Localization and Specificity of the Phospholipid and Actin Binding Sites on the Tail of *Acanthamoeba* Myosin IC

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Abstract. We used bacterially expressed β-galactosidase fusion proteins to localize the phospholipid binding domain of *Acanthamoeba* myosin IC to the region between amino acids 701 and 888 in the NH_2-terminal half of the tail. Using a novel immobilized ligand lipid binding assay, we determined that myosin I can bind to several different acidic phospholipids, and that binding requires a minimum of 5 mol% acidic phospholipid in a neutral lipid background. The presence of di- and triglycerides and sterols in the lipid bilayer do not contribute to the affinity of myosin I for membranes. We confirm that the ATP-insensitive actin binding site is contained in the COOH-terminal 30 kD of the tail as previously shown for *Acanthamoeba* myosin IA. We conclude that the association of the myosin IC tail with acidic phospholipid head groups supplies much of the energy for binding myosin I to biological membranes, but probably not specificity for targeting myosin I isoforms to different cellular locations.

THE myosin I class of single-headed molecular motors is thought to be involved in translocation of phospholipid membranes relative to actin filaments, but the mechanism of the association of myosin I with membranes is only partially characterized. Among the specific functions proposed for myosin I are extension of pseudopodia, movement of intracellular vesicles, projection of neuronal growth cones, and expulsion of contractile vacuole contents in protozoans (for reviews see Korn and Hammer, 1990; Kiehart, 1990; Pollard et al., 1991). Many organisms express myosin I, including multiple isoforms in protozoans (Pollard and Korn, 1973; Maruta et al., 1979; Coté et al., 1985; Titus et al., 1989), yeast (Goodson, H. V., personal communication), chicken (Collins and Borysenko, 1984), cow (Hoshimaru and Nakanishi, 1987), and *Drosophila* (Montell and Rubin, 1988). Microscopic localization of myosin I from many organisms suggests association with microvillar (Matsudaira and Burgess, 1979; Porter et al., 1992) and other membranes (Gadasi and Korn, 1979; Hagen et al., 1986; Drenckhahn and Dermietzel, 1988; Fukui et al., 1989; Baines and Korn, 1990; Yonemura and Pollard, 1992).

Most myosin I's consist of two major functional domains, an NH_2-terminal region similar to the subfragment 1 of myosin that contains the actin-activated ATPase activity and can support motility in in vitro assays, and a nonpolymerizing COOH-terminal extension strikingly different from the tail of myosin II. Indirect evidence implicates this tail region in the association of myosin I with phospholipid membranes (Adams and Pollard, 1989; Hayden et al., 1990). In some myosin I's, the tail is involved in ATP-insensitive actin filament binding as well (Lynch et al., 1986). Some have speculated that the tail also binds to membrane receptors (for review see Pollard et al., 1991), but this has not been demonstrated directly.

Acanthamoeba myosin I binds membranes stripped of peripheral proteins with NaOH (Adams and Pollard, 1989) or KI (Miyata et al., 1989), and both *Acanthamoeba* myosin I (Adams and Pollard, 1989) and the avian brush border myosin I (Hayden et al., 1990) bind purified acidic phospholipids. Indirect evidence suggests that phospholipid binding is mediated by the NH_2-terminal region of the tail (Adams and Pollard, 1989; Miyata et al., 1989). Myosin I's studied to date show no affinity for neutral phospholipids, and binding to acidic phospholipids is eliminated by high salt.

In this study we used bacterially expressed recombinant proteins to identify the membrane binding region of *Acanthamoeba* myosin I in the NH_2-terminal region of the tail. We also used a novel immobilized ligand binding assay to characterize the phospholipid specificity of the interaction of myosin I with membranes.

Materials and Methods

Construction of Plasmids and Cloning of Myosin IC

We used the recombinant DNA methods of Sambrook et al. (1989) unless otherwise noted. We isolated a nearly full-length cDNA comprising bases 252-3504 of *Acanthamoeba* myosin IC with 182 bases of 3′ untranslated sequence from a λgt10 library (Rimm et al., 1989) by screening for hybridization with a radiolabeled restriction fragment derived from the cloned myosin IC cDNA isolated by Jung et al. (1987). This cDNA was originally identified as myosin IB, but was later determined to encode myosin IC (Jung et al., 1989*b*). The 2,980-bp EcoRI fragment was subcloned into Bluescript KS+ (Stratagene Inc., La Jolla, CA). This plasmid is called p4.5L.

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Expression Plasmids

pT627-888. We digested p4.5L DNA with HpaII and isolated the 786-bp fragment corresponding to bases 1877–2663, and subcloned this into ClaI cut and dephosphorylated Bluescript II KS+. This plasmid is called pB627-888. We then digested pB627-888 with SalI and HindIII, isolated the fragment containing the *Acanthamoeba* sequence, and subcloned this into SalI–HindIII–digested pTRB-1 (Bürglin and DeRobertis, 1987), a vector designed for high level expression of recombinant β-galactosidase fusion proteins.

pT701-888. We digested pB627-888 with HincII, isolated the 3,520-bp fragment, and reclosed it. This plasmid, which contains bases 2101–2663 in Bluescript II KS+, is designated pB701-888. We digested pB701-888 with BamHI and HindIII and subcloned this fragment into BamHI–HindIII–digested pTRB-1.

pT388-676. We introduced two new PstI sites at bases 2016 and 2692 by oligonucleotide-directed mutagenesis of p4.5L using the method of Kunkel (1987). The mutated plasmid is designated p4.5LPstAB. After digestion of this plasmid with PstI, we isolated the 837-bp fragment, subcloned this into PstI-digested pTRB-1, and screened for correct orientation of the insert.

pT900-1168. Digestion of p4.5LPstAB with PstI and HindIII yields a 1,026-bp fragment which was subcloned into PstI–HindIII cut pTRB-1.

pT978-1035. We incubated two oligonucleotides, CCCGAGCAGGCGCGTCGC and CGCAAGCTTGCGCGGGATGAG, with p4.5L DNA in a polymerase chain reaction. This reaction yields a fragment consisting of bases 2932–3106 of myosin IC with an additional BamHI site on the 5′ end and HindIII site on the 3′ end. We digested this fragment with BamHI and HindIII and subcloned this into BamHI–HindIII–digested pTRB-1.

