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Analysis of the regulatory phosphorylation site in *Acanthamoeba* myosin IC by using site-directed mutagenesis

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ABSTRACT **The actin-activated ATPase activity of *Acanthamoeba* myosin IC is stimulated 15- to 20-fold by phosphorylation of Ser-329 in the heavy chain. In most myosins, either glutamate or aspartate occupies this position, which lies within a surface loop that forms part of the actomyosin interface. To investigate the apparent need for a negative charge at this site, we mutated Ser-329 to alanine, asparagine, aspartate, or glutamate and coexpressed the Flag-tagged wild-type or mutant heavy chain and light chain in baculovirus-infected insect cells. Recombinant wild-type myosin IC was indistinguishable from myosin IC purified from *Acanthamoeba* as determined by (*i*) the dependence of its actin-activated ATPase activity on heavy-chain phosphorylation, (*ii*) the unusual triphasic dependence of its ATPase activity on the concentration of F-actin, (*iii*) its K_m for ATP, and (*iv*) its ability to translocate actin filaments. The Ala and Asn mutants had the same low actin-activated ATPase activity as unphosphorylated wild-type myosin IC. The Glu mutant, like the phosphorylated wild-type protein, was 16-fold more active than unphosphorylated wild type, and the Asp mutant was 8-fold more active. The wild-type and mutant proteins had the same K_m for ATP. Unphosphorylated wild-type protein and the Ala and Asn mutants were unable to translocate actin filaments, whereas the Glu mutant translocated filaments at the same velocity, and the Asp mutant at 50% the velocity, as phosphorylated wild-type proteins. These results demonstrate that an acidic amino acid can supply the negative charge in the surface loop required for the actin-dependent activities of *Acanthamoeba* myosin IC *in vitro* and indicate that the length of the side chain that delivers this charge is important.**

In recent years, 111 members of the myosin superfamily, grouped into 15 classes, have been identified at the DNA sequence level (1), the atomic structures of actin (2) and the myosin motor domain (3) have been determined, and the actin–myosin interface has been simulated by fitting the atomic structures to reconstructions of cryoelectron microscopic images of the rigor complex (4). However, very little is known of how F-actin activates the myosin ATPase, in part because relatively few of the myosins have been purified and studied biochemically, and those that have are mostly representatives of the two largest classes: conventional, class II myosins, with 46 known members, and class I myosins, with 32 known members (the next largest, class V, contains only 7 known members).

Acanthamoeba myosins IA, IB, and IC were the first unconventional (i.e., non-class II) myosins to be discovered and are the most extensively studied class I myosins. These *Acanthamoeba* myosins are single-headed, nonfilamentous, actin-based mechanoenzymes that appear to play important roles in several aspects of cellular and intracellular motility (refs. 5 and 6; for review, see ref. 7). Their single heavy chains are composed of an ≈80-kDa N-terminal head domain and a ≈50-kDa C-terminal tail (8, 9). The head domains, which are highly homologous to subfragment 1 of conventional class II myosins, contain an ATPase site (10, 11), an ATP-sensitive actin-binding site (12), and a single, putative light-chain binding site (an IQ domain; refs. 8 and 9); the bona fide light chain of *Acanthamoeba* myosin IC (AMIC) has been cloned and sequenced (13). The tail domain contains a positively charged region that interacts with membranes (14) and negatively charged phospholipids (15), a Pro-Gly-Ala-rich region that binds F-actin in an ATP-insensitive fashion (10, 11, 15, 16), and a Src-homology region 3 (SH3) domain (8, 9), whose function is not clear. As a consequence of the membrane- and actin-binding domains in the tail, monomeric *Acanthamoeba* myosins I can crosslink and move actin filaments relative to each other (17) and move actin filaments relative to membranes (18).

