Identification of an Actin-Binding Site in the Basic (Tail Homology-1) Domain

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Wei-Lih Lee‡§, E. Michael Ostap¶, Henry G. Zot†, and Thomas D. Pollard§**

From the ‡Structural Biology Laboratory, The Salk Institute for Biological Studies, La Jolla, California 92037, the ¶Department of Biology, Eastern Michigan University, Ypsilanti, Michigan 48197, the §BCMB Graduate Program, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and the †Pennsylvania Muscle Institute and the Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

The Acanthamoeba myosin-IA heavy chain encodes a 134-kDa protein with a catalytic domain, three potential light chain binding sites, and a tail with separately folded tail homology (TH) -1, -2, and -3 domains. TH-1 is highly resistant to trypsin digestion despite consisting of 15% lysine and arginine. TH-2 is resistant to \( \alpha \)-chymotrypsin digestion. The peptide link between TH-1 and TH-2 is cleaved by trypsin, \( \alpha \)-chymotrypsin, and endo-AspN but not V8 protease. The CD spectra of TH-2/3 indicate predominantly random structure, turns, and \( \beta \)-strands but no \( \alpha \)-helix. The hydrodynamic properties of TH-2/3 (Stokes’ radius of 3.0 nm, sedimentation coefficient of 1.8 S, and molecular mass of 21.6 kDa) indicate that these domains are as long as the whole myosin-I tail in reconstructions of electron micrographs. Furthermore, separately expressed and purified TH-1 binds with high affinity to TH-2/3. Thus we propose that TH-1 and TH-2/3 are arranged side by side in the myosin-I tail. Separate TH-1, TH-2, and TH-2/3 each binds muscle actin filaments with high affinity. Salt inhibits TH-2/3 binding to muscle actin but not amoeba actin filaments. TH-1 enhances binding of TH-2/3 to muscle actin filaments at physiological salt concentration, indicating that TH-1 and TH-2/3 cooperate in actin binding. An intrinsic fluorescence assay shows that TH-2/3 also binds with high affinity to the protein Acan125 similar to the SH3 domain of myosin-IC. Phylogenetic analysis of SH3 sequences suggests that myosin-I acquired SH3 domain after the divergence of the genes for myosin-I isoforms.

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** To whom correspondence should be addressed: The Salk Institute for Biological Studies, 10010 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 619-453-4100 (ext. 1261); Fax: 619-546-0838; E-mail: pollard@salk.edu.

1 The abbreviations used are: TH, tail homology; kb, kilobase pairs; MOPS, 4-morpholinopropanesulfonic acid; GST, glutathione S-transferase; RT-PCR, reverse transcriptase-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; UTR, untranslated region; TFE, trifluoroethanol; HA, hydroxylapatite; Trx, thioredoxin; CAPS, 3-(cyclohexylamino)propanesulfonic acid; bp, base pair.
Fig. 1. Acanthamoeba myosin-IA gene structure. A, genomic DNA sequence is drawn as predicted exons (box) connected by introns (line) and with flanking 5′-UTR (line) and 3′-UTR (line). Two fragments, BamHI and SalI clones, isolated from phage 17A are shown. Sequences coding for different regions of the protein are shaded with the same pattern in C. Genomic sequence of myosin-IA is available in GenBank with accession number AAC35357. B, deduced protein sequence of myosin-IA. The TEDS rule phosphorylation site, threonine 330, is boxed. Sequence flanking the phosphorylation site is bold (22). The three IQ motifs are highlighted. Residues identical to the N-terminal sequences of the 30-kDa α-chymotryptic fragment of myosin-IA are bold (12). The C-terminal SH3-domain is underlined. C, the domain organization of myosin-IA and expression constructs of myosin-IA tail. T1, T2, and T3 are tryptic sites determined in Fig. 3D. Asp1 and Asp2 are endo-AspN proteolytic sites deduced using “Results.” Three RT-PCR products of myosin-IA coding for the tail domains are reverse-transcribed Acanthamoeba mRNA with primer R and subsequently PCR with three separate pair of primers (primer positions shown in A: lane 1, primers 1 and R; lane 2, primers 2 and R; lane 3, primers 3 and R. RT-PCR products are separated by electrophoresis in 1% agarose. Marker sizes are in kb. E, Northern analysis of myosin-IA transcript. Acanthamoeba poly(A) mRNA was separated by electrophoresis and blotted with probe N (shown in A) spanning the IQ motif derived from the genomic BamHI clone. Marker sizes are in kb.

binding and binds tightly to the TH-2/3 domains. Hydrodynamic data suggest that TH-2/3 must fold back onto TH-1. This biochemical evidence is consistent with three-dimensional reconstructions of electron micrographs of myosin-Is attached to actin filaments (19–21).

MATERIALS AND METHODS

Cloning of Acanthamoeba Myosin-IA Gene, cDNA Tail—Degenerate oligonucleotides corresponding to conserved myosin ATP-binding site (QCVIISGESGAGKTEASK) and to myosin-IA-specific phosphorylation site (AGTTYALNLNKM) (22) were used to amplify a 1-kb fragment, SUB01, from an Acanthamoeba genomic library constructed in the λ phage vector (a gift from Dr. Eric Bateman, University of Vermont). SUB01 was used to screen the genomic library for the myosin-IA gene. From eight positive phage clones, we selected the largest fragment containing SUB01 by restriction mapping and Southern blot analysis. A 9-kb BamHI fragment was cloned into pBluescript. This fragment did not span the entire myosin-IA gene. We used a 250-bp SalI-BamHI fragment from the 3′ end of the 9-kb BamHI fragment as a probe to identify an overlapping 1.6-kb SalI fragment from the parent phage. Both 9-kb BamHI and 1.6-kb SalI fragments were subcloned into smaller sizes for sequencing on both strands by primer walking. We used 10% formamide in our dideoxy sequencing reactions (U. S. Biochemical Corp.) to eliminate “stalling” of DNA polymerase at GC-rich sequences (23).

We cloned three cDNA fragments of Acanthamoeba myosin-IA coding for the IQ, TH-1, -2, and -3 domains by RT-PCR using either poly(A) mRNA (data not shown) or total RNA as template (Fig. 1C). Total RNA was isolated from 6 g of Acanthamoeba in mid-log phase. Cells were lysed with a glass-Teflon homogenizer in 80 ml of TRIZOL Reagent (Life Technologies, Inc.) and processed according to the manufacturer. Poly(A) mRNA was isolated from total RNA using the Poly(A) Quik mRNA Isolation kit (Stratagene, La Jolla, CA). Reverse transcription of total or poly(A) mRNA was performed at 42 °C for 1 h using Superscript II RT (Life Technologies, Inc.) with primer R (TTAGATGTCCTCGAG- TagTT). Subsequent PCR reactions were done in 20% glycerol using Elongase Enzyme Mix (Life Technologies, Inc.) with reverse primer R and forward primer 1 (ATAACATATCGGCAACGACC), primer 2 (CT- GCCTGAGAACATTTGGC) or primer 3 (CCGAGTTCAAGAACCTCCTC-GC). RT-PCR products were cloned into pBluescript for restriction analyses and sequencing. Northern blotting of Acanthamoeba RNA was performed as described (18).

