Acanthamoeba myosin IC has a single 129-kDa heavy chain and a single 17-kDa light chain. The heavy chain comprises a 75-kDa catalytic head domain with an ATP-sensitive F-actin-binding site, a 3-kDa neck domain, which binds a single 17-kDa light chain, and a 50-kDa tail domain, which binds F-actin in the presence or absence of ATP. The actin-activated MgATPase activity of myosin IC exhibits triphasic actin dependence, apparently as a consequence of the two actin-binding sites, and is regulated by phosphorylation of Ser-329 in the head. The 50-kDa tail consists of a basic domain, a glycine/proline/alanine-rich (GPA) domain, and a Src homology 3 (SH3) domain, often referred to as tail homology (TH)-1, -2, and -3 domains, respectively. The SH3 domain divides the TH-3 domain into GPA-1 and GPA-2. To define the functions of the tail domains more precisely, we determined the properties of expressed wild type and six mutant myosins, an SH3 deletion mutant and five mutants truncated at the C terminus of the SH3, GPA-2, TH-1, neck, and head domains, respectively. We found that both the TH-1 and GPA-2 domains bind F-actin in the presence of ATP. Only the mutants that retained an actin-binding site in the tail exhibited triphasic actin-dependent MgATPase activity, in agreement with the F-actin-cross-linking model, but truncation reduced the MgATPase activity at both low and high actin concentrations. Deletion of the SH3 domain reduced the MgATPase activity at both low and high actin concentrations. Deletion of the SH3 domain had no effect. Also, none of the tail domains, including the SH3 domain, affected either the $K_m$ or $V_{max}$ for the phosphorylation of Ser-329 by myosin I heavy chain kinase.

The myosin superfamily includes more than 120 different isoforms falling into 15 classes based on the sequences of their catalytic domains and also differing in the structure of their tails (1). Other than the conventional class II myosins, class I myosins are the most numerous, most widely distributed, and most extensively studied. At this writing, the complete DNA sequences of the heavy chains of 30 class I myosins from 13 species including yeast, protozoa, invertebrates, and vertebrates have been determined. The structure, enzymatic properties, and possible functions of many of the class I myosins have been investigated, none more extensively than the three Acanthamoeba myosin Is (IA, IB, and IC), which were the first unconventional (nonclass II) myosins to be discovered (2).

All class I myosins have a single, relatively short (for myosins) heavy chain, one or more light chains, and, unlike class II myosins, do not polymerize into filaments. By sequence analysis (3–5), the masses of the heavy chains of Acanthamoeba myosin IA, IB, and IC are 134, 125, and 129 kDa, respectively, consisting of an N-terminal head (catalytic, motor) domain of $\sim$75 kDa, a short neck domain that binds one (myosin IB and myosin IC (6)) or possibly as many as three (myosin IA (5)) light chains and a nonhelical C-terminal tail domain of $\sim$50 kDa. Only the single light chain of myosin IC has been cloned and sequenced (7); it is a calmodulin-like protein with a mass of $\sim$17 kDa. The tail domains have been subdivided by sequence into three regions: a basic domain (TH-1), a Gly/Pro/Ala-rich (GPA) domain (TH-2), and a Src homology 3 domain (TH-3). These three regions occur sequentially in Acanthamoeba myosins IA (5) and IB (4), but the TH-3 region of myosin IC splits its C-terminal TH-2 domain in two (3). It seems highly likely that specific interactions of these three tail domains play important roles in determining the different localizations and different functions of the three isozymes (8–10).

The bacterially expressed TH-1 domain of Acanthamoeba myosin IC binds acidic phospholipids (11) and, therefore, TH-1 is almost certainly responsible for the ability of the native myosins to bind to acidic lipids (12) and amoeba plasma membranes (13). Experiments with bacterially expressed TH-2 plus TH-3 domains of Acanthamoeba myosin IC (11), with bacterially expressed TH-2 region of Dictostelium myosin IB (14) and with a C-terminal 30-kDa proteolytic fragment of Acanthamoeba myosin IA (15), had indicated that the TH-2 region may be principally responsible for the ability of the amoeba myosin Is to bind to F-actin in the presence of ATP (15–17). As we will discuss later (see “Discussion”), the TH-3 domain is likely to be involved in the localization of myosin Is and the organization of the actin cytoskeleton (18).