The actin-activated ATPase activities of most class II myosins [*Acanthamoeba* myosin II is a notable exception (19)] are regulated by Ca^{2+} interacting with the tropomyosin–troponin complex that is associated with F-actin, Ca^{2+} binding to one of the two pairs of myosin light chains, or phosphorylation of one of the two pairs of light chains by Ca^{2+}-activated myosin light-chain kinase (20). On the other hand, the *Acanthamoeba* myosin I isozymes are activated by phosphorylation of the heavy chain at a single site by a specific *Acanthamoeba* myosin I heavy-chain kinase (21, 22)—a member of the p21-activated protein kinase family (23)—activated by acidic phospholipids and membranes (24, 25) and small GTPases (26). The phosphorylation site, Ser-329 in AMIC (ref. 22; see *Results* for numbering change), corresponds to Glu-411 of chicken skeletal muscle myosin (3), which resides in a surface loop that has been thought to form part of the actin-binding interface in the upper portion of the 50-kDa domain of subfragment 1 (4). Almost all of the other myosins (both conventional and unconventional) that have been sequenced, including most of the class I myosins, have either a Glu or an Asp at this position (1). The myosins known to have Ser or Thr at this site—the TEDS site (27)—are the three *Acanthamoeba*, five *Dictyostelium*, two *Saccharomyces*, one *Aspergillus*, and one *Emericella* class I myosins, *Drosophila*, pig, and human class VI myosins, and a *Tetrahymena* myosin of unknown class (1). These observations suggest that a negative charge at the TEDS site, provided by either an acidic amino acid or a phosphorylated hydroxyamino acid, is required for high actomyosin ATPase activity (27).

What experimental data support this reasonable inference? In *Acanthamoeba*, 20–89% of the myosin I is phosphorylated

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Abbreviation: AMIC, *Acanthamoeba* myosin IC.

Data deposition: The sequence reported in this paper has been deposited in the Genbank database (accession no. AF051353).

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in vivo, depending on the isoform (28). The phosphorylated myosins I are preferentially enriched at motile regions such as pseudopodia and phagocytic cups (28), and phosphorylated myosin IC appears to be required for contractile vacuole activity (28, 29). Wild-type myo3 (one of the two yeast myosins I) and myo3 with Ser-357 replaced by Asp at its TEDS site, rescued the lethal myo3/myo5 double-myosin I mutant of yeast, but myo3 with Ala at the TEDS site did not (30). Similarly, the analogous Ala mutant of a *Dictyostelium* myosin I did not rescue a null cell line that the wild-type protein rescues (31), and the deleterious effects of overexpression of the wild-type myosin were abrogated by the Ala mutant (32). In apparent conflict with these observations, however, *Aspergillus* myosin I with Ser-371 replaced by Ala at its TEDS site fully rescued growth on standard solid media (33).

The *in vitro* biochemical data also are inconclusive. Although the actin-dependent ATPase activities of two of the *Dictyostelium* class I myosins, like the three *Acanthamoeba* myosins I, have been shown to be activated *in vitro* by phosphorylation (34, 35) catalyzed by a p21-activated protein kinase (36, 37), this has not yet been shown for any of the other class I or class VI myosins that have Ser or Thr at the TEDS site. Also, there are exceptions to the TEDS rule: *Drosophila* myosin IA has Asn, two *Acetabularia* class XIII myosins have Gly, and two *Toxoplasmodium* class XIV myosins have Gln at the TEDS site, whereas a *Drosophila* class IV myosin and a *Caenorhabditis elegans* class XII myosin have gaps at this position (1).

From the above summary, it seems possible that the essential functions *in vivo* of some myosins may not, and of a few other myosins cannot, require an acidic residue at the TEDS site. A first step in evaluating these possibilities is to determine the effects of mutations at this site on myosin activities that can be studied *in vitro*. To this end, we generated a full-length cDNA clone of the wild-type heavy chain of AMIC and mutated Ser-329 to either Ala, Asn, Asp, or Glu. The wild-type and mutant heavy-chain cDNAs were coexpressed with the previously isolated, full-length AMIC light-chain cDNA in baculovirus-infected insect cells, and the myosins were purified and assayed for actin-activated ATPase activity and the ability to translocate actin filaments in an *in vitro* motility assay. The results demonstrate directly and quantitatively the requirement for a negative charge at the TEDS site for the actin-activated ATPase and actin filament translocation activities of AMIC and indicate that the length of the side chain that delivers this charge can be important.