Protein Purification

Chimeric β-galactosidase/myosin IC fusion proteins were purified by a modification of the procedure of Koenen et al. (1985). We transformed *Escherichia coli* K-12 71-18 (Δ[lac,pro], F′ lacI^{q}ZΔM15) with the expression plasmids described above, inoculated 50-ml cultures of L broth containing 100 μg/ml of either ampicillin or carbenicillin, and incubated with shaking at 37°C overnight. The 50-ml culture was then used to inoculate a 1-liter culture, which was incubated for 1 h at 37°C. We added isopropyl-thiogalactoside to 50 μM, and incubated at 25°C for an additional 4 h. The bacteria were harvested by centrifugation for 20 min at 7,000 *g*, 4°C. We resuspended the cell pellet in 2 vol of STET buffer (10 mM Tris-HCl, pH 8.0, 8% sucrose, 50 mM EDTA, 0.5% Triton X-100) supplemented with protease inhibitors (1 mM PMSF, 1 μg/ml each chymostatin, leupeptin, aprotinin, and pepstatin), added lysozyme to 0.2 mg/ml, and incubated on ice for 30 min. The cell suspension was then frozen at −70°C for 1–18 h and thawed in ice water. We lysed the cells by sonicating the thawed suspension three times with a microtip sonicator (Sonifier 450; Branson Ultrasonics Corp., Danbury, CT) at power level 4 for 60 s. The suspension was stored on ice for 2 min between each sonication. We centrifuged the lysed cells for 90 min at 100,000 *g*, 4°C and recovered the supernatant. We diluted the supernatant 1:1 with A buffer (10 mM Tris-HCl, pH 7.6, 250 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM PMSF) and applied this to a 10-ml column of *p*-aminophenylthiogalactoside (APTG)[1]-agarose (Steers et al., 1971; Ullmann, 1984) (Sigma Chem. Co., St. Louis, MO) at 4°C. We washed the column with five column volumes of A buffer, eluted the fusion protein with 100 mM Na borate, pH 10.0, collected the fractions containing peak β-galactosidase activity assayed by the method of Miller (1972), and dialyzed them overnight against 100 vol of ME buffer (60 mM potassium glutamate, 10 mM imidazole, pH 6.4, 2 mM EGTA, 2 mM $MgCl_2$, 1 mM ATP). This buffer is identical to that used in the membrane binding experiments of Adams and Pollard (1989) and approximates the intracellular pH and ionic strength of *Acanthamoeba* (Sinard and Pollard, 1989). Occasional preparations that had low molecular weight contaminants after the APTG column were further purified by gel filtration in ME buffer on a Sephacryl S-300 column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). Fusion protein concentrations were determined by the method of Bradford (1976) using β-galactosidase as a standard.

Rabbit muscle actin was purified by the method of Eisenberg and Kielley (1974). Additionally, some preparations were gel filtered as described by MacLean-Fletcher and Pollard (1980). *Acanthamoeba* myosin IB was prepared by the method of Adams and Pollard (1989) and was a gift of Richard Adams.

Membrane Pelleting Assay

Acanthamoeba membranes were stripped of endogenous peripheral proteins with 100 mM NaOH by the method of Zot et al. (1992) and stored at −70°C until use. The fusion protein preparations were centrifuged at 100,000 *g*, 4°C, for 30 min before use. We diluted the membranes in ME buffer supplemented with 100 μg/ml BSA, added fusion protein, and incubated for 30 min on ice. The mixtures were pelleted in a TL-100 rotor (Beckman Instrs., Inc., Fullerton, CA) at 100,000 *g*, 4°C, for 30 min. We assayed the supernatant for β-galactosidase activity and normalized this activity to that of control samples containing no membranes. Since membranes interfered with the assay, we could not directly measure the concentration of β-galactosidase in the pellet. We fit the data to a bimolecular reaction mechanism using a nonlinear regression analysis program (Regression; Blackwell Scientific Software, Oxford) to determine the equilibrium constant. Since the nature of the binding site on the membrane is not known, the concentration of binding sites was determined from the binding isotherms. The phosphate content of the membrane preparations was determined by ashing in $MgNO_3$ followed by inorganic phosphate analysis with malachite green (Stull and Buss, 1977). We calculated the phospholipid content of the membrane preparations by adjusting the phosphate concentration for the presence of lipophosphonoglycan and other phosphate-containing species as reported by Korn and Wright (1973).

Actin Pelleting Assay

Actin filaments and fusion protein were incubated in 50 mM KCl, 10 mM imidazole, pH 6.4, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP on ice for 30 min. We centrifuged the samples at 100,000 *g*, 4°C, for 1 h, collected the supernatant and pellet, and assayed both for β-galactosidase activity. Both measurements were normalized to controls containing no actin, and the concentration bound was calculated for both. We report the average of the supernatant and pellet calculations, which did not differ by more than 7% in all experiments.

