A Conserved Negatively Charged Amino Acid Modulates Function in Human Nonmuscle Myosin IIA

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Received January 20, 2000; Revised Manuscript Received February 22, 2000

ABSTRACT: A myosin surface loop (amino acids 391−404) is postulated to be an important actin binding site. In human β-cardiac myosin, mutation of arginine-403 to a glutamine or a tryptophan causes hypertrophic cardiomyopathy. There is a phosphorylatable serine or threonine residue present on this loop in some lower eukaryotic myosin class I and myosin class VI molecules. Phosphorylation of the myosin I molecules at this site regulates their enzymatic activity. In almost all other myosins, the homologous residue is either a glutamine or an aspartate, suggesting that a negative charge at this location is important for activity. To study the function of this loop, we have used site-directed mutagenesis and baculovirus expression of a heavy meromyosin- (HMM-) like fragment of human nonmuscle myosin IIA. An R393Q mutation (equivalent to the R403Q mutation in human β-cardiac muscle myosin) has essentially no effect on the actin-activated MgATPase or in vitro motility of the expressed HMM-like fragment. Three mutations, D399K, D399A, and a deletion mutation that removes residues 393−402, all decrease both the V_{max} of the actin-activated MgATPase by 8−10-fold and the rate of in vitro motility by a factor of 2−3. The K_{ATPase} of the actin-activated MgATPase activity and the affinity constant for binding of HMM to actin in the presence of ADP are affected by less than a factor of 2. These data support an important role for the negative charge at this location but show that it is not critical to enzymatic activity.

Myosins are known to constitute a diverse superfamily of molecules that have a conserved motor domain which can interact with actin in an ATP-dependent manner. Recent studies reveal at least 15 distinct classes of myosins (*1*). The best studied of these myosins are the myosin II class molecules, which comprise the striated and smooth muscle myosins as well as the conventional filament-forming nonmuscle myosins.

Myosin is thought to have a broad interaction surface with actin, encompassing several discrete areas of its primary sequence. Evidence for some of these interacting sites comes from studies of actomyosin cross-linking and protection of specific regions of myosin against proteolysis following binding to actin (*2*−*5*). One surface loop on myosin has been proposed to interact with actin on the basis of less direct evidence. This loop, corresponding to amino acids 402−418 in chicken skeletal muscle myosin S1, was modeled to be physically near actin by Rayment et al. (*6*). The first mutation described in human β-cardiac myosin associated with hypertrophic cardiomyopathy (HCM)[1] was an arginine to glutamine change at position 403 of the cardiac sequence (corresponding to amino acid 405 of the chicken skeletal muscle sequence) (*7*). This mutation leads to a very malignant form of HCM and myosin isolated from patients who were heterozygous for this mutation translocated actin at a much reduced rate compared to wild-type myosin (*8*, *9*). In addition, recombinant myosin fragments of either human β-cardiac myosin or rat α-cardiac myosin bearing this mutation had lower rates of in vitro motility and actin-activated MgATPase activity compared to wild-type myosin (*10*−*12*). On the basis of these studies, this loop is referred to as the HCM loop.

Myosin I class molecules from lower eukaryotic species such as *Dictyostelium*, *Acanthamoeba*, and *Aspergillus* have a phosphorylation site located on this loop at a position corresponding to amino acid 411 of chicken skeletal muscle myosin (*13*). Phosphorylation of this site by a specific myosin heavy-chain kinase is absolutely required for enzymatic activity of these myosins (*14*). Myosin VI class molecules also have a potentially phosphorylatable serine at the equivalent site, but no biochemical studies have provided evidence for phosphorylation of this site either in vivo or in vitro (*15*, *16*). Virtually all other myosins have either a glutamic or an aspartic acid residue at this site, suggesting that a negative charge at this site is necessary for enzymatic activity. This prompted Bement and Mooseker (*17*) to term this site the TEDS rule site, since in most myosins there was either a threonine (T), glutamic acid (E), aspartic acid (D), or serine (S) residue at this position.

We have sought to test the requirement for a negative charge at the TEDS rule site and, more generally, to explore the functional significance of this loop in myosin's interaction

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[1] Abbreviations: HCM, hypertrophic cardiomyopathy; HMM, heavy meromyosin; S1, subfragment 1; NMHCIIA, nonmuscle myosin heavy chain IIA; LC20, 20 000-Da myosin regulatory light chain; LC17, 17 000-Da myosin essential light chain; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonylflouride; DTT, dithiothreitol.