Splice Site Determinations—We used the cDNA sequences of the RT-PCR products to map intron/exon boundaries in the gene sequence coding for the IQ, TH-1, -2, and -3 domains. We predicted the intron/exon boundaries in the genomic sequence coding for the catalytic domain of Acanthamoeba myosin-IA. First, we took advantage of the fact that myosin-I catalytic domain is highly conserved. We compared the Acanthamoeba myosin-IA gene sequence with those in the data base using tBLASTx function of BLAST (translated DNA query versus translated DNA data base) (25) to identify putative exons coding for the catalytic domain. We then searched for the usually conserved 5′ (GTAC) and 3′ (CCAG) splice site sequences flanking the putative exons. These 5′ donor site and 3′ acceptor site sequences were identified in other cloned Acanthamoeba genes: TFIID (26), myosin-IB (8), myosin-IC (7), actin I (27), and myosin-II (28). We predicted the start codon of Acanthamoeba myosin-IA based on alignment with and what is found for Acanthamoeba myosin-IB and myosin-IC genes, in which the start codon was immediately followed by a 5′ donor splice site.

Expression and Purification of Recombinant Myosin-IA Tail Domains—Constructs of tail domains are shown in Fig. 1C. We chose the N terminus of the A123 construct to be immediately downstream of the third IQ motif at residue 757 using the crystal structure of scallop myosin light chain domain as a reference (30). We chose the N terminus of A23 construct to be residue 985, the same as the N terminus of the 30-kDa α-chymotryptic fragment of native myosin-IA (12). We used the RT-PCR product and appropriate primers to amplify the following constructs: A123 (residues 757–1215), A1 (residues 757–984), A2 (residues 985–1161), and A23 (residues 985–1215). Each PCR product was ligated into pMW172 vector (31) at NdeI and BamHI sites. All final constructs were verified by DNA sequencing. We expressed all constructs in freshly transformed Escherichia coli BL21 (DE3) pLysS strain (Stratagene).
A123 Purification—Cells were grown overnight at 16 °C in 500 ml of LB/carbonicillin to $A_600=0.2$, induced with expression for 1 ml isopropyl-1-thio-$eta$-D-galactopyranoside for 24 h at 16 °C, and harvested. One gram of pelletled cells was resuspended in 25 ml of IPED (IPED, 20 mM imidazole, pH 7.5, 2 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) in 500 ml carbonicillin and then sonicated 4 times for 30 s with a sonicator tip of 1.2 cm in diameter (Sonifier 450, Branson Ultrasonics Corp., Danbury, CT) at level 8 and with 4 min incubation on ice between each burst. Crude lysate was spun at 105,000 x g for 30 min to obtain soluble A123. When bacteria were grown at 37 °C rather than 16 °C, >90% of A123 pellets at 100,000 x g. Soluble A123 was partially purified by gel filtration on a 2.5 x 9 cm column of Sephacryl S300 equilibrated with IPED buffer. Protein was monitored by dye binding (Bio-Rad Protein Assay, Bio-Rad) and A123 by SDS-PAGE followed by Coomassie staining. A123 migrated in the void volume together with polynucleotides (DNA/RNA) as determined by UV absorbance and agarose gel electrophoresis. Fractions containing A123 were pooled and used for assessment of domain boundaries (see below). Further purification of A123 from S300 fraction was difficult due to tightly bound bacterial nucleic acids. Hydroxyapatite chromatography in 50% formamide removed all detectable nucleic acids and yielded about 500 μg of pure A123 from 1 g of bacteria. Upon removal of formamide, purified A123 was only soluble at pH >9.5.

A23 Purification—Unlike A123, cells were grown at 37 °C in 650 ml of LB/carbonicillin for 25 h and harvested at pH 9.5. For A23, 3–5 g of bacteria were lysed by sonication in 60 ml of IPED buffer with 0.1 M KCl, 5 μg/ml aprotinin, and 200 μM leupeptin. After clarification, saturated ammonium sulfate, pH 7.5, was added slowly to 20% saturation with gentle stirring for 15 min at 4 °C. The precipitate was pelleted at 100,000 x g for 15 min and discarded. Similar to the 20–40% fraction, 30% and all of the A23 was resolubilized in 9 ml of lysis buffer. We detected no A23 in ammonium sulfate precipitates below 20% or above 40% saturation by SDS-PAGE. After dialysis in IPED buffer and clarification at 3000 x g for 10 min, the 20–40% fraction was passed through a 1 x 9 cm DEAE-Sepharose (Amersham Pharmacia Biotech) column equilibrated with IPED to remove nucleic acids and other bacterial proteins. 7.5 ml of DEAE flow-through was loaded directly on a 1.5 x 59 cm column of Sephacryl S200 (Sigma) equilibrated with IPED with 0.5 M KCl. Fractions of 2 ml were collected and assayed as above. Fractions containing A23 were pooled and dialyzed versus 20 mM imidazole, pH 7.5, 0.5 M KCl, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT. Dialyzed S200 pool was loaded onto a 0.8 x 9 cm column of hydroxyapatite (Bio-Gel HTP Gel, Bio-Rad) equilibrated with the same buffer. Purified A23 flowed through this hydroxyapatite. Hydroxyapatite flow-through was dialyzed into appropriate buffer and concentrated on a Centricon-3 ultrafilter (Amicon Inc., Beverly, MA). The identity of A23 was confirmed by N-terminal protein sequencing and by immunoblotting with M.17 monoclonal antibody (32). Purified A23 was free of DNA/RNA contaminants as determined by UV absorbance (280/260 nm = 2). We measured the concentration of purified A23 using extinction coefficients determined from analysis of ultracentrifugation experiments: 0.651 $A_{280}$/mg/ml or 0.186 $A_{280}$/mg/ml/cm. A typical preparation yielded about 3 mg of A23 from 3 to 5 g of bacteria cells, compared with a yield of less than 3 mg of purified Acanthamoeba myosin-IA from 1 kg of amoebas (5, 6). Purified A23 was very stable, showing no degradation over 50 h at 23 °C during sedimentation equilibrium experiments. Purification of untagged A23 is crucial for obtaining correct measurement of hydrodynamic properties. A23 with a (His)$_6$-tagged aggregated artificially judging from zero length chemical cross-linking and Superdex-S200 gel filtration experiments in 0.1 or 0.5 M NaCl and 5 mM imidazole, pH 7. Bacterial expression also yields much quantities of pure A23 than digestion of Acanthamoeba myosin-IA with a-chymotrypsin (12).