Immobilized Ligand Binding Assay

100-nm-diam large unilamellar vesicles produced by the extrusion technique (LUVETS) (Mayer et al., 1986) were prepared as described by Machesky et al. (1990). Dioleoylphospholipids, glycerides, and sterols were purchased from Avanti Polar Lipids Inc., Birmingham, AL (phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine); Sigma Chem. Co., St. Louis, MO (phosphatidylserine, phosphatidic acid, cardiolipin, phosphatidylinositol, diolein, triolein, cholesterol, and ergosterol); and Amersham Corp., Arlington Heights, IL (^{14}C-labeled phosphatidylcholine). APTG–agarose beads were incubated with bacterial extracts containing fusion proteins or native β-galactosidase for 10 min at 4°C in ME buffer and washed three times with 20 vol of ME buffer. This mixture was incubated with LUVETS in ME buffer for 10 min at 25°C and centrifuged in microfuge tubes equipped with 0.65-μm filters (Ultrafree-MC, model UFC30DV00; Millipore Corp., Bedford, MA) at 1,000 *g* for 2 min. The beads and any bound lipid retained on the filter were resuspended in 200 μl 100 mM Na borate, pH 10.0, and counted in scintillation fluid. This represents the LUVETS bound to the APTG–agarose via the fusion protein. The flow-through from the filter centrifugation was counted in scintillation fluid and represents the free LUVETS. The ratio of bound cpm to total cpm is taken as the fraction of LUVETS bound to fusion protein. In all experiments, nonspecific binding curves for LUVETS associating with β-galactosidase/APTG–agarose beads were subtracted from the β*g*701-888/APTG–agarose binding results. This nonspecific binding of LUVETS represented as much as 22% of total bound LUVETS, and was reproducible within 10% from experiment to experiment. This background binding was the same in several different low salt (<100 mM) buffers.

Microscopy

100-nm-diam LUVETS composed of 30% phosphatidylserine (PS), 10% dioleoylphosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Avanti Polar Lipids Inc.), and 60% phosphatidylcholine (PC) were prepared as described above and incubated with either β*g*701-888/APTG–agarose beads or β-galactosidase/APTG–agarose beads for 10 min at room temperature. We centrifuged the mixture as described above, and resuspended the beads in 50 μl of ME buffer. This suspension was applied

1. *Abbreviations used in this paper*: APTG, *p*-aminophenyl-thiogalactoside; LUVETS, large unilamellar vesicles produced by the extrusion technique; PC, phosphatidylcholine; PS, phosphatidylserine.

directly to a glass microscope slide, and a coverslip was mounted over the sample. We obtained phase and confocal fluorescence images using an MRC 600 confocal imaging system (Bio-Rad Laboratories, Richmond, CA) on an Optiphot microscope (Nikon Inc. Instrument Div., Garden City, NY). Images were printed with a Mitsubishi CP210U color video copy processor or photographed from a high resolution monitor.

Results

Expression and Purification of β-Galactosidase/Myosin I Fusion Proteins in E. coli

The myosin IC molecule can be divided into four regions based on primary structure (Fig. 1). The myosin head is similar to the subfragment 1 of myosin II. The next 200 amino acids have a high net positive charge and are referred to here as the basic domain. At the COOH terminus, two regions with net positive charge that are rich in glycine, proline, and alanine are separated by 50 amino acids called the SH-3 domain, which has sequence homology to a noncatalytic region of the c-*src* protooncogene (Jung et al., 1989*b*; Stahl et al., 1988).

We constructed plasmid vectors to express in *E. coli* five *Acanthamoeba* myosin IC/β-galactosidase fusion proteins with various parts of myosin I fused to the COOH terminus of *E. coli* β-galactosidase (Fig. 1). The fusion proteins are named by the prefix "βg" denoting the bacterial sequence, followed by numbers denoting the amino acid residues from myosin IC.

We purified the recombinant proteins by affinity chromatography. All of the fusion proteins were soluble upon lysis after growth at 25°C, indicating that negligible amounts of the recombinant proteins were aggregated or incorporated into bacterial inclusion bodies. When the bacteria were incubated at 37°C rather than 25°C, 10–50% of some fusion proteins pelleted in a 100,000-*g* centrifugation. The use of slow growth conditions to reduce or eliminate inclusion body formation is well established (Schein and Noteborn, 1988; Chalmers et al., 1990). The five fusion proteins were expressed at varying levels, constituting 5–25% of soluble bacterial protein as determined by the method of Bradford (1976). After induction, all lysates showed a major band at the appropriate molecular weight; however, some preparations also had one to three bands with mobilities between the fusion protein and native β-galactosidase (Fig. 2). These presumed breakdown products constituted up to 25% of the total eluate from the APTG–agarose column, but the presence of visible breakdown products had no effect on fusion

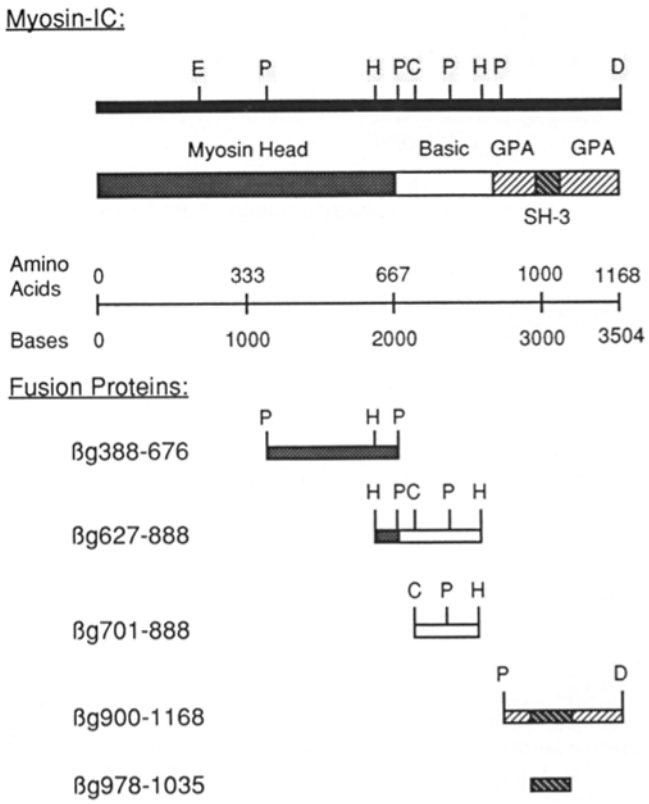

Figure 1. Domain structure of *Acanthamoeba* myosin IC and β-galactosidase fusion proteins. See Materials and Methods for vector construction. *GPA*, glycine-, proline-, and alanine-rich tail regions. *SH-3*, c-*src* homology region 3. Restriction sites: *P*, PstI; *C*, HincII; *H*, HpaII; *D*, HindIII; *E*, EcoRI.

