were monitored (λex = 365 nm; λem = 407 nm). Background fluorescence was subtracted and the corrected numbers were normalized between 0 and 10, with 10 denoting the fluorescence after 24 h of polymerization. Nucleation activity was estimated from the maximal rate of fluorescence increase observed during the first 15 min after initiation of polymerization.

Actin Depolymerization. For depolymerization experiments, portions of pyrene-labeled actin (50 μM, 20% pyrene-labeled) were polymerized in the presence or absence of ponticulin (~60 nM) in Dictyostelium lipid vesicles (~30 μM phospholipid). After 2 h at 24°C, CapZ was added to a final concentration of 60 nM in half the samples, and incubation at 24°C was continued for another 24 h. Initial rates of actin depolymerization were measured by diluting sample tubes 10-fold into a cuvette containing assay buffer and monitoring changes in pyrene fluorescence. Under these conditions, depolymerization should occur only from the pointed ends of the actin filaments. The Journal of Cell Biology, Volume 120, 1993 912

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 50 Ti rotor. The pellets were resuspended in 2% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.4, and compacted in two Beckman airfuge 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 Ultratome E ultramicrotome. Grids were poststained with 4% uranyl acetate in methanol: 70% ethanol (1:1) for 30 min and with Reynolds' 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 were then 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.

Detergent Layer 1
Aqueous Layer 1 (discard)
Pellet 1
Re-extract
Centrifuge
Detergent Layer 2
Aqueous Layer 2 (discard)
Pellet 2

Figure 1. Procedure for the extraction of ponticulin from Dictyostelium cytoskeletons. Ponticulin-enriched fractions are shaded. Note that, unlike a typical TX-114 phase separation in which the detergent forms the lower phase, the detergent layer is less dense than 2 M NaCl.

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, 10 mM Hepes, pH 7.0, final concentrations). The final concentration of OG in the assays was <0.1%. In some experiments, CapZ (60 nM) was premixed with ponticulin (30 nM) in D. discoideum lipid vesicles (14.4 μM phospholipid) before addition of actin. Controls showed that about half the CapZ was inactivated by binding to Dictyostelium lipid under these conditions. Actin (10% pyrene-labeled; 3 μM final concentration) was added to samples in assay buffer, and the initial changes in pyrene-actin fluorescence were monitored (λex = 365 nm; λem = 407 nm). Background fluorescence was subtracted and the corrected numbers were normalized between 0 and 10, with 10 denoting the fluorescence after 24 h of polymerization. Nucleation activity was estimated from the maximal rate of fluorescence increase observed during the first 15 min after initiation of polymerization.

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
Figure 2. Ponticulin partitions into the hydrophobic detergent (TX-114) phase after salt-induced dissociation from cytoskeletons and is the major hydrophobic protein eluted by 2 M NaCl from F-actin columns. Silver-stained gel (A) and autoradiogram of the 15-20-kD region of a protein blot probed with adsorbed R57 IgG followed by 125I-protein A (B) of fractions from an analytical preparation. Lane 1, whole cells; lane 2, cytoskeletal supernatant; lane 3, cytoskeletal pellet; lane 4, detergent layer 1; lane 5, aqueous layer 1; lane 6, pellet 1; lane 7, detergent layer 2; lane 8, aqueous layer 2; and lane 9, pellet 2. All fractions were resuspended to the original volume of suspended cells (20 ml, ~9 mg/ml). 20 µl of each fraction was loaded onto the gel. Because of overloading of the gel, ponticulin is underrepresented in lanes 1-3 of B. Film was exposed for 88 h at ~85°C. Silver-stained gel (C) and autoradiogram of the 15-20-kD region of an antiponticulin immunoblot (D) showing the protein and ponticulin content of fractions from an F-actin affinity column. Lane S, molecular mass standards. Lanes 1-3, run-through fractions (20 µl) from three consecutive passages of the detergent layer through the F-actin column; and lanes 4-6, the corresponding salt-eluted fractions (20 µl) from the F-actin column. Film was exposed for 12 h at ~85°C. Molecular mass standards, in kD, are shown on the left. Asterisks in A and C indicate the position of actin; arrowheads indicate the position of ponticulin.

Table I. Purification of Ponticulin

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (in 10⁶ cells)</th>
<th>Protein (in 10⁶ cells)</th>
<th>Ponticulin (in 10⁶ cells)</th>
<th>% Ponticulin of total protein</th>
<th>Step-fold purification</th>
<th>Total fold purification</th>
<th>Recovery</th>
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<td>Cells</td>
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<td>0.065</td>
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<td>0.58</td>
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<td>8.9</td>
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<tr>
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<td>0.090</td>
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<td>&gt;98</td>
<td>1.30</td>
<td>1508</td>
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* 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 KDa 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 10^10 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 taken 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 prepared 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, v*, of ponticulin was predicted to be 0.716 cm^2/g (the inverse of the density of 1.40 g/ml), which is close to the average v* observed for a wide range of proteins (14). This predicted value for v* should be considered an upper estimate because oligosaccharides, known to be present on ponticulin (16, 103), may reduce v* to 0.68–0.70 cm^2/g (37, 79).