10.1021/bi000133x
Published on Web 04/13/2000

with actin by expressing recombinant fragments of human nonmuscle myosin IIA in the baculovirus/Sf9 system. Mutants were made in which the wild-type D399 residue at the TEDS rule site was mutated to either an alanine or a lysine. Another mutation, R393Q, created the homologous mutation in nonmuscle myosin IIA to the human R403Q present in the β-cardiac myosin from some individuals with HCM. Finally, a mutation was made in which amino acids 393−402 were deleted, which should result in a markedly truncated HCM loop.

EXPERIMENTAL PROCEDURES

Construction of Baculovirus Transfer Vectors. The original human nonmuscle myosin IIA (NMHCIIA) cDNA clones were a generous gift of Dr. D. G. Tenen (Harvard Medical School). Clone L434 containing nucleotides 296−4152 and clone LT-1 containing nucleotides 1−1831 (*18*) were subcloned into the *Eco*RI site of pBluescript (Stratagene). A construct encoding amino acids 1−1337 of the human NMHCIIA, an HMM-like fragment, was constructed by using the unique *Pml*I site in both clones. A pair of PCR primers were used to produce a DNA fragment containing a unique *Sfi*I site at the 5′-end and a FLAG tag (DYKDDDDK) (GACTACAAGGACGACGATGATAAG) followed by a stop codon and an *Xba*I site at the 3′-end. By substitution of this PCR product into the NMHCIIA cDNA, a construct encoding a wild-type human nonmuscle myosin IIA HC HMM-like fragment with a FLAG tag at its C-terminus in pBluescript was generated. The construct was then subcloned into the baculovirus transfer vector pVL1392 (Invitrogen) between the *Eag*I and *Xba*I sites, resulting in the desired cDNA clone for baculotransfection. Four mutants (R393Q, D399A, D399K, and Δ393−402) of the human NMHCIIA HMM-like fragment were made by replacing the fragment between the *Sun*I and *Nar*I sites of the myosin cDNA with PCR products containing the corresponding mutations.

Preparation of Recombinant Proteins. Baculoviruses were produced in a manner similar to that described in Pato et al. (*19*). Briefly, the baculotransfer vectors containing the desired heavy-chain cDNA were transfected into Sf9 cells aided by BaculoGold DNA (PharMingen). The resulting baculoviruses containing the appropriate heavy-chain coding sequence were plaque-purified and then amplified to a titer in the range of 5×10^8 to 2×10^{10} per milliliter, and stored at 4 °C as a stock for further use.

Sf9 cells (about 1 L of culture) were coinfected by the amplified heavy-chain virus along with a recombinant baculovirus expressing the coding sequence for the 20 000 Da myosin regulatory light chain (LC20) and 17 000 Da myosin essential light chain (LC17) (*19*) for 72 h. After collection, the cell pellets were quickly frozen in liquid nitrogen and stored at −80 °C. Partially thawed cell pellets were extracted by homogenization in a ground glass homogenizer in 100 mL of 10 mM MOPS, pH 7.3, 0.2 M NaCl, 2 mM ATP, 10 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 5 μg/L leupeptin, and 1 mM dithiothreitol (DTT). The mixture was stirred continuously at 4 °C for 20−30 min and sedimented at 47000*g* for 10 min. The supernatant was mixed with 10−15 mL of anti-FLAG M2 affinity gel (Eastman Kodak) and gently rocked for 20 min to overnight to ensure the binding of the FLAG-tagged human nonmuscle myosin IIA HMM to the affinity gel. This mixture was then sedimented at 1500*g* for 5 min. The gel resin was washed in buffer A (0.5 M NaCl, 10 mM MOPS, pH 7.0, 0.1 mM EGTA, 1 mM DTT, and 0.1 mM PMSF) containing 1 mM ATP and 5 mM $MgCl_2$ and again in buffer A alone The washed resin was resuspended in buffer A and packed into a gel-filtration column. The FLAG-tagged human nonmuscle myosin IIA HMM was eluted by the addition of 0.3 mg/mL FLAG peptide in buffer A. Fractions from the elution were analyzed on a 12.5% polyacrylamide−SDS gel. Those containing the FLAG-tagged human nonmuscle myosin IIA HMM were pooled, precipitated by addition of $(NH_4)_2SO_4$ to 60% saturation, and sedimented at 47000*g* for 10 min. The resulting pellet was solubilized in 1−10 mL of buffer A and dialyzed against 1 L of the same buffer overnight with two changes to remove residual ATP.