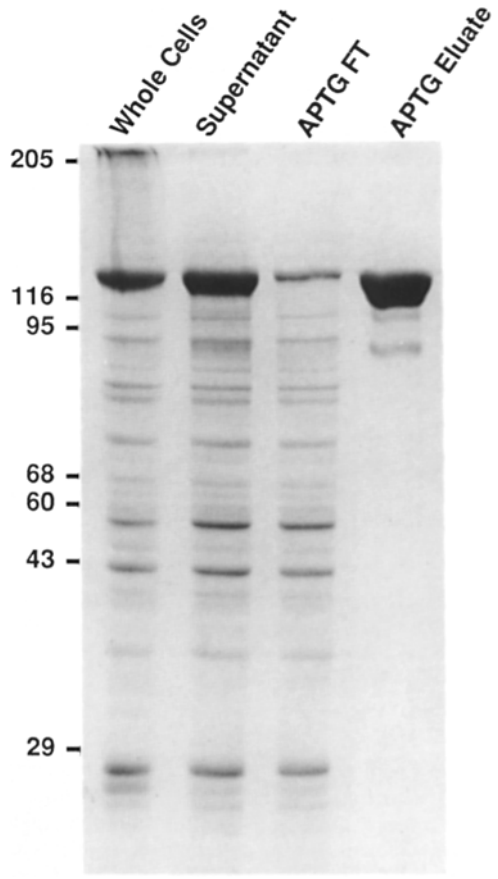

Figure 2. SDS-PAGE analysis of β-galactosidase–myosin IC fusion proteins. *Whole Cells*, whole *E. coli* 71-18 carrying pT701-888 after induction with isopropyl-thiogalactoside. *Supernatant*, 100,000-*g* supernatant after lysis and centrifugation of cells. *APTG FT*, proteins eluting from APTG–agarose column with A buffer (10 mM Tris-HCl, pH 7.6, 250 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM PMSF). *APTG Eluate*, proteins eluting from APTG–agarose column with 100 mM Na borate, pH 10.0. Note presence of minor breakdown products (see Results).

protein behavior in pelleting assays. β-Galactosidase is active as a tetramer, and we suspect that heterotetramer products formed between full-length and degraded proteins, resulting in at least one intact binding site per β-galactosidase fusion protein. SDS-PAGE of supernatants and pellets from pelleting assays of preparations containing breakdown products show equivalent amounts of full-length and smaller products (data not shown).

Binding of Fusion Proteins to Stripped Membranes

Recombinant molecules containing residues 701–888 (βg677–888 and βg701–888) pellet with membranes (Fig. 3), and proteins lacking this region do not. The fusion proteins containing the COOH-terminal 30 kD of the myosin IC tail (βg900–1168) and the SH-3 region alone (βg978–1035) show no affinity for stripped membranes in these experiments (Fig. 3).

The amount of βg627–888 and βg701–888 binding to stripped membranes depends on the concentrations of fusion protein (Fig. 3) and of stripped membranes (Fig. 4) and all of the data is consistent with a dissociation equilibrium constant of 200 nM. This value is similar to those previously reported for intact myosin I binding to these membrane preparations (Adams and Pollard, 1989). The number of binding sites determined by nonlinear regression analysis varied <10% in all membrane preparations used, and the saturation capacity of the membranes was ~1 μg fusion protein per μg membrane protein, or one fusion protein binding site per 400 phospholipid molecules. The fusion proteins do not bind to stripped membranes in 500 mM NaCl (data not shown).

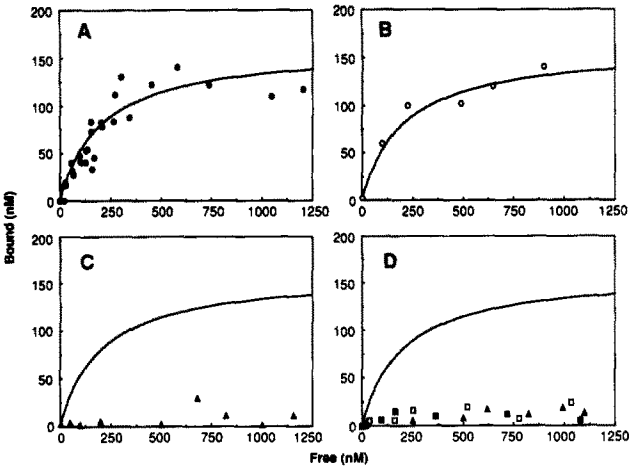

Figure 3. Binding of recombinant proteins to stripped membranes. Dependence on the concentration of fusion protein. Conditions: NaOH-stripped membranes containing 640 nM total phospholipids were incubated with the following fusion proteins in ME buffer with 100 μg/ml BSA: (*A*) βg627–888 (●); (*B*) βg701–888 (○); (*C*) *E. coli* β-galactosidase (△); (*D*) βg388–676 (■), βg900–1168 (□), and βg-978–1035 (▲). Bound and free fusion protein were determined enzymatically after centrifugation. Total recovery was between 90 and 100% for all experiments. The curves shown are theoretical binding isotherms for K_d = 200 nM (assuming four binding sites per fusion protein or β-galactosidase tetramer) and a membrane binding site concentration of 160 nM. The binding site concentration for each membrane preparation was determined from the saturation point of the isotherms.