MATERIALS AND METHODS

Full-Length *Acanthamoeba* Myosin IC Heavy-Chain cDNA. A ≈3-kb cDNA clone that begins at the *Eco*RI site at nucleotide 729 (nucleotides are numbered according to the revised sequence described in this paper) and ends at the Poly(A)$^+$ tail was kindly provided by Thomas Pollard (Salk Institute, La Jolla, CA). To obtain the remaining ≈800 bp of 5′ sequence, we constructed an *Acanthamoeba* cDNA library by using Poly(A)$^+$ mRNA (Qiagen, Chatsworth, CA), Superscript RNase H$^-$ reverse transcriptase (BRL), and a mixture of oligo(dT), random hexamer, and gene-specific primers (the latter corresponding to nucleotides 846–863 in AMIC cDNA: CGGCAAGGGCGGCTCCAC). cDNAs were cloned into λ ZAPII (Stratagene) and screened with a probe generated by reverse transcription–PCR (BRL) using *Acanthamoeba* mRNA, a 5′ primer corresponding to nucleotides −24 to −1 of AMIC cDNA, and a 3′ primer corresponding to nucleotides 281–300 of the AMIC cDNA: ACCAATGCGTCATCATTTCT. The PCR product was ^{32}P-labeled by using a random primer labeling kit (Amersham). Four clones were isolated from approximately 1 million plaques, excised to pBluescript SK(-) plasmid, and their ≈1-kb inserts sequenced on both strands by the dideoxy chain termination method. The four clones were identical and contained 170 bp of 5′ noncoding sequence and 840 bp of 5′ coding sequence that matched (with a few small exceptions; see *Results*) the 5′ end coding sequence of genomic clones (9). This 5′-end fragment was then ligated to the rest of the AMIC cDNA by using the *Eco*RI site at nucleotide 729 to generate a full-length myosin IC heavy-chain cDNA. Some of the 3′ noncoding sequence (including the *Eco*RI site at the 3′ end) was deleted by *Exo*III/mung-bean nuclease digestion (Stratagene) to aid in joining the two cDNA clones.

Construction of the Myosin IC Heavy-Chain Phosphorylation Site Mutants. Ser-329 in the AMIC heavy chain was changed to Ala, Asn, Asp, or Glu by using overlap-extension PCR (38). The two outside primers were GTGAAGGACGTCATCCTCGAG (nucleotides 391–411) and GTAGAAGTCGAGAATACCGGTGG (nucleotides 1127–1149 in the antisense direction). The inside primers, which encompassed the mutation site (967-CAGGGCCGCGGCCGCTCG*TCC*GTCTACTCCTGCCCGCAGGAC-1008) with the codon for Ser-329 (italicized) replaced by GCC for Ala, AAC for Asn, GAC for Asp, and GAG for Glu were synthesized as a pair of complementary sense and antisense primers for each desired mutation. To construct each mutant cDNA, a PCR reaction was performed by using plaque-forming unit DNA polymerase (Stratagene), the wild-type AMIC heavy chain cDNA as a template, and one outside and one inside primer in two separate reactions. The two PCR products (618-bp and 183-bp) were then purified, denatured, annealed, and subjected to overlap-extension PCR by using the outside primers and plaque-forming units. The 758-bp product was purified, digested with *Stu*I and *Bsr*gI and ligated into *Stu*I/*Bsr*gI-cut wild-type AMIC cDNA. Each mutation was confirmed by dideoxy chain termination sequencing.

Epitope Tagging. We placed a Flag epitope tag (Sigma) at the extreme N terminus of the AMIC heavy chain to provide a mechanism for the rapid purification of wild-type and mutant AMIC molecules. The synthetic nucleotide sequence ATGGCCGACTACAAAGACGATGACGACAAAGCC, which codes for the Flag peptide MADYKDDDDYA, was inserted into a *Nco*I site of the 5′ end of coding sequence, resulting in 11 more amino acid residues at the N terminus of each myosin IC heavy chain.

Construction of Transfer Vectors. The wild-type, S329A, and S329E AMIC heavy-chain cDNAs were released from pBluescript by digestion with *Bam*HI and subcloned into the *Bam*HI site of the transfer vector PVL 1393(PharMingen), and the S329N and S329D cDNAs were subcloned into the *Bam*HI site of the transfer plasmid pBlueBac 4.5 (Invitrogen). The myosin IC light-chain cDNA obtained previously (13) was subcloned into the *Eco*RI site of the transfer plasmid PVL 1393 (PharMingen).