A2 Purification—Similar to A23, cells were grown and lysed, and crude lysate was ammonium sulfate-fractionated and passed through a column of DEAE-Sepharose in IPED buffer. However, the DEAE flow-through was loaded on a 1 x 9 cm column of CM-Sepharose (Amersham Pharmacia Biotech) equilibrated in IPED buffer. After elution with a linear gradient of 0–500 mM NaCl in 1.5-ml column of hydroxyapatite equilibrated with 6 mM urea, 0.5 M NaCl, 10 mM imidazole, pH 7.5, and 1 mM DTT to remove nucleic acids and other bacterial proteins, HA flow-through was loaded on a 1.5 x 75 cm column of Sephacryl S200 equilibrated with 6 mM urea/IPED. Pure A2 was pooled and refolded by dialysis in five sequential steps of decreasing urea concentration into a final buffer containing 10 mM imidazole, pH 7.5, 1 mM EDTA, and 1 mM DTT. We clarified refolded A2 at 105,000 x g for 30 min. The identity of soluble A2 was confirmed by N-terminal protein sequencing. A2 was free of DNA and RNA contaminants as determined by UV absorbance. The yield from 1 g of bacteria was 650 μg of A2 at a concentration of 1 μM. A2 soluble at concentrations ≤1 μM but aggregated at higher concentrations. A2 aggregated during storage at 0 °C, so binding assays described below were performed within 2 days of urea removal.

Expression and Purification of Recombinant Trx-AD3 and Trx-AD3A (977–994) —The expression and purification of Trx-AD3 and Trx-AD3A
(977–994) were modified to increase purity. Soluble bacterial lysate was fractionated with 20–40% saturated ammonium sulfate, pH 7. The precipitate was solubilized, dialyzed, and gel-filtered in 20 mM imidazole, pH 7.5, 300 mM KCl, 2 mM EDTA, and 1 mM DTT on a 1.5 x 74-cm column of Sephacryl S300. Fractions containing thioredoxin fusion proteins were pooled and dialyzed versus 10 mM imidazole, pH 7.5, and 300 mM KCl. Next, sample was loaded onto a 1.5 x 9-cm column of hydroxylapatite equilibrated with the same buffer and eluted with a 0–0.2 M gradient of potassium phosphate, pH 7.4. Hydroxylapatite fractions containing thioredoxin fusion proteins were dialyzed versus 50 mM NaH2PO4, 300 mM KCl and 20 mM imidazole, pH 5, for nickel chromatography as described by Xu et al. (16).

Controlled Proteolytic Digestion of Recombinant A123—Pooled S300 fractions of A123 (0.5 mg/ml) were treated for 10, 20, 30, or 60 min with proteolytic enzymes in 20 mM imidazole, pH 7.5, and 1 mM DTT (20 ng/µl trypsin (Sigma) at 0 °C, 4 ng/µl endo-AspN (Roche Molecular Biochemicals) at 0 °C, 10 ng/µl V8 protease (Sigma) at 23 °C, or 10 ng/µl α-chymotrypsin (Sigma) at 25 °C). Reactions were terminated with 10 mM Pefabloc and 10 mM EDTA and frozen immediately with liquid nitrogen. Boiling sample buffer was subsequently added, and digestion products were separated by SDS-PAGE and stained with Coomassie or transferred to Immobilon-P (Millipore Corp., Bedford, MA). Bands on polyvinylidene difluoride were visualized by Amido Black staining and excised for N-terminal sequencing by automated Edman degradation by Dr. W. Fischer of the Salk Institute.

Stokes’ Radius Measurement of Recombinant A23—Stokes’ radius (Rg) was measured by gel filtration in 0.5 mM KCl, 20 mM imidazole, pH 7.5, 1 mM DTT, and 2 mM EDTA on a 1.4 x 96-cm column of Sephacryl S200 calibrated with blue dextran (void volume), bovine serum albumin (6.8 nm), trypsinogen A (2.09 nm), cytochrome c (1.3 nm), ovalbumin (3.05 nm), carbonic anhydrase (2.36 nm), chymotrypsinogen (2.09 nm), cytochrome c (1.7 nm), and ATP (salt volume). Stokes’ radius of A23 was obtained from plot of erfc⁻¹ of the partition coefficients versus Stokes’ radius according to Ackers (33).

Actin Filament Co-pelleting Assay—Acanthamoeba (34) or rabbit skeletal muscle (35) actin was polymerized for 2 h at room temperature in 1 mM MgCl2, 1 mM EGTA, 10 mM Tris, pH 7.5, and 0, 20, 40, or 60 mM KCl. Purified A23 at 0.1–0.3 µg was incubated with various concentrations of polymerized actin in final 1 mM MgCl2, 1 mM EGTA, 10 mM Tris, pH 7.5, and 0, 20, 40, or 60 mM KCl. The mixtures were centrifuged at 95,000 rpm (rotor TLA 100.2, Beckman Optima TLX ultracentrifuge, Beckman Instruments, Fullerton, CA) for 30 min at 23 °C. The supernatants were transferred to 1.5-ml tubes and evaporated to dryness in an Eppendorf Concentrator (model 5301, Brinkmann Instruments, Inc., Westbury, NY). Each sample was resuspended in sample buffer and separated by SDS-PAGE with a range of A23 standards and stained with Coomassie. A23 was quantified by densitometry of wet gels and analyzed with NIH image software. Binding results were plotted as bound A23 versus total actin concentration, and fit by Equation 1.

\[
\frac{[A23]_{\text{bound}}}{[A23]} = \frac{([K_a + [A23] + [A]] - ([K_a + [A23] + [A]]^2 - 4[A23][A])^{1/2}}{2}
\]

where \([A23]_{\text{bound}}\) is the concentration of A23 bound to actin filaments, \([A]\) is the total actin concentration, \([A23]\) is the total A23 concentration, and \(K_a\) is the dissociation equilibrium constant.

A1 × A23 pelleting with muscle actin filaments was done in 1 mM MgCl2, 1 mM EGTA, 10 mM Tris, pH 7.5, and 60 mM KCl. Purified A1 at about 0.02 µg was pelleted with various concentrations of polymerized actin. After centrifugation, the pellets or air-dried supernatants were analyzed as above.