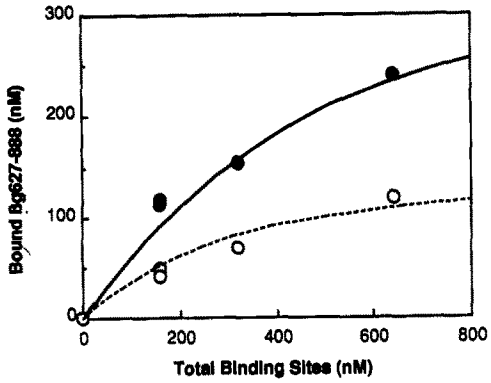

Figure 4. Binding of recombinant proteins to stripped membranes. Dependence on the concentration of membranes. Conditions: βg677–888 at 150 nM (○) or 350 nM (●) in ME buffer were incubated with stripped membranes. Bound and free fusion protein concentrations were determined by centrifugation and assaying the supernatant for β-galactosidase activity. Total recovery was 95–100% for all experiments. The curves shown are theoretical binding isotherms for K_d = 200 nM.

βg627–888 competes with *Acanthamoeba* myosin IB for stripped membrane binding sites (Fig. 5). The amount of bound fusion protein in the presence of varying concentrations of myosin IB is consistent with the K_d for β627–888, the concentration of binding sites determined by cosedimentation in the absence of myosin IB, and the K_d for myosin I reported by Adams and Pollard (1989). Neither βg388–676 nor βg900–1168 competed with myosin IB for binding to stripped membranes (data not shown).

Binding of Unilamellar Vesicles to Immobilized βg701–888

Fluorescent LUVETS containing 30% PS bind to βg701–888 immobilized on APTG–agarose beads, but not to immobi-

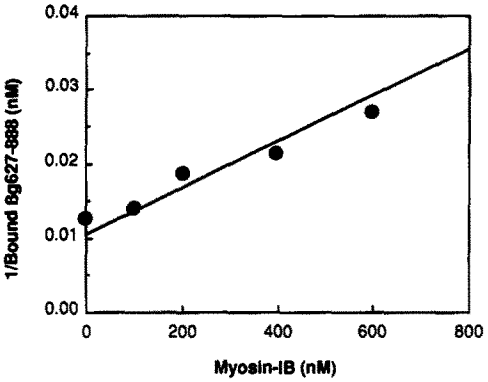

Figure 5. Dixon plot of competition between βg677–888 and *Acanthamoeba* myosin IB for binding to stripped membranes. βg677–888 was added to increasing amounts of myosin IB in the presence of stripped membranes containing 640 nM total phospholipids. After centrifugation, bound and free fusion protein concentrations were determined by β-galactosidase assay of the supernatant. The curve shown is a theoretical line calculated assuming K_d = 200 nM for binding of βg677–888 to membranes and K_d = 170 nM for binding of myosin IB to membranes.

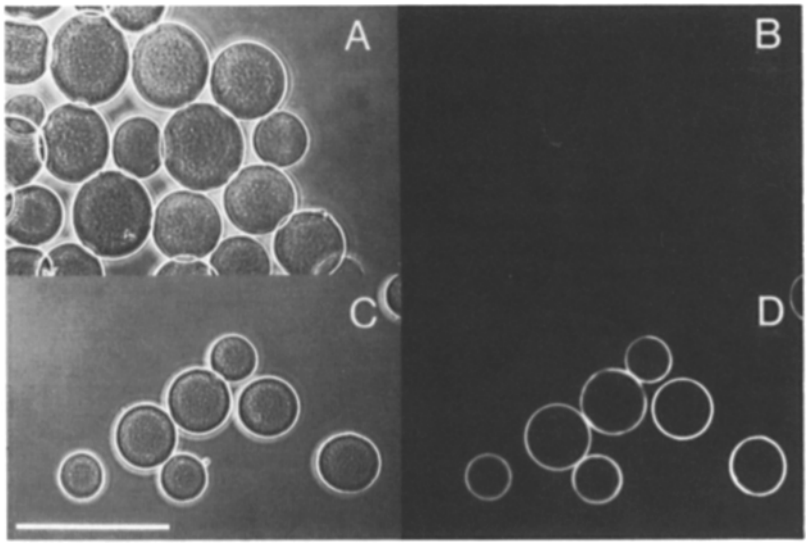

Figure 6. Confocal phase-contrast (*A* and *C*) and fluorescence (*B* and *D*) micrographs of LUVETS containing 30 mol% phosphatidylserine and 10 mol% rhodamine-labeled phosphatidylethanolamine after incubation with ligand immobilized on APTG agarose beads. (*A* and *B*) β-galactosidase/APTG-agarose; (*C* and *D*) βg701-888/APTG-agarose.

lized β-galactosidase (Fig. 6). Fluorescent LUVETS were excluded from the interior of the agarose beads, as expected from the pore size of Sepharose 4B and the diameter of the LUVETS.

PS/PC LUVETS containing 5–30% PS bind to βg701-888 immobilized on APTG-agarose beads (Figs. 7 and 8). We determined the bound and free LUVET concentrations by radioactive labeling of the vesicles with ^{14}C-PC and after correction for background binding calculated the apparent K_d by nonlinear regression analysis as described above. A minimum concentration of 5 mol% PS is required for vesicle binding to immobilized fusion protein. The apparent K_d values for PS/PC LUVETS binding to βg701-888/APTG-agarose in this assay are much less than 10 nM for 10 mol% to 30 mol% PS, while LUVETS containing 2.5 mol% PS have an apparent K_d greater than 200 nM, which is beyond the sensitivity of this assay. LUVETS with 5–7.5 mol% PS have intermediate affinities.