Sf9 Cell Transfection and Purification of Heavy- and Light-Chain Recombinant Viruses. Sf9 cells (2×10^6) were transfected with 2–4 μg of transfer plasmid DNA and either 0.5 μg of Baculo Gold vector DNA (PharMingen) for the wild-type, S329A, and S329E cDNAs or 1 μg of Bac-N-Blue vector DNA (Invitrogen) for the S329N and S329D cDNAs. Transfections were performed in 1 ml of Grace's medium (Life Technologies, Gaithersburg, MD) by using 20 μl of liposome solution as described by the manufacturer. The transfection of the myosin IC light-chain cDNA mirrored that of the wild-type heavy-chain cDNA. Recombinant viruses were identified either as occlusion-negative plaques (PharMingen method) or blue plaques (Invitrogen method) and were confirmed by using SDS/PAGE analysis of the infected cell lysates followed by immunoblot analysis using the anti-Flag (M2) mAb (Kodak). Viral stock amplification was carried out according to the manufacturer's manual, and the titers were determined by Paragon Biotechnology.

Production and Purification of Recombinant AMIC Molecules. Tissue-culture plates (100 × 15 mm; Falcon no. 1029) containing 2×10^7 Sf9 insect cells each were inoculated with both light- and heavy-chain viruses at a combined multiplicity of infection of ≈10. The infected cells were grown in insect medium (Paragon Biotech) for 72 hr at 27°C, released with a rubber policeman, and harvested by centrifugation at 5,000 × *g* for 10 min. The pellet was washed with PBS and either used immediately or quick frozen in liquid nitrogen and stored at −80°C. To purify the protein, 3 g of cell pellet (from ≈12 dishes) was homogenized in 30 ml of buffer containing 20 mM imidazole (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 5 mM ATP, 200 mM NaCl, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride and 2 tablets of protease inhibitor (Boehringer Mannheim) by using a ground-glass Dounce homogenizer. The lysate was stirred gently for 40 min and centrifuged at 200,000 × *g* for 1 hr. The supernatant was loaded onto a column containing 1 ml (settled volume) of anti-Flag M2 affinity gel (Kodak) that had been equilibrated with 1 mM ATP, 5 mM $MgCl_2$, and 7% (wt/vol) sucrose in PBS (column buffer). After washing with column buffer, Flag-tagged AMIC was eluted with column buffer supplemented with 0.3 mg/ml free Flag peptide. The fractions that contained the purified protein were pooled and dialyzed against 20 mM Tris (pH 7.5), 100 mM KCl, 1 mM DTT, and 50% (vol/vol) glycerol and stored at −20°C. All purification steps were carried out at 4°C.

Phosphorylation and ATPase Assays. Wild-type and mutant AMIC molecules were phosphorylated as described (25) by using either purified AMIC heavy-chain kinase (21) or the catalytic domain of this kinase expressed in baculovirus (23). Actin-activated ATPase assays were performed as described (39). The dependency of the actomyosin ATPase activity on the concentration of ATP was measured by a coupled assay in the presence of an ATP-regenerating system (40). Reactions were performed at 22°C in 25 mM Tris (pH 8.0), 25 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM DTT, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 60 units/ml lactate dehydrogenase, 60 units/ml pyruvate kinase, 80 nM Flag-tagged AMIC, 2 μM F-actin, and various concentrations of ATP. The rate of ADP release was enzymatically coupled to the oxidation of NADH, which was monitored by the change in absorbance at 340 nm. An extinction coefficient of 6,220 M^{-1} cm^{-1} for NADH was used.

***In Vitro* Motility Assays.** Motility assays were performed by using the modified procedure of Sellers *et al.* (41). Flag-tagged AMIC molecules were bound in the presence of Mg^{2+}-ATP to a glass surface coated with F-actin filaments to encourage the binding of myosin IC to the surface via its ATP-insensitive actin-binding site in the tail. After washing away unbound myosin, rhodamine phalloidin-labeled actin filaments were added and their translocation initiated by the addition of motility buffer containing 40 mM KCl, 20 mM Mops (pH 7.0), 5 mM $MgCl_2$, 0.1 mM EGTA, 50 mM DTT, 2.5 mg/ml glucose, 0.1 mg/ml glucose oxidase, 0.02 mg/ml catalase, and 0.7% methylcellulose at 30°C. The rates of filament translocation were determined as described (41).