The three Acanthamoeba myosin Is have similar catalytic activities. All have high ATPase activity in the presence of EDTA and either NH$_4^+$ or K$^+$ and low activity in the presence of Mg$^{2+}$ that is substantially activated by F-actin, a diagnostic characteristic of the myosin superfamily. However, the MgATPase activity of these amoeba myosin Is has an unusual triphasic dependence on the F-actin concentration (17, 19, 20), i.e. substantial activation at low F-actin concentrations peaking at about 2 $\mu$M, followed by a decrease in the activity and then normal hyperbolic activation that begins to plateau at about 80 $\mu$M F-actin. A variety of experimental data and computer modeling strongly support the conclusion that this triphasic actin dependence is caused by cooperative cross-linking of actin fil-

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‡The abbreviations used are: TH, tail homology; GPA, glycine, proline, and alanine-rich; MIF-GCK, myosin I heavy chain kinase; Pak, p21-activated kinase; SH3, Src homology 3; PCR, polymerase chain reaction; bp, base pairs; DTT, dithiothreitol; BSA, bovine serum albumin.
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lements by myosin I at high ratios of myosin I to actin (17, 19, 21, 22). The ability of these myosins to cross-link actin filaments can be explained by the presence of two actin-binding sites, the ATP-sensitive actin-binding site in the catalytic domain that is common to all myosins of all classes and the ATP-insensitive actin-binding site(s) in the tail that occurs in some (but not all) class I myosins including, in addition to the three Acanthamoeba myosin Is, two of the five Dictyostelium myosin Is (23, 24).

The act-activated MgATPase activities of the myosin Is from Acanthamoeba (25), Dictyostelium (26), and Aspergillus (27) are activated by phosphorylation of a Thr or Ser at a position (Ser-329 in Acanthamoeba myosin IC (28)) in a conserved actin-binding surface loop (29) where almost all other myosins have a Glu or Asp residue (1, 30, 31). The Acanthamoeba (32) and Dictyostelium (33) myosin I heavy chain kinases (MIHCK) that phosphorylate the Ser and Thr residues are members of the p21-activated kinase (Pak) family. Like other Paks, the 97-kDa MIHCK is activated by Rac and Cdc42 (34) and lipids (35). The SH3 domains have important roles in the activity and function of the Acanthamoeba myosin Is. However, the properties of the tail domains have been characterized mostly by studying the properties of bacterially expressed peptides. In the work described in this paper, we compared the properties of expressed wild type Acanthamoeba myosin IC and truncated and deletion tail mutants to define more quantitatively the actin-binding region in the tail, to confirm that the triphasic actin dependence of the MgATPase activity is caused by the second actin-binding site in the tail, and to evaluate the possibility that the SH3 domain of Acanthamoeba myosin IC may interact with the Pro-rich region of MIHCK. After these studies were completed, Lee et al. (5) reported interesting results, which we will discuss later in this paper, on the actin-binding properties of bacterially expressed peptides corresponding to the TH-1 and TH-2 plus TH-3 regions of Acanthamoeba myosin IA.

MATERIALS AND METHODS

Generation of Mutant cDNAs—Standard methods were used for all DNA manipulations (36). The myosin I heavy chain cDNA (accession number AF013153) cloned into pBluescript plasmid (3, 6) served as the template for the PCR reactions described below. Fig. 1 shows the mutants constructed in this study.

The plasmid containing the TH-3 deletion mutant (ΔSH3) was constructed utilizing a unique SacI site located upstream of the SH3 domain of myosin IC and an XbaI site in the vector located downstream of the myosin IC stop codon. Two primers were synthesized for PCR. The sense primer (primer A) 5'-GCGCAGCGACGCGTTAGATGAGTTCGACG-3' (bp 3141–3169); for T-6, 5'-GGGTCTTGCGGAGGAATCTCACGCG-3' (bp 2977–3012); for T-5, 5'-GTCATACAGCGCAGCCACGGGTCCC-3' (bp 2471–2511); for T-4, 5'-GCCCGCGCTCCAGAGCCCTCATGGCACTGGC-3' (bp 2661–2703 of myosin IC), which introduced a new stop codon (in bold) after the truncation point and an MluI restriction site (underlined) immediately downstream of the stop codon. The antisense primer was: for T-2, 5'-AGGGCGCAACCGCGCGCTTATGTTGAGCAGCTGATT-3' (bp 2124–2181); for T-3, 5'-AGCTGTACAGACGGCTGTTGTTGTGCG-3' (bp 2574–2611); for T-4, 5'-GCCCGCGCTCCAGAGCCCTCATGGCACTGGC-3' (bp 2661–2703 of myosin IC), which introduced a new stop codon (in bold) that was introduced immediately after the truncation point and an MluI restriction site (underlined) immediately downstream of the stop codon.