F-actin (3 μM) stabilized with phalloidin (5 μM) was added to the dialyzed expressed HMM, and the solution was sedimented for 15 min at 470000*g* to pellet the F-actin−HMM complex. The resulting pellet was solubilized in 0.5−5 mL of buffer A containing 1 mM ATP, 5 mM $MgCl_2$, and 3 μM phalloidin and resedimented to release the bound myosin from actin. The supernatant contained the expressed HMM-like fragment, which was dialyzed against 10 mM MOPS (pH 7.0), 0.1 mM EGTA, 1 mM DTT, and 0.1 mM PMSF or passed through a Sephadex G-50 fine column (Pharmacia Biotech) to remove ATP. The HMM-like fragment was used within 1 week of purification or was flash-frozen and stored for several months in liquid N_2.

Measurement of Actin-Activated MgATPase Activity. The actin-activated MgATPase activity of recombinant HMMs was measured by the method of Pollard and Korn (*20*) in a buffer containing 10 mM MOPS (pH 7.0), 2 mM $MgCl_2$, 1 mM ATP, and 0.1 mM EGTA at 35 °C. The regulatory light chain of HMM was phosphorylated prior to conducting the assay by incubation in a buffer containing 10 mM MOPS (pH 7.0), 5 mM $MgCl_2$, 0.5 mM [^{32}P]ATP, 0.1 mM EGTA, 0.2 mM $CaCl_2$, 0.2 μM calmodulin, and 40 ng/mL myosin light-chain kinase. The actin concentration was varied between 2 and 20 μM. The rate in the absence of actin (less than 0.02 s^{-1}) was subtracted from the actin-activated rates. To determine the kinetic constants V_{max} and K_{ATPase}, the data were fitted to the Michaelis−Menten equation.

Measurement of the Rate of in Vitro Motility. The rate of in vitro motility of actin filaments sliding over the expressed myosin fragments was measured as previously described in a buffer consisting of 80 mM KCl, 20 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, 200 nM tropomyosin, 50 mM DTT, 2.5 mg/mL glucose, 2 μg/mL catalase, and 0.1 mg/mL glucose oxidase at 30 °C (*21*). The myosin was applied at a concentration of 0.2 mg/mL to a nitrocellulose-coated surface. The sample was phosphorylated on the surface by incubation in a buffer containing 80 mM KCl, 20 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, 0.2 mM $CaCl_2$, 10^{-7} M calmodulin, and 0.4 μg/mL myosin light-chain kinase for 2 min at room temperature. This procedure resulted in full phosphorylation of the regulatory light chains as observed by urea extraction of the proteins on the cover slips followed by glycerol gel electrophoresis and silver staining. Furthermore, control experiments were performed in which HMM was first

Chicken Skeletal Muscle	ALCYP**R**VKVGN.....**E**FVTKGQ
Human β-Cardiac Muscle	GLCHP**R**VKVGN.....**E**YVTKGQ
Acanthamoeba IC	QALLY**R**TITTGEQGRGRS**S**VYSCPQ
Dictyostelium IC	TALEF**R**QMET.RHGNQRG**T**QYNVPL
Mouse Myosin VI	VSLTT**R**VMLTTAGG.TKG**T**VIKVPL
Human Nonmuscle IIA	GILTP**R**IKVGR.....**D**YVQKAQ
	393 399

FIGURE 1: Alignment of sequences of the HCM loop regions of different myosins. The amino acid sequences of chicken skeletal muscle myosin (P13538), human β-cardiac myosin (P12883), *Acanthamoeba* myosin IC (P10569), *Dictyostelium* myosin IC (L35323), mouse myosin VI (U49739), and human nonmuscle myosin IIA (M81105) were aligned with the Clustal W program. The portion of each surrounding the HCM loop is shown. The accession number for each sequence is given in parentheses. Dots are used to insert gaps in the alignment.