Amino acid analyses also were used to assess the accuracy of the BCA protein assay for ponticulin. Trace amounts of 125I-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).
HIC-purified ponticulin binds F-actin. Supernatants (S) and pellets (P) from sedimentation assays containing ~15 nM 125I-ponticulin with (lanes 1 and 2) and without (lanes 3 and 4) 4.6 µM 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 125I-ponticulin pelleted with F-actin (Fig. 4, lane 2). By contrast, only 18 ± 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 µM. However, the stochastic aggregation of the 125I-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 (v*) and the sedimentation coefficient (S20,w) were derived from the sedimentation behavior of these complexes in sucrose density gradients in H2O and D2O (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 D2O than in H2O (Fig. 5 A). HIC-purified ponticulin exhibited an apparent sedimentation coefficient of 2.5 ± 0.2 S (n = 4) in H2O gradients, while the apparent sedimentation coefficient was only 1.4 ± 0.3 S (n = 2) in D2O gradients. This difference in apparent sedimentation rate indicates that the ponticulin–OG micelle has a larger v* than those of the soluble protein standards which average 0.74 ± 0.02 cm3/g (see Materials and Methods). Specifically, v* for the ponticulin–OG micelle was calculated to be 0.825 ± 0.015 cm3/g (theoretically equivalent to a density of ~1.21 g/cm3), a value larger than that (~0.716 cm3/g) predicted from amino acid analyses of the protein alone, but comparable to values (0.82–0.84 cm3/g) obtained for other integral membrane proteins in detergent micelles (14, 24, 30, 39, 67). Using the calculated value for v* to convert the apparent sedimentation coefficients to standard conditions (H2O at 20°C (17, 18), we obtained a value of 2.42 ± 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-
diameter, $R_m$, of 3.6 ± 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 ± 0.4 nm ($n = 2$) (Fig. 5 B).

Using the values $R_m = 3.6$ nm, $s = 2.42$ S, and $v^* = 0.825$ cm$^3$/g, we calculate a molecular mass of 56 ± 6 kD for the ponticulin–OG micelle. This size and $v^*$ are those expected for a ponticulin monomer of 13–17 kD in an OG micelle of 30–40 kD, 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 cm$^3$/g) (75), is inconsistent with the presence of ponticulin dimers or oligomers in OG micelles. For instance, a 26-kD ponticulin dimer in a micelle with 30-kD of OG should exhibit a $v^*$ of [26/56(0.716) + 30/56(0.859)] = 0.792, a number well outside the experimental range. Thus, the micellar detergent-to-protein mass ratio appears to be at least 2.3 mg OG per mg ponticulin. 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 excluded.

Although tightly bound lipid also would increase $v^*$, its possible presence should not affect the conclusion that ponticulin is monomeric in OG micelles. To account for our experimental values, ~66% of the OG in a dimer-containing micelle would have to be replaced by endogenous phospholipid (average $v^* = 0.95$ cm$^3$/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 excess (2).

The calculated frictional ratio, $ff_o$, for a 56-kD ponticulin–OG micelle is 1.26, assuming a hydration factor (6) 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, $f_f$ 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, $ff_o$ = 0.98, indicating an essentially spherical protein–detergent micelle.

**Reconstitution of Bilayer Structure**

Ponticulin was readily incorporated into lipid bilayers made with either with lipids extracted from *Dictyostelium* plasma membranes 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. 6 A, fractions 4–10), compared with ~28% of ponticulin reconstituted with DMPC (Fig. 6 B, 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. 6 A), $^{125}$I-labeled ponticulin sedimented a shorter distance into the sucrose gradient than after reconstitution into DMPC (Fig. 6 B), but further than ponticulin diluted in the absence of lipid (P, Fig. 6 A), indicating that the protein's final position in the gradient depended 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 μm, with most between ~0.2 and ~0.5 μm. 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), perhaps reflecting local differences in lipid or amounts of incorporated protein, possibly induced by glutaraldehyde treatment.

**Actin Nucleation Activity**

HIC-purified ponticulin reconstituted into *Dictyostelium* plasma membranes was highly active in inducing actin filament formation. Activity was dosedependent, and the 50% effective concentration ($EC_50$) was calculated as 0.45 μM.
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. 8A). Neither Dictyostelium lipid vesicles 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. 8A) 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 phosphatidylethanolamines (PCs) with low gel-fluid chain melting temperatures (T_c's), such as dioleoyl-L-α-PC (T_c = -21°C) and egg PC (T_c = -15 to -5°C), nor relatively high-melting PCs, such as DMPC (T_c = 23.5°C) and dipalmitoyl-L-α-PC (T_c = 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 PE bilayers composed of low lamellar-to-hexagonal phase transitions (T_n's), such as dioleoyl-L-α-PE (T_n = -16°C, T_m = 12°C) and the plasmalogens form of bovine brain PE (T_n = 3°C, T_m = 18°C). (T_c and T_n 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-diocatanoil-sn-glycerol (10 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) \(n = 5\) (Fig. 8 B). The comparable rate for Dic-
tyostelium plasma membranes in the absence of exogenous disaccharide was ~0.013 min \(^{-1}\) (\(\mu g/ml\) protein) \(^{-1}\) (80). Thus, the specific activity of purified ponticulin is ~112 times larger than that observed for isolated membranes, which is about the enrichment in specific activity expected for a protein that constitutes ~0.7 ± 0.4 % of plasma membrane protein (103). Second, actin nucleation activity copurified with ponticulin during both gel filtration chromatography (Fig. 9 A) and HIC (Fig. 9 B). In both chromatographic steps, fractions containing the most ponticulin also exhibited the most actin nucleation activity, after reconstitution with Dic-
tyostelium lipid. Because the 15- and 19-kD polypeptides are present in only trace amounts, their contributions to the total nucleation activity are unclear.