The antisense primers were: for T-2, 5'-GAGGGCAGACCCCGCGCTTATGTTGAGCAGCTGATT-3' (bp 1234–1267 of myosin IC heavy chain), which contains a PstI site (underlined); antisense primer 5'-GGGTCTTGCGGAGGAATCTCACGCG-3' (bp 2977–3012) introduced a new stop codon (in bold) after the truncation point and an MluI site (underlined) immediately downstream of the stop codon. The PCR product was subcloned into pBluescript T-2 digested completely with MluI and partially with SacI (because the vector DNA has a single SacI site).

T-1 was constructed utilizing the PstI site located within the head domain of the myosin IC heavy chain and the MluI site in the T-2 plasmid. Primers used for PCR were: sense primer 5'-CAACCTCGT-GAAGGTGCGACGATTTCTC-3' (bp 1324–1367 of myosin IC heavy chain), which contains a PstI site (underlined); antisense primer 5'-GGGTCTTGCGGAGGAATCTCACGCG-3' (bp 2977–3012) introduced a new stop codon (in bold) after the truncation point and an MluI site (underlined) immediately downstream of the stop codon. The PCR product was subcloned into pBluescript T-2 digested completely with MluI and partially with PstI (because the vector contains another PstI site). This produced truncated DNA encoding T-1 (Fig. 1). The sequences of all the mutant DNAs were confirmed.

The wild type and mutant heavy chain DNAs were subcloned from pBluescript into the expression vector PVLI3939 (PharMingen) by digestion with BamHI. A Flag epitope tag, MADYKDDDDY, was placed at the N terminus of all of the heavy chains to provide a means for rapid purification of the type II and mutant myosins.

Culture of SF-9 Cells and Production of Myosin Heavy Chain Recombinant Viruses—SF-9 cells were cultured in suspension using Grace's medium supplemented with 10% fetal calf serum (Life Technologies, Inc.). Transfection was achieved by mixing 2–5 μg of plasmid DNA with 0.5 μg of BaculoGold vector DNA (PharMingen) according to the protocol provided by the manufacturer. Recombinant viruses were identified as occlusion-negative plaques. Viral stock was amplified according to the manufacturer's protocols and kept at 4 °C. The myosin IC light chain (6), the 35K catalytic domain (32), and full-length MIHCK viral stocks were prepared previously in this laboratory.

Expression and Purification of Proteins—Wild type and mutant myosins were produced by co-infection of SF-9 cells (2 × 10⁶ cells/ml) with heavy chain and light chain viral stocks. All purification procedures were carried out at 4 °C. Wild type and mutant myosins were purified as described previously (6) with some modification. Briefly, SF-9 cells were harvested 48 h after infection. About 2 g of cells were homogenized in 20 ml of extraction buffer (200 mM NaCl, 4 mM MgCl₂, 2 mM ATP, 1 mM DTT, and 10 mM Tris, pH 7.5) containing 0.1 mM phenylmethylsulfonyl fluoride and 1 tablet of protease inhibitor mixture (Roche Molecular Biochemicals). The lysate was centrifuged at 45,000 rpm in a Beckman Ti70 rotor for 1 h, and the resultant supernatant was added to 1 ml of packed anti-FLAG antibody resin (Sigma) that had been washed with 0.1 M glycine (pH 3.5) and equilibrated with the extraction buffer. The resin was washed with 20 ml of extraction buffer, and myosin was eluted with 2.5 ml of extraction buffer containing 0.15 M NaCl and 0.5% β-mercaptoethanol. After dialysis of the sample was clarified by spinning at 15,000 rpm for 10 min at 4 °C, and the final product was kept in liquid nitrogen until use.

The myosins were partially phosphorylated during expression in SF-9 cells. To prepare unphosphorylated myosins for use as substrates for

*C. Tan, E. D. Korn, and H. Brzeska, unpublished data.
MIHCK, wild type myosin IC and the T-2 mutant were dephosphorylated while bound to the FLAG affinity resin as an added step in the purification procedure. The resin with bound myosin was washed with ~20 volumes of extraction buffer and then with one volume of phosphate buffer (New England Biolabs, Beverly, MA). About 4000 units of lambda protein phosphatase (New England Biolabs) in 1 ml of phosphate buffer (New England Biolabs) was added to ~1 ml of the resin-bound myosin. Dephosphorylation was carried out at room temperature for 15 min with occasional resuspension of the resin by pipetting. The resin was then washed with the extraction buffer, and myosin was eluted and collected as described above.