LUVETS containing 20 mol% of either of two acidic phospholipids, phosphatidylinositol or phosphatidic acid, bind to βg701-888/APTG-agarose with apparent K_d values of 5.0 and 6.8 nM, respectively (Table I). These results compare quite closely with the apparent K_d of 5.0 nM for binding of 20 mol% PS. Neutral phospholipids bind weakly, if at all, with apparent K_d values of 140–150 nM.

LUVETS with the same phospholipid composition as *Acanthamoeba* plasma membranes (47.2% phosphatidylethanolamine, 26.9% PS, 18.6% PC, 5.0% phosphatidic acid, 2.6% cardiolipin, and 0.2% PI; Ulsamer et al., 1971) bind βg701-888/APTG-agarose with an apparent K_d of 5.2 nM (Table I). Within the sensitivity of the assay, addition of diglycerides and triglycerides in the molar ratios determined by Ulsamer et al. (1971) had no effect on the apparent K_d, nor did addition of up to 30 mol% cholesterol or 10 mol% ergosterol, the maximum that could be incorporated into the LUVETS.

Binding of Fusion Proteins to Actin Filaments

βg900-1168, which includes the COOH-terminal 30 kD of the myosin IC tail, binds to actin filaments with an uncorrected K_d of 560 nM (Fig. 9). The K_d was determined by nonlinear least squares analysis of the binding data. This assay is complicated by relatively high background binding due to β-galactosidase–actin interactions. When corrected for actin binding to β-galactosidase alone, the dissociation constant is ~100 nM. This is similar to the K_d of <500 nM reported by Lynch et al. (1986) for binding of a COOH-terminal 30-kD proteolytic fragment of *Acanthamoeba* myosin IA to actin filaments.

βg978-1035, which includes the SH-3 domain but not the surrounding sequences, shows no binding above background

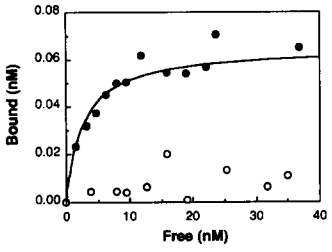

Figure 7. Binding of 30 mol% phosphatidylserine LUVETS to βg701-888/APTG-agarose. Dependence on the concentration of phosphatidylserine. LUVETS containing 30 mol% (●) or 0 mol% (○) phosphatidylserine were incubated with βg701-888/APTG-agarose and centrifuged through 0.65-μm filters. The amounts of bound and free LUVETS were determined by counting the flow-through and the retentate in a scintillation counter. The curve shown is an isotherm calculated assuming K_d = 2.85 nM.

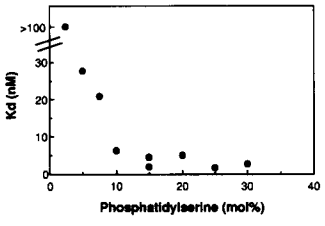

Figure 8. Dependence of the dissociation constant for binding of PS/PC LUVETS to βg701-888/APTG-agarose on the mole fraction of phosphatidylserine. LUVETS containing 2.5 to 30 mol% PS were incubated with βg701-888/APTG-agarose and centrifuged through 0.65-μm filters. The amounts of bound and free LUVETS were determined by counting the flow-through and the retentate in a scintillation counter. Dissociation constants were determined by nonlinear least squares analysis.

Table I. Apparent Dissociation Constants for Binding of LUVETS to Bg701–888/APTG–Agarose: Dependence on the LUVET Composition

LUVET Composition	Apparent K_d
	nm
Acidic Phospholipids	
20% PS	5.0
20% PA	6.8
20% PI	5.0
Neutral Phospholipids	
100% PC	140
44% PE	145
Plasma Membrane Compositions	
PM	5.2
PM/Glycerides	3.6
PM/Glycerides/30% Cholesterol	5.5
PM/Glycerides/10% Ergosterol	5.7

20%PS, 20 mol% PS/80 mol% PC; *20%PA*, 20 mol% PA/80 mol% PC; *20%PI*, 20 mol% PI/80 mol% PC; *44%PE*, 44 mol% PE/56 mol% PC; *PM*, LUVETS composed of phospholipids in the molar ratios determined for *Acanthamoeba* plasma membranes (47.2% PE, 26.9% PS, 18.6% PC, 5.0% PA, 2.6% cardiolipin, 0.2% PI; Ulsamer et al., 1971); *PM/Glycerides*, PM LUVETS with di- and triglycerides added in the molar ratios determined by Ulsamer et al. (1971); *PM/Glycerides/30% Cholesterol*, PM/Glycerides LUVETS with 30 mol% cholesterol added; *PM/Glycerides/10% Ergosterol*, PM/Glycerides LUVETS with 10 mol% ergosterol added.

in this assay (Fig. 9). Elimination of the SH-3 domain from βg900–1168 reduces actin binding to background levels (data not shown); however, this may be due to improper folding of the surrounding sequences rather than direct interaction between the SH-3 sequence and actin filaments. Alternatively, the surrounding glycine-, proline-, and alanine-rich domains may be necessary for proper folding of the SH-3 polypeptide. βg627–888 and βg388–676 also show no affinity above background for actin filaments in this assay.