Circular Dichroism Measurements—CD spectra were collected in an Aviv model 62DS spectropolarimeter (Aviv Associates, NJ) using the manufacturer’s 60DS software. Purified A23 samples at 0.263 or 0.132 mg/ml in 5 mM sodium phosphate, pH 7.6, ± 250 mM NaF, ± 50% TFE were placed in a 0.5-mm cell at 22 °C and scanned 4 times between 260 and 185 nm with an integration time of 1 s, a bandwidth of 1.5 nm, a collection frequency of 1 nm/point, and a photomultiplier tube voltage of <390 V at 185 nm. No post collection smoothing was applied to the data. Buffer spectra were subtracted from sample spectra. The raw data were converted to mean residue ellipticity using the mean residue weight calculated from the composition and the concentration determined by extinction coefficient at 295 nm. Spectral deconvolutions by the method of Yang et al. (36) employed the program PROSEC (DOS version) supplied by Aviv. Spectral deconvolution by the method of Bohm et al. (37) employed CDNN (version 2.0c).
Analytical Ultracentrifugation—Sedimentation velocity and equilibrium were carried out at 23 °C using An-60 Ti rotor in a Beckman Optima XLI ultracentrifuge (Beckman Instruments). For sedimentation equilibrium, we loaded A23 in IPED or IPED with 0.5 mM KC1 into six-hole, charcoal-filled Epon centerpieces and centrifuged them to equilibrium for 20 h at 100,000 g. We monitored absorption at 280 nm collected data sets every hour, calculated the root mean square deviation of each data set from the final using the Matchv7 software (Jeff Lary, National Analytical Ultracentrifuge Facility, Storrs, CT), and considered equilibrium attained when there was no change in root mean square deviation in consecutive data sets. We used the program Reedlft (Jeff Lary, National Analytical Ultracentrifuge Facility) to truncate and save the data for individual samples into separate data sets. Determination of the effective reduced molecular weight of A23 from all data sets by the method of Johnson et al. (38) employed the program Winnoln. We also fit each data set to an exponential function defined by Equation 2.

$$A = Ce^{(r - r_0)/r_e}$$  
(Eq. 2)

where $A$ is 280 nm absorbance, $C$ is a fitting constant, $r$ is radial position, $r_0$ is the radius of the meniscus, and $r_e$ is the effective reduced molecular weight. From these fits we calculated the molecular mass of A23 using a partial specific volume of 0.7164 cm$^3$/g.

For sedimentation velocity, we loaded A23 into two-sector, charcoal-filled Epon centerpieces and centrifuged at 45,000 rpm for approximately 4 h. We monitored sedimentation of A23 by fringe displacement and absorption at 280 nm and collected data sets every 2 min. Sedimentation velocity of A23 was carried out in three different conditions as follows: IPED; 5 mM sodium phosphate, pH 7.6, or IPED with 0.5 mM KC1. Determination of $s_v$ from sedimentation velocity data by the method of Philo (39) and Stafford (40) employed the programs SVEDBERG and DCDT, respectively. The partial specific volume of 0.7164 cm$^3$/g and hydration of 0.4171 g/g were calculated from the amino acid composition using the method of Philo (39) and Stafford (40) employed the programs SVEDBERG and DCDT, respectively. The partial specific volume of 0.7164 cm$^3$/g and hydration of 0.4171 g/g were calculated from the amino acid composition and used in hydrodynamic modeling employing the program SEDNTERP (41). We calculated the extinction coefficients from data sets collected near the end of the run using Equation 3.

$$e = \frac{\Delta A}{\Delta F} \left( \frac{1}{1.17 \text{ cm}} \right) \left( 3.3 \text{ fringes/mg/ml} \right)$$  
(Eq. 3)

where $e$ is the extinction coefficient, $\Delta A$ and $\Delta F$ are the changes in magnitude of absorbance and fringe displacement, respectively, from the meniscus to the plateau.

Intrinsic Fluorescence Measurements—Interactions of recombinant A23 with recombinant Acan125 C-terminal 39-kDa fragment (Trx-A23) were measured by intrinsic tryptophan fluorescence. Measurements were carried out in KMEM buffer (KMEM, 20 mM MOPS, pH 7.6, 0.1 M NaF, and 50% TFE) and 0.263 mg/ml A23 in 5 mM sodium phosphate, pH 7.6, and 250 mM NaF. Trx fusion protein at a fixed level of 0.1 mM was incubated with 5 mM GST, GST-C3, or GST-A23 in KMEM buffer. 200 mM of glutathione beads equilibrated in KMEM was added and subsequently incubated for 30 min at 4 °C. The mixture was pelleted at 16,000 $\times$ g for 10 min. Proteins in the supernatants were precipitated with methanol/chloroform, separated by SDS-PAGE, and stained with Coomassie. We aligned the amino acid sequence of the catalytic domain of selected myosin-IA using the multiple alignment function of ClustalW (42). We chose the last residue of the catalytic domain as the 15th residue after the invariant phenylalanine (Phe-672 in Acanthamoeba myosin-IA) in the usually conserved sequence TKIFIR. The alignment was used to generate a bootstrapped tree using the neighbor-joining method of ClustalW. We used NPlot to display the unrooted tree as a dendrogram, drawn to scale based on sequence divergence.

We aligned the amino acid sequence of the SH3 domain of Acanthamoeba myosin-IA to selected SH3 sequences from various SH3-containing proteins. We chose the first residue of the SH3 domain to be the 33rd residue N-terminal to the invariant proline (Pro-1195 in Acanthamoeba myosin-IA), and the last residue of the SH3 domain to be the 7th residue after the invariant proline (Pro-1208 in Acanthamoeba myosin-IA). The alignment was used to generate a database for a bootstrapped tree using default parameters of ClustalW. The unrooted tree was drawn using default parameters of the PHYLIP program and manipultated in Canvas program.

We used the program MODELLER (43) to generate atomic models of the myosin-IA and myosin-IC SH3 domains based on the structure of other SH3 domains. Templates with high protein sequence identities to myosin-IA or myosin-IC SH3 were used individually or in combination for comparative modeling: Protein Data Base Codes 5hck (44), 1gfc (45), 1hsq (46), and 1neb (47) templates (see Fig. 10 legend) are 41, 44, 44 and 50% identical to myosin-IA SH3, respectively. Template sequences were aligned with myosin-IA or myosin-IC SH3 by ClustalW before input for automated comparative modeling by MODELLER. The best myosin-IA or myosin-IC SH3 model was selected when spatial restraints derived from the template structures were satisfied as well as possible.

RESULTS

Primary Structure of Acanthamoeba Myosin-IA—Acanthamoeba myosin-IA was the first unconventional myosin discovered (5) but resisted cloning until now. We mapped the exon/intron junctions in the myosin-IA gene by three criteria as follows: comparison with other myosin-Is, a search for consen-
sus splice donor/acceptor sites, and the sequences of the available cDNAs. Acanthamoeba myosin-IA gene consists of 22 exons of varying sizes (mean of 166 bp and standard deviation of 87 bp) and 21 introns of approximately uniform sizes (mean of 97 bp and standard deviation of 21 bp) (Fig. 1A). Northern blot of Acanthamoeba poly(A) mRNA using myosin-IA-specific DNA probe detected a single band of 4 kb (Fig. 1E), consistent with a size predicted from splicing all exons plus 3'5' bp of 3' and 5' UTR.