For expression of maximum actin-dependent MgATPase activity, the myosin must be fully phosphorylated on Ser-529. The wild type and mutant myosins were incubated with activated (autophosphorylated) 35K catalytic domain of MIHCK at a molar ratio of 1 to 8 in buffer containing 50 mM imidazole, pH 7.0, 2.5 mM ATP, 3.5 mM MgCl2, and 2 mM EGTA (and 50 mM NaCl and 25% glycerol derived from the myosin storage buffer) at 30 °C for 10 min. As determined by the incorporation of 32P from [γ-32P]ATP, phosphorylation was complete (1 mol/mol).

The 35K catalytic domain and full-length MIHCK were purified using a nickel-nitriilotriacetic acid resin column as described (32). Purified kinase was dialyzed against 50% glycerol, 10 mM Tris, pH 7.5, and 1 mM DTT and kept in liquid nitrogen until use. The kinase and 35K catalytic domain were essentially homogeneous as determined by SDS-polyacrylamide gel electrophoresis (data not shown). To obtain fully active autophosphorylated kinases, the purified 35K catalytic domain and full-length MIHCK (both at 0.2 mg/ml) were first incubated for 30 min at 30 °C in 50 mM imidazole (pH 7.0) containing 2.5 mM ATP, 3.5 mM MgCl2, and 2 mM EGTA and BSA (0.2 mg/ml).

Rabbit skeletal muscle actin was prepared from rabbit skeletal muscle acetone powder according to Spudich and Watt (37). Acanthamoeba myosin IC, which was kindly provided by Dr. Kirsten Remmert (National Heart, Lung, and Blood Institute), was prepared as described (38). The concentrations of myosins and kinases were determined by the Bradford method using BSA as the standard (39). Actin concentrations were determined spectrophotometrically using an extinction coefficient of 0.62 cm2/ml at 290 nm.

**Kinase Assay.—**Assays were performed essentially as described (40). The MgATPase and Ca2+-activated MgATPase activities of wild type and mutant myosins, as substrates, in 50 mM imidazole (pH 7.0) containing 2.5 mM [γ-32P]ATP (30,000 cpm/nmol), 3.5 mM MgCl2, and 2 mM EGTA and BSA (0.2 mg/ml). At all substrate concentrations, the rate of phosphorylation was linear with time for the period of incubation.

**Actin-binding Assay.—**The binding of wild type and mutant Acanthamoeba myosin IC to F-actin was assayed in solutions containing 10 mM Tris, pH 7.5, 3.5 mM MgCl2, 1 mM EGTA, 0.2 mg/ml BSA, with or without 2.5 mM ATP as indicated, and NaCl at the concentrations indicated in the figure legends. Myosins were mixed with various concentrations of dephosphorylated wild type and T-2 mutant myosins, as substrates, in 50 mM imidazole (pH 7.0) containing 2.5 mM [γ-32P]ATP (120 cpm/nmol), 3.5 mM MgCl2, and 2 mM EGTA and BSA (0.2 mg/ml). At all substrate concentrations, the rate of phosphorylation was linear with time for the period of incubation.

**ATPase Assays.—**Steady state ATPase activities were determined at 30 °C by measuring the radioactivity of P released from [γ-32P]ATP as described (2). The reaction mixtures for the assay of MgATPase activity contained 20 mM imidazole, pH 7.5, 4 mM MgCl2, 1 mM EGTA, 1 mM DTT, 3 mM [γ-32P]ATP (120 cpm/nmol) with or without F-actin as indicated. The reaction mixtures for the assay of NH_EDTA-ATPase activity contained 25 mM Tris (pH 7.5), 400 mM NH4Cl, 35 mM EDTA, 1 mM DTT, and 3 mM [γ-32P]ATP (120 cpm/nmol). The reactions were started by the addition of myosin at 30 °C.

**Electrophoresis—**SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (41). The separating gel consisted of two layers; the upper half contained 7.5% acrylamide and the lower half contained 13% acrylamide.