Determination of Molecular Mass. Purified wild-type AMIC was dialyzed into buffer containing 10 mM Tris (pH 7.5), 100 mM KCl, 2 mM 2-mercaptoethanol, and 0.1 mM EGTA (final concentration of AMIC, 0.5 mg/ml) and centrifuged at 44,000 rpm for 1 hr in a Beckman Optima XL-I analytic ultracentrifuge. UV absorption (280 nm) scans were conducted 15 times at 4-min intervals. The data were analyzed by using the Optima XL-A data analysis software (Beckman).

Miscellaneous. Actin was prepared from rabbit skeletal muscle acetone powder according to Spudich and Watt (42) and stored on ice. *Acanthamoeba* myosin I heavy-chain kinase and its expressed catalytic domain (kindly provided by Joanna Szczepanowska, National Heart, Lung, and Blood Institute) were purified as described (21, 23). The 8-residue Flag peptide (DYKDDDDK) was synthesized by Angela Murphy (National Heart, Lung, and Blood Institute). NADH, phosphoenolpyruvate, lactate dehydrogenase, and pyruvate kinase were from Sigma. Protein concentrations were determined by using the Bio-Rad assay with BSA as standard.

RESULTS

Expression and Purification of Recombinant AMIC. The cloned full-length AMIC heavy-chain cDNA spans 3,558 bp and encodes a heavy-chain polypeptide of 1,186 amino acid residues (GenBank accession code AF051353). The deduced amino acid sequence matches the one predicted from genomic clones (GenBank accession code J02974) with only three minor differences. As a result of small errors in the choice of splice codons and mapping digestion fragments, the revised sequence has an insert of 6 residues between original amino acids 135 and 136 and an insert of 12 residues between original amino acids 287 and 288. A frameshift caused by addition of one nucleotide and a compensating deletion 30 nucleotides downstream changed the sequence of 9 amino acids originally numbered 201–209. The two insertions increase the sequence similarity of AMIC to muscle myosin II, and the frameshift increases the sequence similarity to *Acanthamoeba* myosin IB. As a result of the additional 18 amino acids, the phosphorylated serine is renumbered as Ser-329. The deduced heavy-chain sequence has a single IQ motif in the neck region (694-IQRFLRKTAMRKYYY-708) the presumptive light-chain binding site.

Highly purified, baculovirus-expressed wild-type and mutant AMIC can be obtained in one working day by using affinity chromatography (Fig. 1) with a yield of 1.5–2 mg from 3×10^8 infected cells. The coexpressed light chain copurifies with the epitope-tagged heavy chain (Fig. 1). A single symmetrical peak with a center at 6.22 s in the time derivative g(s*) versus s* plot was obtained by using sedimentation analysis of purified wild-type myosin IC in 100 mM KCl (data not shown), indicating a single component in solution with a molecular mass of 149 kDa. This result is in excellent agreement with the calculated mass of 147.5 kDa based on one light chain (16.7

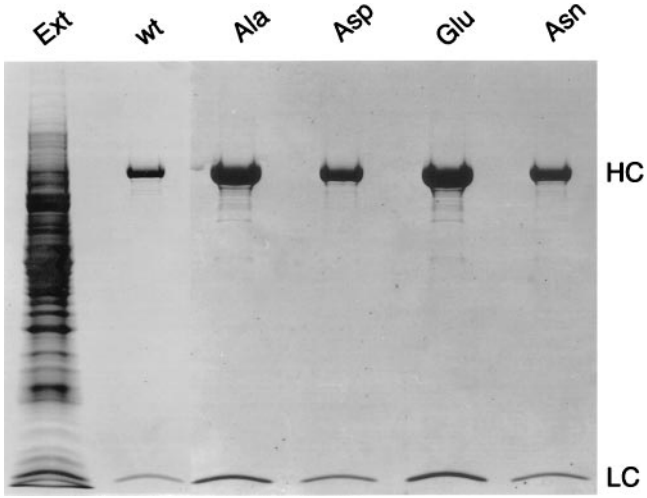

FIG. 1. Purification of recombinant wild-type and Ser-329 mutants of *Acanthamoeba* myosin IC. SDS/PAGE of the total cell extract (200,000 × *g* supernatant) of cells expressing wild-type AMIC that was applied to the affinity column (Ext) and the purified wild-type AMIC (wt) and mutants S329A (Ala), S329D (Asp), S329E (Glu), and S329N (Asn). The positions of the heavy chain (HC) and light chain (LC) are indicated. Approximately 3 μg of wild-type myosin and 6 μg of the mutant myosins were analyzed. The gels were stained with Coomassie blue. The extract and wild-type myosin were on one gel and the mutant myosins on another. For details, see *Materials and Methods*.

kDa) and one Flag-fused heavy chain (130.7 kDa; the calculated mass of the heavy chain alone is 129.5 kDa).