Acanthamoeba myosin-IA heavy chain gene encodes a protein of 134 kDa (Fig. 1B) consisting of a catalytic domain (residues 1–687), three IQ motifs (residues 688–756), a basic region (TH-1, residues 757–984), GPA-rich region (TH-2, residues 985–1161), and SH3 domain (TH-3, residues 1162–1215) (Fig. 1C). Our genomic sequence codes for Ala at residue 354 where Brzeska et al. (22) reported Ser in the peptide phosphorylated by myosin-I heavy chain kinase. The TEDS rule phosphorylation site, Thr-330, is located 16 residues N-terminal to the conserved DALAK sequence. The basic TH-1 domain has a calculated pI of 9.72. TH-2 is rich in Gly (39%), Pro (28.8%), and Ala (11.9%). TH-3 is a small 55-residue src homology 3 domain.

The sequence of the catalytic domain of Acanthamoeba myosin-IA is more similar to Dictyostelium myosin-IC than any other known myosin, including Acanthamoeba myosin-IB and myosin-IC (Fig. 2A and Table I). The sequence following the catalytic domain of Acanthamoeba myosin-IA, residues 688–756, is strikingly similar to that of Dictyostelium myosin-IC (Fig. 2B) and includes three potential light chain binding sites. Acanthamoeba myosin-IA residues Ile-733 to Val-756 are a recognizable but imperfect IQ motif. The sequences Asn-688 to Cys-711 and Val-712 to Glu-728 are similar to each other and to the first IQ motif identified in Aspergillus nidulans myoA (residues 730–753) (2), but neither conforms to a classic IQ motif.

**TABLE II**

| Mass | \(s^*\) | \(\beta_f\) | Ellipsoid models |
|------|---------|-----------|----------------|
|      |         | \(nm\)    | Prolate | Oblate       |
| A23  | 21.6    | 1.8       | 1.7     | 17.2\(a\)  | 9.0\(a\)  |
|      |         |           |         | 2.1\(b\)  | 0.9\(b\)  |
| A2   | 14.6    | 1.2       | 1.9     | 19.5       | 9.2\(a\)  |
|      |         |           |         | 1.6        | 0.6        |
| A123 | 48.8    | 2.9       | 1.6     | 20.5       | 11.2       |
|      |         |           |         | 2.9        | 1.4        |

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Mass = 21.6 kDa. D, residuals from fitting of 20,000 rpm data in C.
Expression and Purification of Myosin-IA Tail Domains—We expressed several constructs of the myosin-IA tail in E. coli and purified them by column chromatography (Fig. 1C and Fig. 3). Of these constructs, A23 was the best behaved, yielding large amounts of soluble protein that allowed detailed characterization. A2 was also easy to work with. A1 was insoluble in bacterial lysates but could be solubilized and purified by chromatography in 6 M urea. Purified A1 could be refolded by removing urea but was not soluble at concentrations above 1 mM, limiting the range of studies. A123 was partially soluble in bacterial lysates, and purification was challenging due to tightly associated nucleic acids. Hydroxylapatite chromatography in 50% formamide removed all detectable nucleic acid, but after removal of formamide, the protein was only soluble at high pH (> 9.5).

Domain Boundaries in the Myosin-IA Tail—Trypsin digestion of partially purified A123 yielded 34- and 29-kDa peptides (T1 and T2, Fig. 3D) that were slowly converted to a 27-kDa peptide (T3, Fig. 3D). By Edman degradation, all had the same N terminus as A123. Given the molecular weights, the C-terminal cleavage sites were most likely at Arg-1084 (for T1) and Arg-1000 (for T2), followed by further cleavages at Arg-996, Arg-993, Arg-987, or Arg-985. This behavior indicates that TH-1 is a stably folded, trypsin-resistant domain despite its remarkably high content of basic residues. Intact TH-2/3 was not recovered as a stable trypsinic peptide, but a tryptic peptide of 15-kDa reacted with monoclonal antibody M1.7 (Fig. 3D), which has an epitope in the C-terminal half of TH-2. α-Chymotryptic digestion rapidly yielded two peptides, one with the same mobility as TH-1 and the other with the mobility of the A23 construct, as expected from the cleavage of myosin-IA at Arg-985 (12). The protease endo-AspN, which cuts the peptide bond on the N-terminal side of aspartic residues, nicked the link between TH-1 and TH-2, creating a peptide of 30 kDa with the same N terminus as A123. Given the molecular weight, the cleavage site was most likely at Asp-1008 (Asp2 site, Fig. 1C). Endo-AspN also cut A123 before Asp-927 in the middle of TH-1 (Asp1 site, Fig. 1C), creating a C-terminal peptide of 35 kDa that reacted with monoclonal antibody M1.7. A123 was resistant to digestion by V8 protease. These experiments established that recombinant A23 is stably folded and that the link between TH-1 and TH-2 is sensitive to proteolytic digestion. We used this knowledge of the proteolytic sites to design the domain constructs used in the following experiments.

Physical Properties of A23—The CD spectrum of A23 in 5 mM sodium phosphate, pH 7.6, ± 250 mM NaF shows no evidence of α-helical structure (Fig. 4). The spectra are interpreted as predominantly β-turn, and random structure by both PROSEC (5 mM sodium phosphate, pH 7.6: 4% α, 26% β, 19% turn, 51% random; 5 mM sodium phosphate, pH 7.6, with 250 mM NaF: 0% α, 45% β, 11% turn, 44% random) and CDNN analyses (data not shown). The lack of helix is consistent with the high content of glycine and proline in TH-2 and the known structures of SH3 domains (44–47). Not even 50% TFE promoted significant helical content, so A23 is most likely stably folded.

The hydrodynamic properties of recombinant A23 show that it is a well behaved monomer with an asymmetric shape. A23 sediments as a single homogenous peak at 1.80 ± 0.06 S (n = 5) independent of concentration between 35 and 234 mM (Fig. 5B). Sedimentation in imidazole or sodium phosphate buffer with high (0.5 M KCl) or no salt yielded similar results. The molecular mass of A23 was 21.6 kDa by sedimentation equilibrium at three different speeds and at starting concentrations of 9.6 and 19.2 μM (Fig. 5C). Sedimentation equilibrium at higher concentration (38.4 μM) and in high (0.5 M KCl) or no salt buffer gave similar results. The molecular mass of A23 calculated from the amino acid composition is 21,271 Da, but the protein migrates anomalously on SDS gels at 27–29 kDa. The sedimentation coefficient and molecular weight yielded a frictional ratio of 1.7 and a Stokes’ radius of 3.04 nm, which is in good agreement with the value of 3.0 nm obtained by gel filtration on Sephacryl S200 in 0.5 M KCl, 10 mM imidazole, pH 7.5, 1 mM DTT, and 1 mM EDTA, and 1 mM DTT (Fig. 5A).