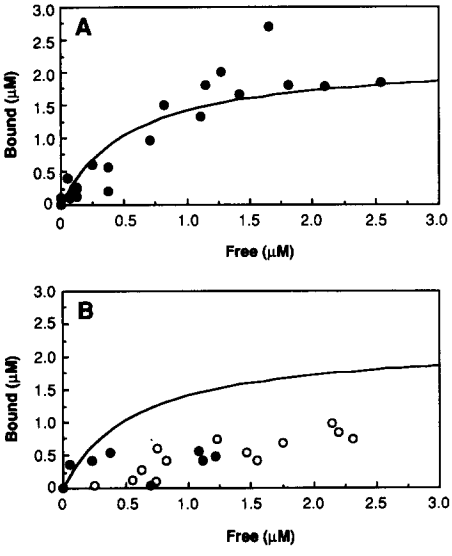

Figure 9. Binding of fusion proteins to actin filaments. Dependence on the concentration of fusion protein. Conditions: (*A*) βg900-1168 (●) and (*b*) *E. coli* β-galactosidase (○) or βg978–1035 (●) were added to actin filaments in 50 mM KCl, 10 mM imidazole, pH 6.4, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP, and 1% BSA. The actin filaments were pelleted by centrifugation and the amounts of bound and free fusion protein were determined by assaying for β-galactosidase activity. The curve shown is an isotherm for $K_d = 560$ nM.

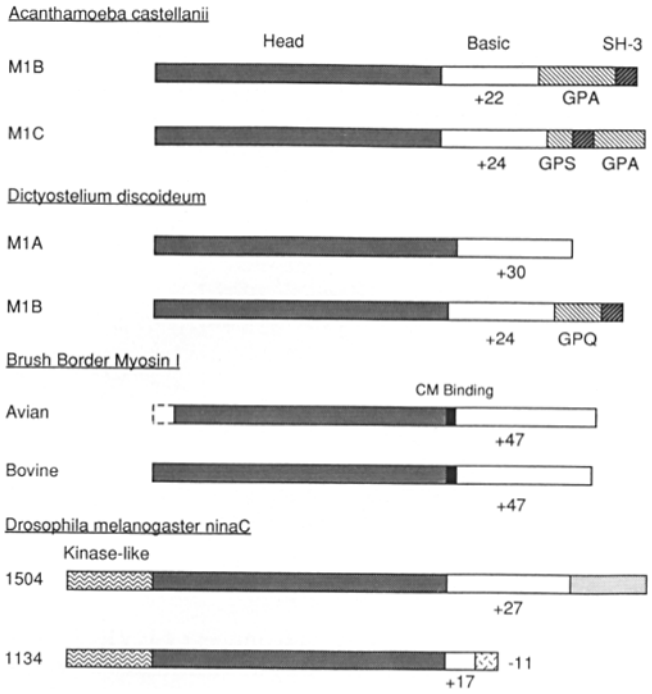

Figure 10. Domain structure of myosin I isoforms. Note concentrations of positive charges in basic domain COOH-terminal to the myosin head. Based on sequence data from Garcia et al. (1989) (avian brush border myosin I), Hoshimaru and Nakanishi (1987) (bovine brush border myosin I), Jung et al. (1987) (*Acanthamoeba* myosin IC), Jung et al. (1989*a*) (*Dictyostelium* myosin IB), Jung et al. (1989*b*) (*Acanthamoeba* myosin IB), Montell and Rubin (1988) (*Drosophila ninaC*), and Titus et al. (1989) (*Dictyostelium* myosin IA).

Discussion

We used recombinant fusion proteins as both free and immobilized ligands to map functional domains and to study lipid specificity in the binding of myosin IC to anionic phospholipids. This system has the advantages of versatility in protein construction, ease of protein purification, and experimental accessibility with both stripped natural membranes and synthetic phospholipid vesicles.

In addition to traditional pelleting assays with isolated membranes, we used a new assay for association of phospholipid head groups with peripheral membrane proteins. This method has three advantages over a conventional pelleting assay with soluble proteins and non-LUVET lipid vesicles. First, LUVETS are much more homogeneous than most other phospholipid preparations. The narrow size distribution and absence of multilamellar vesicles makes quantitation of binding results considerably more accurate. Second, immobilization of the ligand on agarose beads facilitates rapid, clean separation of the bound and free lipid by a simple centrifugation step. This is particularly important with LUVETS that have approximately the same density as the buffer and hence cannot be pelleted away from the free pro-

tein. Third, we purified and immobilized the fusion protein in the same step by binding it to beads coupled to APTG. The coupling method described here using the nonhydrolyzable substrate analogue APTG to bind β-galactosidase is by no means the only useful method. We expect that nonsubstrate affinity resins such as antibody-coupled agarose or calmodulin–agarose would be equally convenient. Covalent binding of proteins to agarose beads using CNBr-activated agarose might also be productive, although less convenient because of the need to purify the protein before coupling.

In spite of many advantages, both of the assays used in this study have some limitations. The analysis of the stripped membrane pelleting assay is complicated by the tetrameric nature of β-galactosidase, which prohibits an exact measurement of the dissociation constant. The immobilized ligand assay also yields apparent rather than actual values for the dissociation constants, since the exact nature of the fusion protein binding site on the vesicles (and therefore the number of binding sites per vesicle) is unknown. Further, the relative size of the LUVETS and agarose beads make it likely that one binding event will sterically hinder other fusion protein molecules on the beads from interacting with other LUVETS. Nevertheless, the values determined in both assays are useful as a relative measure of binding affinity, if not an exact determination of the protein/lipid binding site dissociation constant. Because of these limitations, the dissociation constants determined from these two assays are not directly comparable.

The results described here localize the phospholipid binding site of *Acanthamoeba* myosin IC to the region between amino acids 701 and 888, in the NH_2-terminal half of the tail. This is reasonable based on previous indirect evidence showing that neither the head domain of skeletal muscle myosin nor the COOH-terminal 30-kD fragment of the myosin IA tail bind lipids (Adams and Pollard, 1989). This region may not be the minimum essential phospholipid binding site, but it certainly contains this site within its boundaries.