Characterization of Expressed Wild-Type and Mutant AMIC Molecules. As expected, expressed wild-type AMIC was a good substrate for myosin I heavy-chain kinase being phosphorylated to ≈1 mol per mol of heavy chain in 5 min (Fig. 2). The four mutant proteins were phosphorylated at <10% the initial rate of phosphorylation of the wild-type protein and only to ≈0.3 mol per mol even after 60 min. These results are consistent with earlier observations that no Ser or Thr other than Ser-329 is phosphorylated to a significant extent by myosin I heavy-chain kinase (21, 22).

As described previously for native *Acanthamoeba* myosin I, the actin-activated ATPase activity of phosphorylated, wild-type recombinant AMIC exhibited triphasic dependence on the concentration of F-actin (Fig. 3). The ATPase activity of 80 nM AMIC increased with the concentration of F-actin up to 2 μM, decreased as the F-actin concentration was raised to 30 μM, and increased again as the F-actin concentration continued to be raised to 80 μM. As discussed in detail previously (43), these data provide indirect evidence for a second actin-binding site in the tail domain of myosins I from *Acanthamoeba*. The actin-activated ATPase activities of the Ala and Asn mutants were very low—indistinguishable from that of unphosphorylated wild-type protein (Fig. 3 and Table 1)—and unaffected by the low level of nonspecific phosphorylation by myosin I heavy-chain kinase (data not shown). On the other hand, the ATPase activity of the Glu mutant was indistinguishable from that of phosphorylated wild-type protein. It had the same triphasic dependence on the concentration of F-actin (Fig. 3) and the same maximal activity, which was about 16-fold higher than the activities of unphosphorylated wild-type protein and the Ala and Asn mutants (Table 1). The ATPase activity of the Asp mutant also showed triphasic actin dependence (Fig. 3), but its maximum activity was only about half that of the Glu mutant and the phosphorylated wild-type protein (Table 1). In contrast to the differences in their rates of ATP hydrolysis, the K_m values (for ATP) of the recombinant myosins were very similar (Fig. 4 and Table 1).

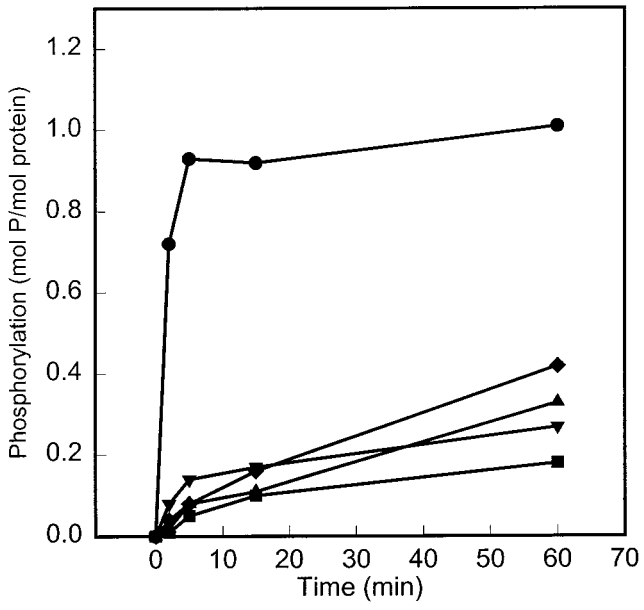

FIG. 2. Phosphorylation of recombinant wild-type and Ser-329 mutants of *Acanthamoeba* myosin IC by *Acanthamoeba* myosin I heavy-chain kinase. After incubation with [γ-^{32}P]ATP at 30°C for the indicated times, the heavy chains were separated by SDS/PAGE, located by autoradiography, excised, and their radioactivity was measured by scintillation counting. For details, see *Materials and Methods*. ●, wild type; ■, S329A; ▲, S329D; ▼, A329E; ♦, S329N.