Two models are typically used to approximate nonspherical particles in hydrodynamic studies, a prolate ellipsoid (cigar-shaped) and an oblate ellipsoid (disc-shaped). If A23 were a prolate ellipsoid the axial ratio would be 8.2:1 with length of 17.2 nm and diameter of 2.1 nm, and if it were an oblate ellipsoid the axial ratio would be 1:10 with thickness of 0.9 nm and diameter of 9 nm (Table II). The lengths of Acanthamoeba myosin-IB (20) and myosin-IC (21) tails are approximately 8.3 and 5.5 nm, respectively. Therefore, the hydrodynamic data indicate that the size of A23 spans the whole length of the myosin-1 tail (see “Discussion”).

A2 and A123 are also asymmetric (Table II). Analysis of A123 was possible only at high pH, under which condition it is monomeric with molecular mass of 48.8 kDa (Table II). The
Interaction of A23 with A1—Consistent with the above results, purified A1 binds A23 with high affinity in physiological salt conditions. Soluble A1 bound a fusion protein GST-A23 (GST fused to the TH-2/3 domain of Acanthamoeba myosin-IA) immobilized on beads in 60 mM NaCl, 10 mM imidazole, pH 7.5, 1 mM DTT, and 1 mM EDTA (Fig. 6A). Neither GST nor control glutathione beads depleted soluble A1 from the supernatant. The dependence of A1 depletion on the concentration of GST-A23 immobilized on beads gave a \( K_d \) of 0.25 \( \mu M \) (Fig. 6B).

Interaction of Myosin-IA Tail Domains with Actin Filaments—A23 binds Acanthamoeba actin filaments with approximately the same affinity at all salt concentrations tested as follows: \( K_d \) of 0.05 \( \mu M \) at no salt; 0.20 \( \mu M \) at 20 mM KCl; and 0.09 \( \mu M \) at 60 mM KCl (Fig. 7A). In contrast, salt inhibits binding of A23 to rabbit skeletal muscle actin filaments. Although A23 binds muscle actin filaments with a \( K_d \) of 0.2 \( \mu M \) in no KCl with 1 mM MgCl\(_2\), 1 mM EGTA, and 10 mM Tris, pH 7.5, the affinity of A23 for muscle actin filaments is markedly lower in higher salt. The \( K_d \) is 2.6 \( \mu M \) in 20 mM KCl and \( \approx 50 \mu M \) in 40 and 60 mM KCl (Fig. 7B). The actin-binding site resides in TH-2, since A2 without the SH3 domain binds muscle actin filaments with \( K_d \) of 0.2 \( \mu M \) in the no-salt buffer (Fig. 7C), just like A23. Binding of A2 to muscle actin filaments was also inhibited by 60 mM KCl.

Interestingly, purified A1 binds muscle actin filaments with high affinity in salt, where A23 does not bind. In 60 mM KCl, A1 binds muscle actin filaments with a \( K_d \) of \( \sim 0.1 \mu M \) as assayed by depletion from the supernatants (Fig. 8A) and accumulation in the pellets (Fig. 8B). When pellets are assayed, A23 does not bind muscle actin filaments in the same 60 mM KCl buffer (Fig. 8C), just like when supernatants are assayed in Fig. 7B (closed circles). However, A23 binds muscle actin filaments in 60 mM KCl if A1 is also present at equimolar concentration (Fig. 8D). Thus, the presence of A1 increases the affinity of A23 for muscle actin filaments in salt by 50-fold, from \( K_d \approx 50 \mu M \) in the absence of A1 (Fig. 7B, closed circles) to \( \sim 1 \mu M \) in the presence of A1 (Fig. 8D, open circles). In the presence of an excess A1 over A2, A23 binds muscle actin filaments with \( K_d \) of 0.3–0.5 \( \mu M \) (other experiments, \( n = 2 \)). Similar to A23, A2 does not bind muscle actin filaments under the same conditions (60 mM salt). The presence of excess A1 over A2 enhances the binding of A2 to muscle actin filaments. However, at the highest actin concentration tested (5 \( \mu M \)) only 40% of A2 pellets (data not shown), indicating that the A1-mediated enhancement of binding to muscle actin filaments is weaker for A2 than A23.

Interaction of A23 with Acan125—Two assays established that Acanthamoeba Acan125 binds the tail of myosin-IA with high affinity, similar to its known interaction with the SH3 domain of Acanthamoeba myosin-IC (16). In the first assay, GST-A23 immobilized on beads bound a fusion protein Trx-AD3 containing the putative SH3-binding sites (PXXP motifs) of Acan125 (Fig. 9A). This assay was originally used to demonstrate binding of GST-C3 (GST fused to the SH3 domain of Acanthamoeba myosin-IC) to Trx-AD3. Neither GST nor glutathione beads alone deplete Trx-AD3 from the supernatant. Neither GST-A23 nor GST-C3 bound Trx-AD3 (977–994), an Acan125 construct lacking the putative SH3-binding sites. A
small amount of “receptor” (GST, GST-C3, or GST-A23, data not shown) was soluble, which could be due to denatured fusion proteins or contaminating beads in the supernatant.

Intrinsic fluorescence provided a second, more quantitative assay for the interaction of purified recombinant A23 and Trx-AD3. The tryptophan fluorescence of A23 is three times higher than equimolar Trx-AD3. Mixtures of the two proteins are less fluorescent than the sum of the two components. This quenching measures the extent of interaction during a titration of A23 with Trx-AD3 (Fig. 9B). The dependence of quenching (ΔF) on Trx-AD3 concentration fit a theoretical curve with a K_d ≈ 20 nM (Fig. 9C). Other experiments yielded K_d ≈ 10 nM. Trx-AD3-(977–994) did not quench the fluorescence of A23 (Fig. 9C), suggesting that PXPF motifs of Trx-AD3 mediate binding to...
A23.

To establish the physiological relevance of this affinity, we measured the concentration of Acan125 protein in amoeba. We solubilized whole cells in boiling SDS sample buffer, ran these extracts on the same gel as a range of GST-AD3 standards, and used immunoblotting with a polyclonal antibody to Acan125 (generated against AD3 fragment) to compare the unknowns and standards. The concentration of Acan125 is approximately 2–5 μmol/liter of packed cells (n = 3); this is 2–5 times the total concentration of 1 μM for all isoforms of myosin-IA combined (48).