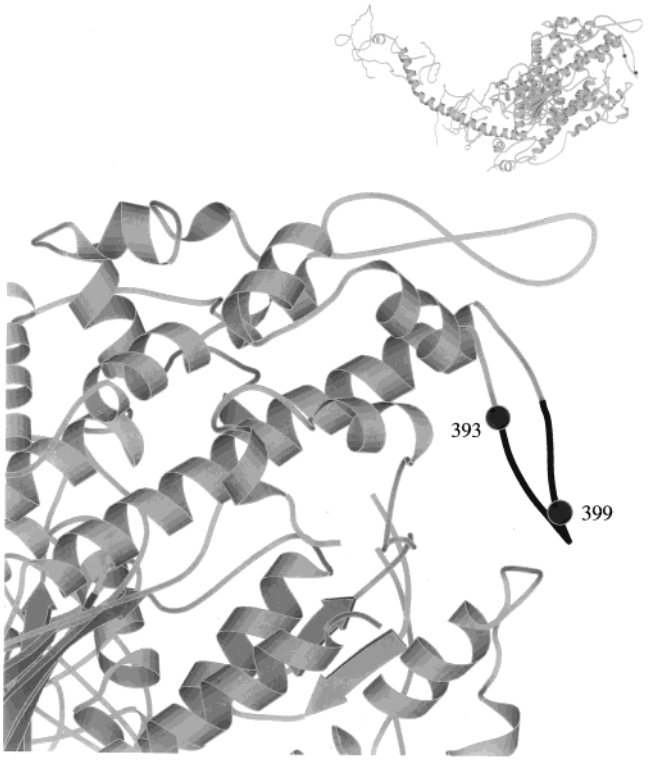

FIGURE 2: Model of the positions of R393 and D399 in human nonmuscle myosin IIA. The homologous residues for human nonmuscle myosin R393 and D399 in chicken skeletal muscle are R405 and E411, respectively. The positions of these residues in the structure of chicken skeletal muscle myosin subfragment 1 are marked by spheres labeled with the human nonmuscle myosin IIA numbers. The figure was prepared with Molscript (*39*).

phosphorylated in solution and then applied to the cover slip. Indistinguishable rates of in vitro motility were observed when the solution-phosphorylated sample was compared with samples phosphorylated while bound to the surface. The rhodamine phalloidin- (Molecular Probes) labeled actin filaments were imaged with a Videoscope microchannel plate intensifier coupled with a Hamamatsu Neuvicon camera and recorded on sVHS videotape (Panasonic). The rate of actin filament sliding was quantified with the Cell Trak system (Motion Analysis) as previously described (*21*).

Stopped-Flow Measurement of the Actin Binding Affinity. The binding of HMM to pyrene-labeled actin filaments was measured in a SF-2001 stopped-flow apparatus (KinTek Corp.) by the method of Kurzawa and Geeves (*22*). Briefly,

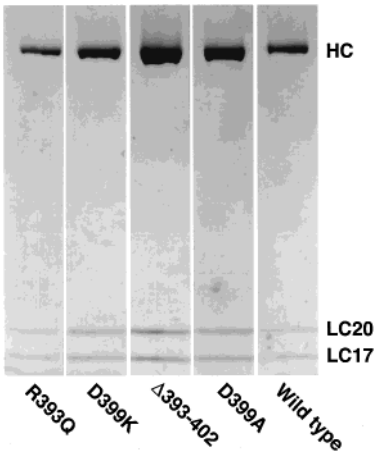

FIGURE 3: Polyacrylamide (12.5%)−SDS gels of expressed human nonmuscle myosin IIA HMM-like fragments. The positions of the HMM heavy chains (HC) and of LC20 and LC17 are denoted.

Table 1: Actin-Activated MgATPase Activity at 35 °C

human IIA	V_{max} (s^{-1})	K_{ATPase} (μM)
wildtype	1.2 ± 0.3	7.4 ± 1.3
R393Q	1.0 ± 0.3	4.4 ± 1.4
D399K	0.14 ± 0.05	4.5 ± 1.4
D399A	0.12 ± 0.02	5.3 ± 2.5
Δ393−402	0.12 ± 0.05	4.6 ± 1.0

one syringe contained 30 nM phalloidin-stabilized pyrene-labeled F-actin, 50 μM ADP, and varying concentrations of HMM. The other syringe contained 100 μM ATP. Upon mixing, ADP dissociates from HMM and is replaced by ATP, which results in a rapid dissociation of HMM from actin. This is measured as an increase in pyrene fluorescence, as HMM binding is known to quench the fluorescence of pyrene-labeled actin. The transient is fitted to a single-exponential function and the amplitude of the fit is plotted as a function of HMM concentration.