The major distinguishing characteristic of this region is the high concentration of the basic amino acids lysine, arginine, and histidine. There is some sequence similarity between the myosin IC membrane binding domain and the other protozoan isoforms (Hammer, 1991), and, although this sequence is not well conserved among myosin I's in higher eukaryotes, the net positive charge in the NH_2-terminal region of the tail is a common feature of many myosin I's. The net charge in the 200 amino acids after the head domain range from +22 for *Acanthamoeba* myosin IB to +40 for the avian brush border myosin I (Fig. 10). We suggest that the basic domains of these molecules are indeed homologous, i.e., evolutionarily related, despite the low level of sequence similarity. Several gene duplication events could have given rise to the family of myosin I genes. If there is no selection for exact sequence in the basic domain, but rather a general selection for positively charged amino acids on the tail surface, we might expect to see charge conservation without sequence similarity in this region, even as the head and remainder of the tail domains remain relatively well conserved.

Our experiments with stripped *Acanthamoeba* membranes and β-galactosidase fusion proteins yield an apparent dissociation constant of 200 nM. This measurement is in good agreement with previous observations. Adams and Pollard (1989) reported a K_d of 139 nM in binding experiments with intact *Acanthamoeba* myosin IA and IB and membrane preparations identical to those used in this study. In experiments using highly purified plasma membranes stripped of peripheral membrane proteins by KI, Miyata et al. (1989) reported a lower K_d of 40 nM. The higher molar fraction of acidic phospholipids in plasma membranes compared with the unfractionated membrane preparations used here may contribute to the difference in affinity. The plasma membrane phospholipids of *Acanthamoeba* are 34.7% acidic phospholipids (Ulsamer et al., 1971), while total *Acanthamoeba* phospholipids are only 21.8% acidic (Ulsamer et al., 1969). Our lipid preparations are undoubtedly closer to the "total" composition, while the membranes prepared by Miyata et al. (1989) should have a higher acidic phospholipid content and therefore a higher affinity for myosin I.

The ratio of phospholipid molecules to myosin I binding sites on the stripped membranes is ~400:1. The actual number of acidic phospholipid head groups per binding site is certainly much lower than 400, since the membranes used in this assay are multilammellar and only ~25% of the phospholipids are acidic.

The interaction between positively charged amino acids on the myosin I tail and negatively charged phospholipids in the membrane appears to be primarily electrostatic. There is no evidence for a transmembrane domain in myosin I, and no suggestion of any acyl modifications. Binding of the myosin IC fusion proteins to lipid bilayers requires ~5% acidic phospholipids and is inhibited by high salt. All negatively charged phospholipids tested support binding. The conservation of net positive charge in the NH_2-terminal region of the tails of many myosin I's suggests that the affinity for acidic phospholipids may be mediated by a common mechanism in these molecules. The binding appears to be relatively forgiving of variations in lipid head structure and protein primary sequence.

Association of the myosin I tail with phospholipids may be the primary interaction that concentrates myosin I at the membrane. This conclusion is supported by the ability of pure lipid bilayers to support myosin I–based motility of actin filaments (Zot et al., 1992).

Nevertheless, the presence of many isoforms of myosin I from different genes in both *Acanthamoeba* (Jung et al., 1987,1989*b*) and *Dictyostelium* (Jung et al., 1989*a*; Titus et al., 1989) and the strikingly different localization of the two *ninaC* isoforms in the *Drosophila* photoreceptor cell (Porter et al., 1992) suggest that membranes may also have protein receptors that lend some specificity to the interaction of these isoforms with various membranes. The hypothetical interactions of myosin I with protein receptors might be relatively weak since phospholipid binding may concentrate myosin I at the membrane. Local differences in phospholipid composition may contribute to the differential localization of myosin I isoforms, but the relative promiscuity of myosin I for all acidic phospholipids makes this less likely.

Pelleting experiments prove the presence of an ATP-insensitive actin binding site in the COOH-terminal 30 kD of myosin IC, similar to that found directly in myosin IA (Lynch et al., 1986) and indirectly in myosin IB (Brzeska et al., 1988). This site, between residue 900 and the COOH terminus at residue 1168, binds actin with a submicromolar K_d. When corrected for actin–β-galactosidase interactions,

the dissociation constant for the myosin IC fusion proteins is ~100 nM, similar to the value of 120–340 nM for the COOH-terminal 30-kD proteolytic fragment of myosin IA (Lynch et al., 1986). The multivalent fusion proteins may cross-link actin filaments in this assay, but we could not detect this by falling ball viscometry (data not shown).

Protease protection assays provided evidence for the localization of the ATP-sensitive actin binding site of *Acanthamoeba* myosin IA in the NH_2-terminal 62 kD of the molecule (Brzeska et al., 1988). Since βg388–676 (which includes the region corresponding to the myosin IA ATP-sensitive actin binding site) does not bind to actin filaments, sequences in the NH_2-terminal 40 kD of myosin IC may be necessary for the folding and/or activity of this site. The NH_2-terminal 40-kD region includes the polyphosphate binding site and the phosphorylation site necessary for activation of the ATPase activity.

The SH-3 domain of myosin IC lies within the region reported here as the actin binding region. This sequence motif has been speculated to be either a membrane binding site (Rodaway et al., 1989) or an actin binding site (Drubin et al., 1990). Our results can neither support nor explicitly disprove these hypotheses. The lack of affinity for actin by fusion proteins containing only SH-3 sequences suggests that this sequence either may not interact directly with actin or may require the surrounding glycine-, proline-, and alanine-rich domains to fold correctly. It is also possible that the SH-3 domain is shielded from the actin by some portion of the β-galactosidase molecule. We suspect that the SH-3 domain may be involved in myosin I–receptor interactions, since many SH-3 proteins are transiently associated with membranes in vivo (Clark et al., 1990; Hamaguchi and Hanafusa, 1987; Todderud et al., 1990) and do not appear to have appreciable affinity for actin or phospholipids alone.

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