**RESULTS**

**Expression and Purification of Myosin IC Wild Type and Mutants—**The 129-kDa heavy chain of myosin IC can be divided into a head domain, residues 1–693; a light chain-binding neck domain, residues 694–720; and four tail domains: the basic TH-1 domain, residues 721–940; the TH-3 (SH3) domain, residues 997–1051; and the two segments of the GAPA TH-2 domain, GPA-1, residues 941–996, and GPA-2, residues 1052–1186, that are separated by the SH3 domain (Fig. 1). As described under “Materials and Methods,” we constructed six truncated mutants and one deletion mutant (Fig. 1): T-1, the head only; T-2, the head and neck; T-3, the head, neck, and one-half of TH-1; T-4, the head, neck, and entire TH-1; T-5, the head, neck, TH-1, and GPA-1; T-6, the head, neck, GPA-1, and SH3; and ΔSH3, the entire heavy chain except for the deleted SH3 region. The wild type heavy chain and seven mutant heavy chain constructs were individually co-expressed with the light chain in SF-9 cells. All of the heavy chains were expressed very well (see Fig. 2, first lane, for expression of wild type heavy chain). All of the myosins were readily purified by the procedure described under “Materials and Methods” (Fig. 2), except for T-3, the mutant that was truncated within the TH-1 domain, which we were unable to solubilize and, therefore, could not study further.

**Binding to F-actin—**We compared the binding of the wild type and six mutant myosins to an excess of muscle F-actin in the presence or absence of ATP. As expected, because the head domain of all myosins has a high affinity, ATP-sensitive actin-binding site, all of the expressed myosins bound to actin essentially quantitatively in the absence of ATP (Fig. 3). The wild type and ΔSH3 myosins also bound to F-actin in the presence of ATP but T-1 and T-2 did not, T-4, T-5, and T-6 had intermediate behavior. Each bound extensively to F-actin in the presence of ATP but not as well as in the absence of ATP. These results are consistent with the recent report by Lee et al. (5) that bacterially expressed peptides corresponding to the TH-1, TH-2, and TH-2/3 domains of Acanthamoeba myosin IA bind to F-actin in the presence of ATP.

Next, we quantified the relative affinities for F-actin of wild type myosin and the T-4, T-5, T-6, and ΔSH3 mutants in the presence of ATP (Fig. 4). The measured Kd values were: wild type, 22 nm; ΔSH3, 35 nm; T-6, 122 nm; T-5, 120 nm; T-4, 164 nm (Fig. 4). The similar affinities for F-actin of wild type and ΔSH3 and of T-6 and T-5 are consistent with earlier data that the SH3 region is not directly involved in actin binding. However, the slightly higher Kd values for the constructs that lack the SH3 domain (ΔSH3 compared with wild type and T-5 compared with T-6) may indicate that the SH3 domain has a small modifying effect on binding to F-actin. The higher Kd values for the truncated mutants T-6, T-5, and T-4 indicate that the GAPA2 region (Fig. 1) contributes significantly to actin binding by the myosin IC tail.

Lee et al. (5) reported that bacterially expressed peptide TH-2 of Acanthamoeba myosin IA bound much more weakly to...
muscle F-actin in 60 mM KCl than in the absence of KCl, but that salt did not affect the binding of TH-2 to Acanthamoeba F-actin and that the binding of expressed TH-1 peptide to both actins was unaffected by the ionic strength. We found that in the presence of ATP, i.e., when binding occurs only through the tail site(s), both T-4 and wild type bound equivalently to muscle and Acanthamoeba F-actin. In contrast to the findings of Lee et al. (5) for peptides, we found that the binding of both myosins to both actins was very, and approximately equally, sensitive to the ionic strength (Fig. 5).

ATPase Activities of Wild Type and Mutant Myosins—The MgATPase activities in the absence of F-actin of fully phosphorylated (on Ser-329) wild type and the six mutant myosins were very similar, ~0.05 s⁻¹, as were their NHL/EDTA-ATPase activities, ~20 s⁻¹ (Table I). Thus, neither deletion nor truncation within the tail domain affected these basal activities.