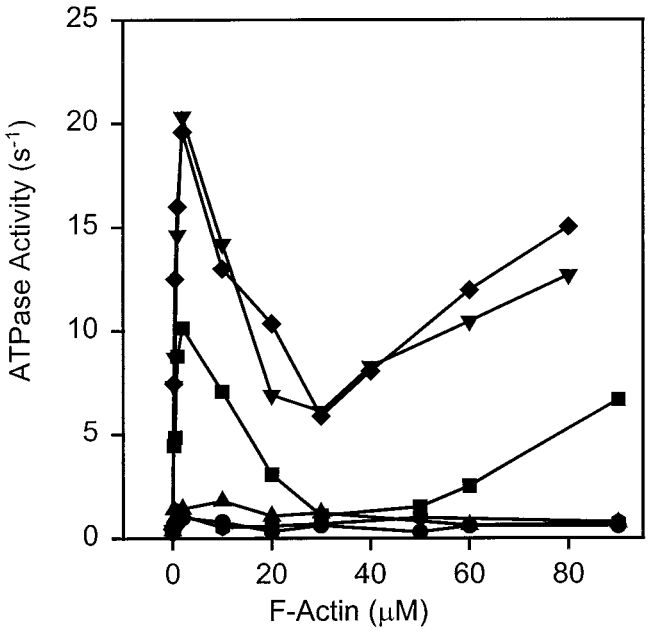

FIG. 3. Dependence of ATPase activity of recombinant wild-type and Ser-329 mutants of *Acanthamoeba* myosin IC on the concentration of F-actin. The reaction mixtures contained 80 nM myosin IC and F-actin at the indicated concentrations. The amount of $^{32}P_i$ released from [γ-^{32}P]ATP was determined after incubation for 3 min at 30°C. For details, see *Materials and Methods*. The experiments with wild-type, S329E, and S329D myosins were repeated four or five times, and the results of typical experiments are shown. ♦, phosphorylated wild type; ▼, S329E; ■, S329D; ▲, S329N; ●, S329A; ●, unphosphorylated wild type.

The abilities of the recombinant wild-type and mutant myosins to translocate actin filaments *in vitro* paralleled their actin-activated ATPase activities (Table 1). Unphosphorylated wild-type myosin and the Ala and Asn mutants were incapable of moving actin filaments, whereas the Glu mutant translocated actin filaments at the same velocity, and the Asp mutant at 50% the velocity, as wild-type myosin.

DISCUSSION

Whereas hydrodynamic studies of myosin IA and IB purified from *Acanthamoeba* provided clear evidence that each consists of a single heavy and a single light chain, the hydrodynamic mass of purified myosin IC had seemed to be more consistent with a stoichiometry of one heavy chain and two light chains (44). The data in this paper, which show that recombinant AMIC with a single light chain (consistent with a single IQ domain in the heavy chain) is biochemically indistinguishable

Table 1. Comparison of the properties of wild-type and Ser-329 mutants of *Acanthamoeba* myosin IC

Myosin	Actomyosin ATPase: Specific activity, s^{-1}	Actomyosin ATPase: K_m(ATP), μM	*In vitro* motility, μm/s
Wild type			
Unphosphorylated	1.37	10.4	0
Phosphorylated	19.85	12.4	0.11
S329A	0.98	8.3	0
S329N	1.40	—	0
S329E	20.29	13.7	0.098
S329D	9.92	11.1	0.047

The specific activities are the values in Fig. 3 at 2 μM F-actin. The values for K_m(ATP) are from Fig. 4. The *in vitro* motility activities were determined as described in *Materials and Methods*.