**DISCUSSION**

We studied the tail of *Acanthamoeba* myosin-IA, because the tails of the various classes of myosins appear to be major determinants of their functions, and relatively little is known about the structures of the tails of myosin-Is or other unconventional myosins. Previous studies established three regions of sequence homology among myosin-I tails and provided evidence that TH-1 binds acidic phospholipids (11, 49), TH-2 binds actin filaments (11, 13, 14) and TH-3 binds Acan125 (16) (or verprolin Vrp1p in budding yeast (18)). Our determination of the primary structure of the tail of amoeba myosin-IA is important because it has been used in many previous biochemical studies (3, 5, 12, 22, 50–53). The size and shape of the tails of amoeba myosin-IB (20) and -IC (21) and of brush border myosin-IA (19) were known from reconstructions of electron micrographs of decorated actin filaments. The tails of amoeba myosin-Is (with TH-1, -2, and -3) are more massive but not much longer than brush border myosin-IA (with TH-1 alone). However, it was not clear from previous work how the tail homology regions are arranged in the tail or whether these domains cooperate in any ligand binding activities. In the following analysis, we assume that the organization of the tail of myosin-IB (for which we know the overall size and shape) is the same as myosin-IA, since the primary structures of the tails are similar in size (459 residues for myosin-IA and 437 residues for myosin-IB) and the order of their tail homology regions.

Several lines of evidence support the hypothesis that the tail of myosin-IA is composed of three domains, with a TH-1 domain lying side by side with the elongated TH-2/3 domain. We call the domains A1, A2, and A3 to distinguish them from the tail homology regions of myosin-IB and -IC. The tail homology regions are real domains in the sense that they fold independently and can be separated by proteolysis (Ref. 12 and this paper). Several proteases (α-chymotrypsin, trypsin, and endo-Asn but not V8 protease) cleave the link between A1 and A2. A3 is a typical SH3 domain. A1 and A2 can be expressed and purified separately. Although it consists of 15% lysine and arginine, A1 is highly resistant to trypsin digestion. A23 is resistant to α-chymotrypsin, and its CD spectrum indicates predominantly turns and β-structure. Our observations are consistent with earlier studies of proteolytic digestion of native myosin-IA (12, 51, 52).

We were surprised that the hydrodynamic properties of A23 indicate that this domain alone is as long as the whole myosin-IB tail observed in reconstructions of electron micrographs. In the reconstructions the tail of myosin-IB is a flattened structure 8.2 nm long, 4.0 nm thick, and 4.8 nm wide (20). The hydrodynamic properties of recombinant B23 are the same as A23.2 Neither oblate ellipsoid nor prolate ellipsoid models are realistic models for the myosin-I tail, but both ways of modeling the hydrodynamic data indicate that A23 is highly asymmetric with one dimension between 9.0 and 17.2 nm. If A23 extends the length of the tail, A1 must lie side by side, rather than the tail domains being arranged in series. Consistent with this idea, monomeric A23 is asymmetric with maximum length in one dimension between 11.2 and 20.5 nm (Table II), thus A23 fits within and spans the size of A123. Additional evidence supports this side-by-side interaction hypothesis.

First, separately purified A1 and A23 bind each other with submicromolar affinity under physiological conditions. Given this high affinity, one might ask how Lynch et al. (12) separated A23 from the rest of myosin-I after α-chymotrypsin digestion. In their work, A23 eluted separately from other digested products of native myosin-IA in 150 mM KCl on Mono Q anion exchange chromatography at pH 8.8. Thus this tight interaction may be disrupted by high salt and high pH.

Second, the EM reconstructions show that the tail of brush border myosin-IA (with only TH-1) is less robust but nearly as long as myosin-IB (with TH-1, -2, and -3). The tail of brush border myosin-IA is 6.1 nm long, 2.0 nm thick, and 4.9 nm wide (19). A plausible explanation for myosin-IB being twice as thick as brush border myosin-IA is that TH-2/3 folds back onto the TH-1 domain.

Given this side-by-side model for the tail of long-tailed myosin-Is, we considered the possibility that the C-terminal SH3 might substitute for the 80 residues missing at the N terminus of the catalytic domain of myosin-Is. The 80 N-terminal residues of myosin-II are folded similar to an SH3 domain and are located near the light chain domain.3 This is close to where the C terminus of a folded back myosin-I tail might be located. However, the EM reconstruction of myosin-IB (20) lacks the density occupied by the N-terminal SH3-like domain of myosin-II. Thus the C-terminal SH3 domain does not fill the space made available by the missing N-terminal 80 residues.

**Interaction of Myosin-I Tail with Actin Filaments**—The existence of an ATP-insensitive actin-binding site in the tail of myosin-IA was first suggested by the ability of the protein to cross-link actin filaments (observed by electron microscopy) and by the biphasic activation of the Mg2+-ATPase by actin filaments (50, 54). Lynch et al. (12) confirmed the existence of this actin filament binding site and located it near the C terminus of the tail by cleaving myosin-IA with α-chymotrypsin and isolating a C-terminal actin binding domain of about 30 kDa (shown here to actually be 21 kDa). We have characterized this A23-binding site in more detail.

First, we showed that A3 is not required for actin binding, since A2 binds actin filaments the same as A23. Recombinant TH-2 from *Dictyostelium* myosin-IB (14) and myosin-IC (13) also binds actin filaments without an SH3.

Second, we found that in physiological salt concentrations A23 binds *Acanthamoeba* actin filaments but not muscle actin filaments. *Acanthamoeba* acotphorin (55) and profilin (56) also bind amoeba actin better than muscle actin. Lynch et al. (12) did not report on the binding of proteolytic A23 to muscle actin filaments in physiological salt concentrations, only in low salt.

Like amoeba A23, salt also inhibits the binding of TH-2 of *Dictyostelium* myosin-IB to muscle actin filaments (14); the *Kd* values are 1.3 μM in no salt, 7.2 μM in 20 mM KCl, and 49 μM in 100 mM KCl. It would be interesting to know if this myosin also binds better to its own actin independent of salt concentration.

Third, we found that A1 also binds muscle actin filaments, even in physiological salt conditions. In a previous study (11), the TH-1 domain of myosin-IC (C1) fused to β-galactosidase did not bind actin filaments, but fusion to the bulky, tetrameric β-galactosidase (4 times 90 kDa) may have interfered or myosin-IC may differ in this regard. The 100-kDa fragment isolated by cleaning myosin-IA with α-chymotrypsin bound actin filaments tightly both in the presence (*Kd* = 0.5 μM) and absence of salt.

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2 W.-L. Lee and E. M. Ostap, unpublished observations.