The actin dependence of the MgATPase activities of the wild type and mutant myosins are shown in Fig. 6. Wild type and ∆SH3 myosins had the triphasic actin dependence characteristic of native Acanthamoeba myosin I (Fig. 6A). The actin-dependent MgATPase activities of the T-4, T-5, and T-6 mutants were qualitatively similar to wild type (Fig. 6B) but neither the T-1 nor T-2 mutant had significant MgATPase activity at low actin concentrations (Fig. 6A). These results are consistent with the earlier proposal that the triphasic actin-dependent MgATPase activity of the Acanthamoeba myosin I results from the ATP-insensitive actin-binding sites in the tail. However, note that the T-4, T-5, and T-6 mutants were less active than wild type and ∆SH3 at low actin concentration (Fig. 6 and Table I) and less active than wild type, ∆SH3, T-1, and T-2 at high actin concentrations (Fig. 6 and Table I).

Albanesi et al. (21) found the triphasic actin dependence of the MgATPase activities of the Acanthamoeba myosin I to be a function of the molar ratio of myosin to F-actin, i.e., activation occurs at low actin concentration when the concentration of myosin is sufficient to cross-link cooperatively the actin filaments thus increasing the effective concentration of actin for the myosin catalytic domains. The data in Fig. 7 are consistent with this proposal. Although, the peak specific activities of 40 and 120 nm wild type myosin IC at low F-actin concentration were the same, the activity peak was much narrower for 40 nm myosin (Fig. 7A). The specific activity at low F-actin concentration of T-5, whose tail had a substantially lower affinity for F-actin than wild type tail (Fig. 3), was very much less at 40 nm than at 141 nm (Fig. 7).

Comparison of Wild Type and Mutant Myosins as Substrates for Myosin I Heavy Chain Kinase—As one test of the possible interaction between the SH3 domain of Acanthamoeba myosin I and the PXXP motifs in the proline-rich region of MIHCK, we determined the $K_m$ and $V_{max}$ for the phosphorylation by full-length MIHCK of wild type myosin IC and the T-2 mutant, which lacks the entire tail domain including the SH3 region. The data were very similar for the two substrates (Fig. 8) with a $K_m$ of ~6.6 μM and a $V_{max}$ of ~15.5 s⁻¹, indicating that nothing in the myosin I tail, including the SH3 domain, affects the kinetics of phosphorylation. Consistent with these results, we found that wild type myosin IC did not bind to a bacterially expressed peptide corresponding to the entire Pro-rich region of the kinase (data not shown).

**DISCUSSION**

We have used baculovirus-expressed wild type and truncated and deletion mutants of Acanthamoeba myosin IC to gain further insights into the ability of the Acanthamoeba myosin I to bind to F-actin in the presence of ATP, the unusual triphasic actin dependence of their MgATPase activities and the possibility that the SH3 domain in the tail of the myosin I might interact with the PXXP motifs in the proline-rich region of Acanthamoeba myosin I heavy chain kinase.

The functions of the SH3 domain of amoeba myosin I are not well understood (18). The SH3 domain binds to the PXXP motif of Acan125 (42, 43) and to an analogous Dictyostelium protein (44), which may serve as a scaffold for proteins, including myosin I, involved in the assembly of the actin cytoskeleton (44). The observation (45) that Dictyostelium myosin IB lacking the SH3 domain is unable to rescue the defects in growth and endocytosis of myosin IB null cells may be the physiological expression of these biochemical interactions. Similarly, the Saccharomyces myosin I is has been found to bind through the SH3 domain to the PXXP regions of verprolin (46, 47) and Bee1p (47, 48), proteins involved in the assembly of

![Fig. 2](image1.png)

**Fig. 2.** SDS-polyacrylamide gel electrophoresis analysis of purified recombinant Acanthamoeba myosin IC wild type and mutants. The FLAG-tagged T-1, T-2, T-4, T-5, T-6, and ∆SH3 mutants and wild type (WT) were purified from the cell extracts (the extract shown is wild type) in one step using an anti-FLAG antibody affinity resin. The T-3 mutant was expressed but was insoluble and, therefore, could not be purified. The gels were stained with Coomassie Blue.

![Fig. 3](image2.png)

**Fig. 3.** Effect of ATP on the binding of Acanthamoeba myosin IC wild type and mutants to rabbit muscle F-actin. Myosin (0.8 μM) in 10 mM Tris, pH 7.5, 1 mM EGTA, 3.5 mM MgCl₂, 0.2 mg/ml BSA, and 22 mM NaCl (from the myosin stock solutions) and with or without 10 μM F-actin and 2 mM ATP was centrifuged for 30 min at 4°C at 100,000 rpm in a Beckman TL centrifuge. Equivalent amounts of supernatant (S) and pellet (P) were analyzed by SDS-polyacrylamide gel electrophoresis.
actin, and the SH3 domain is required for the polymerization of actin (48) and localization of the myosins to actin patches in yeast (46). In contrast to these observations, however, deletion of the SH3 region of Aspergillus myoA, the only myosin I in this organism, had no phenotypic effect (49).