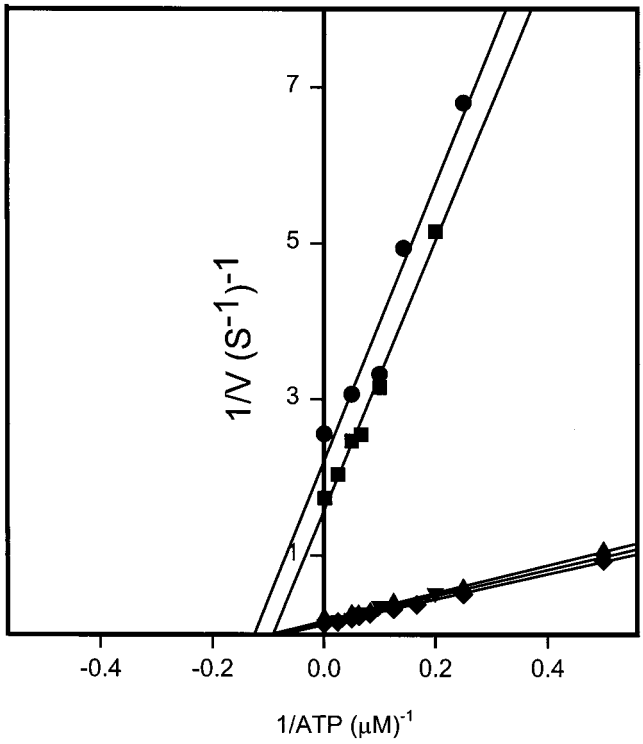

FIG. 4. Double-reciprocal plots of the rate of ATP hydrolysis by recombinant wild-type and Ser-329 mutants of *Acanthamoeba* myosin IC as a function of ATP concentration. The reaction mixtures, which were incubated at 24°C, contained 80 nM myosin IC, 2 μM F-actin, ATP as indicated, and an ATP regenerating system that coupled the hydrolysis of ATP to the oxidation of NADH, which was quantified by the change in absorbance at 340 nm. For details, see *Materials and Methods*. The experiments with wild type, S329E, and S329D were repeated four or five times, and the results of typical experiments are shown. ●, S329A; ■, unphosphorylated wild type; ◆, S329E; ▼, phosphorylated wild type; ▲, S329D.

from the enzyme purified from the amoebae, make it highly likely that the native form of AMIC contains one heavy and one light chain.

The finding that recombinant *Acanthamoeba* myosin IC with Ser-329 replaced by either Glu or Asp is constitutively active, whereas the Ala and Asn mutants are constitutively inactive, establishes the importance of an acidic residue at this position in this myosin, a position that is occupied by Glu or Asp in most myosins. As discussed in the Introduction, however, there are native and mutated myosins that have neither an acidic nor a phosphorylatable residue at this position yet seem able to carry out their essential functions *in vivo*. Perhaps other differences in the primary amino acid sequence of the surface loop compensate for the absence of an acidic residue at the TEDS site, or perhaps the activities of these myosins are regulated by a different posttranslational modification. It would be very interesting to study the biochemical properties of such myosins *in vitro*.

In the x-ray structure of chicken subfragment 1 (3), Glu-411, which corresponds to Ser-329 in AMIC, is located in the loop between Arg-405 and Lys-415 of the upper 50-kDa domain that has been proposed (4) to form a close contact with residues Pro-332–Glu-334 of actin (but see ref. 45 for a different interpretation). The corresponding sequences in *Acanthamoeba* myosins IB and IC have seven more amino acid residues, indicating the possibility of a bigger loop in these unconventional myosins (1). The putatively larger size of this loop may be related to the observation that the S329D mutant of *Acanthamoeba* myosin IC is only 50% as active as the S329E mutant. It would be of interest to determine the effects of interchanging Glu and Asp in myosins where one or the other is normally present.

How might an acidic residue at this position affect actomyosin ATPase activity? Studies in this laboratory have demonstrated that the protection by F-actin of a trypsin-sensitive site very near the TEDS site of myosin IA (22) is substantially enhanced by phosphorylation of the Thr at the TEDS site (46) but not by phosphorylation in the absence of actin, even though unphosphorylated and phosphorylated myosin I bind to F-actin approximately equally well (10). This result suggests that either the negative charge itself or a conformational change in the loop induced by the negative charge changes the nature, but not the affinity, of the F-actin-myosin I interaction. If so, this difference may be revealed by cryoelectron microscopy of the complexes of F-actin with the constitutively active S329E and the constitutively inactive S329A mutants of *Acanthamoeba* myosin IC. The results of such a study are reported in the accompanying paper (47).

We thank Dr. Michal Zolkiewski and Dr. Ann Ginsburg (National Heart, Lung, and Blood Institute) for conducting and analyzing the results of the analytical ultracentrifugation experiment and Dr. Andrej Sobota for preparing the AMIC that was used.

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