3 R. A. Milligan, personal communication.
RT-loop on myosin-IA and myosin-IC SH3 models are black. Remarkably high. The P motif of A23 domain of myosin-IA binds Trx-AD3. Given that the concentration of A23 exceeds that of all myosin-Is in amoeba, we expect that most of the myosin-I is bound to Acan125 in the cell unless regulated by other cellular factor(s).

Both myosin-IA and myosin-IC bind Acan125. Models of the SH3 domains of myosin-IA and myosin-IC provide the structural basis for this interaction (Fig. 10). The SH3 domains of both myosin-IA and myosin-IC have the hydrophobic residues critical for binding poly-L-proline ligands as follows: Tyr-1168, Tyr-1170, Trp-1195, Pro-1208, and Tyr-1211 for myosin-IA and Tyr-985, Phe-987, Trp-1013, Pro-1026, and Tyr-1029 for myosin-IC. Both myosins have acidic residues in the variable RT loop, a region believed to confer ligand specificity in other SH3 domains (60, 61): Glu-1174, Asp-1176, and Glu-1177 for myosin-IA and Glu-990, Asp-993, and Glu-994 for myosin-IC. In the crystal structure of crk-N SH3 complexed with a polyproline ligand (61), PPAPLPPKKK, three acidic residues in the RT loop coordinate a lysine in the +2 position (underlined) relative to the second P of the ligand, just like the PXPPP sequence of the two tandem motifs of Acan125. Attempts to model the SH3 domain of Acanthamoeba myosin-IB failed since the sequence did not satisfy several spatial parameters defined by the SH3 templates used for modeling.

The properties of Acan125 suggest that it may organize myosin-I into higher order structures. This may be essential for function since the kinetic properties of myosin-Is show that single myosin-I cannot move along actin filaments alone (10). Acan125 has not only two tandem PXPPP motifs that could bind two myosin-Is but also 16 tandem leucine-rich repeats in the N-terminal half of the molecule. Leucine-rich repeats in other proteins contribute to intermolecular interactions, serving either directly as a ligand-binding site or as a second site to enhance affinity and/or specificity (62). The leucine-rich repeats of Acan125 do not bind the SH3 domain of Acanthamoeba myosin-IC in vitro but may link myosin-1 into a larger complex.

SH3 domains assemble protein complexes in other systems. For instance, SH3 domains of yeast myosin-Is, Myo3p and Myo5p, bind verprolin Vrp1p, a proline-rich protein localized at budding sites (18, 63). Similarly, the yeast cytoskeletal protein Abp1p binds adenyl cyclase-associated protein Srv2p and actin-associated protein Rvs167p through SH3-ligand interactions to form a protein complex required for actin patch formation (64).

Duplication and Evolutionary Divergence of Myosin-1 Genes—Phylogenetic analysis of the sequences of myosin catalytic domains established that all classes of myosin had a common origin. Duplication and divergence of myosin genes established several classes of myosin in primitive eukaryotes before the branching of higher eukaryotes (65). Some higher eukaryotes including budding yeast have lost the genes for some myosins. As the various eukaryotes radiated away from each other over more than a billion years, selective pressures kept all of the myosins within each class more similar to each other than the myosins of any other class. The myosin-I family is no exception to this pattern. The sequences of the catalytic, IQ, and TH-1 domains of amoeba myosin-IA are more similar to Dictyostelium myosin-IC than any other myosin, including the other amoeba myosin-Is (Table I), so they are probably functional homologs that evolved from the same ancestral gene.

Given the ancient origin and conservation of the myosin-I family, we were surprised when our sequence analysis indicated that the myosin-1 genes may have acquired their SH3 domains relatively late, after the separation of contemporary organisms from their common ancestors. Our phylogenetic analysis of SH3 sequences reveals two distinct patterns (Fig. 11). For most protein families the SH3 domains have orthologous relationships. For example, the SH3 domains of Src tyrosine kinases are related most closely to the SH3 domains of other Src tyrosine kinases. The same is true of the SH3 domains of adaptor proteins, PLCγs and α-spectrins. In contrast, the SH3 domains of the various myosin-Is (from yeast, amoebas, Dictyostelium, Caenorhabditis elegans, and mouse) do not form a closely related cluster. In the case of Acanthamoeba myosin-IA, the SH3 domain is closer to other Acanthamoeba...
Properties of the Tail of Acanthamoeba Myosin-IA

myosin-Is than to Dictyostelium myosin-IC, the closest neighbor phylogenetically of the catalytic domain. This pattern suggests that myosin-Is acquired their SH3 domains after the divergence of the genes for myosin-I isoforms. The repertoire of myosin-I genes in the separate ancestors of Acanthamoeba and Dictyostelium may have picked up SH3 domains within these myosin-I genes in the separate ancestors of animals ranging from the invertebrates to the vertebrates and sequenced (9). Given the differences in the IQ motifs of myosin-IA and myosin-IC, it seems unlikely that they share an orthologous relationship. Indeed, so these orthologous relationships can be tested more stringently as more data become available.

One could argue that phylogenetic analysis of short protein sequences like SH3 domains may not be significant. However, we observed tight clustering of protein families (e.g. tyrosine kinases, adaptor proteins, phospholipase Cγ, and α-spectrin) based solely on their SH3 sequences. Each cluster is represented by species ranging from the invertebrates to the vertebrates. The number of sequences from unicellular organisms is limited, so these orthologous relationships can be tested more stringently as more data become available.

**Distinguishing Features of Amoeba Myosin-Is—Acanthamoeba myosin-IA and -IB are similar in many respects, but the sequence of myosin-IA revealed three putative IQ motifs. If all are occupied by light chains, amoeba myosin-IA may have a larger working stroke than amoeba myosin-IB or myosin-IC, which have similar catalytic properties (10) but a single light chain. A longer working stroke may adapt myosin-IA for activities in the cytoplasm where it is thought to function in vesicle transport and phagocytosis (3). However, the number of myosin-IA light chains is unknown. A light chain of 17 kDa and a variable quantity of a 14-kDa peptide (always in less than 1 mol/mol of heavy chain of myosin-IA) co-purify with Acanthamoeba myosin-IA (6). The 16.7-kDa light chain of Acanthamoeba myosin-IC has been isolated and sequenced (9). Given the differences in the IQ motifs of myosin-IA and myosin-IC, it seems unlikely that they share this light chain.**

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Organization and Ligand Binding Properties of the Tail of *Acanthamoeba* Myosin-IA: IDENTIFICATION OF AN ACTIN-BINDING SITE IN THE BASIC (TAIL HOMOLOGY-1) DOMAIN

Wei-Lih Lee, E. Michael Ostap, Henry G. Zot and Thomas D. Pollard

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