Given that the myosin I SH3 domain reacts with the PXXP motifs of Acan125, that MIHCK has four PXXPXXP motifs that are the minimum requirements for interaction of the SH3 domain of Saccharomyces myosin Is with BEEp and verprolin (47), and that mammalian Pak1 (a Pak) binds to the SH3 domain of target proteins (50, 51); it may be surprising that deletion of the SH3 domain from the tail of Acanthamoeba myosin IC had no effect on the kinetics of its phosphorylation by MIHCK and that wild type myosin IC did not bind to an expressed peptide that contains the PXXPXXP motifs of the kinase. However, the absence of any detectable reaction in vitro between the SH3 domain of myosin IC and the Pro-rich region of MIHCK does not necessarily preclude such interaction in vivo.

The actin-binding data show, as expected, that truncated mutants that contained only the head domain or only the head and neck domains did not bind to F-actin in the presence of ATP. Truncated mutants that, in addition to the head and neck domains, also contained the basic TH-1 domain or the TH-1 domain of target proteins (50, 51), bound to F-actin. The actin-binding data show, as expected, that truncated mutants that contained only the head domain or only the head and neck domains did not bind to F-actin in the presence of ATP. Truncated mutants that, in addition to the head and neck domains, also contained the basic TH-1 domain or the TH-1 domain of target proteins (50, 51), bound to F-actin.
domain and all or part of the GPA TH-2 domain did bind to F-actin in the presence of ATP but not as well as wild type myosin IC or the deletion mutant, ΔSH3. These data for the myosin IC mutants are consistent qualitatively with the earlier data for bacterially expressed peptides (11, 14, 15).

Recently, Lee et al. (5) reported $K_d$ values of 0.02–0.20 μM for the binding at low ionic strength of peptides corresponding to Acanthamoeba myosin IA domains TH-2 and TH-2/3 to Acanthamoeba F-actin and for the binding of TH-2/3 to rabbit muscle F-actin. The affinities of the peptides for Acanthamoeba F-actin were unaffected by KCl concentrations up to 60 mM but the $K_d$ values of the TH-2-containing peptides for muscle actin increased to >50 μM in 60 mM KCl. Peptide TH-1, however, bound to muscle F-actin at 60 mM KCl with $K_d$ ~0.1 μM, and TH-1 bound to TH-2/3 and enhanced its binding to muscle F-actin in 60 mM KCl.

In our experiments, wild type myosin IC and the ΔSH3 deletion mutant (both of which have intact TH-1 and TH-2 regions) had much higher affinities for muscle F-actin at low ionic strength ($K_d$ values of 22 and 35 μM, respectively) than Lee et al. (5) found for the TH-1 and TH-2/3 peptides of Acanthamoeba Myosin IA. The T-6 myosin IC mutant (which lacks three-fourths of the TH-2 domain), T-5 (which additionally lacks the TH-3 (SH3) domain), and T-4 (which lacks all of the TH-2 and TH-3 domains but has all of the TH-1 domain) bound to muscle F-actin at low ionic strength with $K_d$ values of 122, 120, and 164 nM, respectively, similar to the affinities of the expressed myosin IA peptides. Like the myosin IA peptides, the myosin IC mutants had similar affinities for muscle and Acanthamoeba F-actin but, unlike the expressed TH-2 and TH-2/3 myosin IA peptides, binding of the truncated mutants of myosin IC to both actins was similarly inhibited by ionic strength being reduced by about 50% in 80–100 mM NaCl and by about 80% in 150 mM NaCl. We do not know if the differences between our results and the interesting observations of Lee et al. (5) reflect differences between expressed myosins (this paper) and expressed peptides (5) or between Acanthamoeba myosin IC (this paper) and Acanthamoeba myosin IA (5).