As has been documented for actin filaments nucleated by Dic-
tyostelium plasma membranes (80), both ends of fila-
ments nucleated by ponticulin appear to be available for monomer assembly and disassembly. The fast-polymerizing "barbed" ends of ponticulin-induced actin nuclei are normally free to elongate because the rate of pyrene–actin polymer-
merization is decreased by 68 ± 21 % (\(n = 3\)) in the presence of CapZ (13, 15), a protein that binds and blocks barbed fila-
ment ends (data not shown). Because this decreased rate is about the same as that observed for CapZ-nucleated actin po-
lymerization, ponticulin-nucleated actin filaments also ap-
parently elongate from their slow-polymerizing "pointed" ends. Accessibility of the pointed ends was confirmed by de-
polymerization experiments in which ponticulin-nucleated, CapZ-blocked filaments were rapidly diluted to a final actin concentration of 0.5 \(\mu M\), a concentration at which depoly-
merization can occur only from the pointed filament ends (72). Initial depolymerization rates were linear and rapid with ~10% of the actin depolymerizing within the first min-
ute (data not shown). Initial rates were identical for CapZ-
capped filaments nucleated with \(-50 ± 7 \text{ nM actin min}^{-1}\), \(n = 3\) and without \(-52 ± 8 \text{ nM actin min}^{-1}\), \(n = 3\) pon-
ticulin, indicating that ponticulin does not block the pointed ends of actin filaments. Thus, both the barbed and pointed ends of ponticulin-nucleated actin filaments are free.

**Ponticulin Acts As a Monomer**

Based on theoretical considerations, we and others have pro-
posed that external factors may generate new membrane-
associated actin nucleation sites by clustering transmem-
brane proteins, each with a single actin-binding site (9, 32, 77, 78). As an initial test of whether the nucleation activity of ponticulin is dependent upon dimer formation in the mem-
brane, we assayed the nucleation activity of ponticulin reconstituted with Dic-
tyostelium lipid at a wide range of lipid-to-protein ratios. In such an assay, actin assembly is expected to be linearly dependent on protein concentration for a func-
tional monomer and to be proportional to the square of the protein concentration for a dimer in equilibrium with mono-
mer (24). In our experiments, fold increases in the maximal rates of actin polymerization were linearly dependent on the ponticulin concentration, but essentially independent of the concentration of Dic-
tyostelium lipid (Fig. 10). Similar nucle-
ation activities were observed for vesicles reconstituted from phospholipid-to-protein mole ratios varying from 1:10 (1.4 μM phospholipid:0.5 μg/ml ponticulin) to ~6000:1 (70 μM phospholipid:0.2 μg/ml ponticulin). Only for mole ratios exceeding 10:1 (140 μM phospholipid and 0.2 or 0.3 μg/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 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, m-maleimidobenzoyl-N-hydroxysulfo-succinimide ester, ethylene glycol bis(sulfosuccinimidyl succinate), 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, ~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 ± 0.01% of the total cell protein (Table I). Given that 10^8 amebas contain ~0.5 g total protein (Table I), there is ~0.26 mg ponticulin per 10^9 cells, or ~9.1 × 10^5 copies/cell, with an upper estimate of 1.2 × 10^6 copies/cell (Table I). Thus, on a per cell basis, ponticulin is about as abundant as Band III (1.2 × 10^5/erythrocyte) and Thy-1 glycoprotein (1 × 10^5/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% (~4 × 10^9 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 ~1,400 μm^2/cell (Swaisgood, M., and T. L. Steck, University of Chicago, personal communication), there are ~300 ponticulin monomers per μm^2 of plasma membrane. This surface density compares favorably with the 250-400 actin oligomers and ~700 ankyrins found per μm^2 in the erythrocyte membrane skeleton, although it is much less than the ~2,000 Band III tetramers/μm^2 (potential ankyrin attachment sites) (5, 10-12, 98). The surface density of ponticulin also is less than the ~1,200 molecules/μm^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 ~0.75-μm high with diameters of ~3 μm [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 ~3.6 × 10^1 actin filaments with unblocked pointed ends; ~96% of these filaments average 0.22 μm in length and are proposed to populate the submembrane cortical mesh (71). This filament number is intriguingly close to the 4 × 10^5 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 v* of the ponticulin–OG micelle 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 water-soluble 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 discussion 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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