The very similar $K_d$ values for wild type and the ΔSH3 myosins and for the T-6 and T-4 constructs indicate, in agreement with the earlier data for peptides, that the SH3 (TH-3) domain does not contribute significantly to actin binding. The substantial difference in the $K_d$ values for F-actin between wild type myosin and T-6 implies either that the GPA-2 domain binds more tightly than the TH-1 domain or that the TH-1 and TH-2 domains cooperatively enhance binding of the full-length tail to F-actin. This latter possibility may be related to the observation by Lee et al. (5) that the TH-1 peptide bound to the TH-2/3 peptide and increased its affinity for F-actin in 60 mM KCi by about 50-fold. The small difference in the F-actin affinities of T-4 and T-5 found in our experiments may reflect a contribution of the GPA-1 domain to the binding of T-5.

Our finding that only wild type myosin and mutant myosins with tail regions containing one or more ATP-Insensitive actin-binding sites (T-4, T-5, and T-6) exhibit triphasic actin dependence of their MgATPase activities is consistent with the proposal (17, 19, 21, 22) that these unusual kinetics are the consequence of the ATP-insensitive actin-binding site(s) in the tail. The MgATPase activities of two Dictyostelium myosin Is (23) and rat myr3 (21) have also been shown to have triphasic actin dependence. Like the Acanthamoeba myosin Is, Dictyostelium myosin IB has been shown to have an ATP-Insensitive F-actin-binding site in its tail region (24), but Stöffler and Bähler (52) did not detect binding of rat myr3 to muscle F-actin in the presence of ATP and concluded, therefore, that a second actin-binding site in the tail is not responsible for the triphasic kinetics of myr3. However, those binding studies were done in

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**Table I**

| Myosin       | MgATPase Actin (μM) | NH₄/EDTA-ATPase |
|--------------|---------------------|-----------------|
|              | 0                   | 2               | 80              |
| Wild type    | 0.04                | 22.33           | 12.82           |
| T-1          | 0.04                | 0.62            | 11.33           |
| T-2          | 0.06                | 1.07            | 13.01           |
| T-4          | 0.06                | 5.03            | 3.90            |
| T-5          | 0.03                | 9.12            | 4.80            |
| T-6          | 0.05                | 2.57            | 3.89            |
| ΔSH3         | 0.04                | 21.47           | 13.23           |

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**Fig. 7.** Effect of myosin concentration on the triphasic actin dependence MgATPase activity of Acanthamoeba myosin wild type and T-5 mutants. A, wild type: 120 nM (●), 40 nM type (○). B, T-5: 141 nM (●), 40 nM (○). Incubations were for 3 min at 30 °C.

**Fig. 8.** Kinetics of the phosphorylation of Acanthamoeba myosin IC wild type and T-2 mutant by Acanthamoeba myosin IC heavy chain kinase. Wild type myosin (●) and the T-2 mutant (○) were incubated at the indicated concentrations with 4.5 nM myosin I heavy chain kinase (fully activated by autophosphorylation) for 20 s at 30 °C, and the extent of phosphorylation was determined as described under “Materials and Methods.” The maximum concentration of myosin was limited by its solubility to about 8 μM. The data are plotted as the reciprocal of the specific activity of the kinase versus the reciprocal of the myosin concentration. The insets are direct plots of the same data.
150 mM NaCl. At this ionic strength, the amoeba myosin I tails bind very weakly to F-actin and, in addition, myosins, including myr3 (52), have greatly reduced actin-dependent MgATPase activity under these conditions. For these reasons, binding of proteolytically cleaved myr3 to F-actin should be re-evaluated at low ionic strength where the MgATPase activity of myr3 shows maximal triphasic actin dependence (52).

It is interesting that the actin-dependent MgATPase activities of myosin mutants that lacked some or all of the TH-2 domain (T-4, T-5, and T-6) were lower than the activities of mutants that had intact TH-1 and TH-2 domains (wild type and ∆SH3) and also of mutants that lacked the entire tail (T-1 and T-2). Lee et al. (5) have suggested that the tails of native Acanthamoeba myosin IIs fold back on themselves stabilized by the interaction of the TH-1 and TH-2/3 domains (the expressed peptides interacted with a K_d of ~250 nM (5)). Possibly, the absence of any such interaction in the T-4, T-5, and T-6 mutants allowed the partially truncated tails of these mutants to interfere with the interaction between F-actin and the catalytic site in the head domain. In contrast to our results, proteolytic truncation at the C terminus of rat myr3 was reported to interfere with the interaction between F-actin and the catalytic site in the head domain. In contrast to our results, proteolytic truncation at the C terminus of rat myr3 was reported to increase its MgATPase activity (51). We hope that crystallography and cryoelectron microscopy of the myosin I constructs described in this paper will clarify some of these issues.

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