RESULTS

Five recombinant carboxyl-terminal FLAG-tagged fragments of human nonmuscle myosin IIA were expressed in Sf9 cells by baculoviral infection. A wild-type HMM-like fragment was produced by truncation at amino acid 1337, which gives a two-headed structure with approximately 500 amino acids of coiled-coil α-helix. The other four recombinant HMM-like fragments had mutations in the HCM loop (amino acids 391−404) (Figures 1 and 2). The recombinant HMM-like fragments were expressed at approximately 2−5 mg of protein/L of Sf9 cell culture, except for the D399A and the deletion mutants, whose expression levels were much poorer. All HMM molecules were purified by a combination of FLAG-affinity column chromatography and ATP-dependent binding to actin (Figure 3). The wild-type HMM-like fragment had enzymatic properties similar to those of proteolytically produced HMM from purified human platelet nonmuscle myosin IIA (*23*). The V_{max} of the actin-activated MgATPase was 1.2 s^{-1} with a K_{ATPase} of 7.4 μM (Table 1). The MgATPase activity in the absence of actin of wild-type HMM was less than 0.02 s^{-1}. The rate of in vitro motility was 0.29 ± 0.03 μm/s (Figure 4).

R393 of human nonmuscle myosin IIA was mutated to a glutamine in order to mimic one of the mutations in human β-cardiac myosin (R403Q) found to cause HCM. Mutation

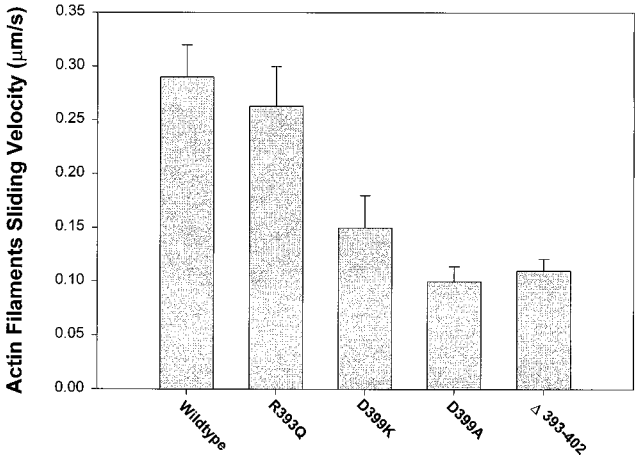

FIGURE 4: In vitro motility of expressed human nonmuscle myosin IIA HMM-like fragments. The conditions are as given under Experimental Procedures. The error bars indicate the standard deviation of the mean.

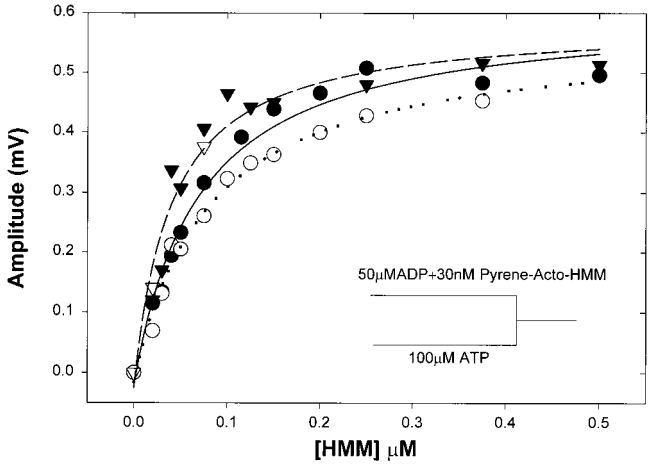

FIGURE 5: Binding of wild-type and mutant myosins to actin in the presence of ADP. The binding of wild-type and mutant HMM molecules to pyrene-labeled actin was measured in a stopped-flow spectrofluorometer by the method of Kurzawa and Geeves (*22*). One syringe (see inset diagram) contained 50 μM ADP and 30 nM phalloidin-stabilized pyrene-labeled F-actin with varying concentrations of HMM. The other syringe contained 100 μM ATP. The amplitude of the single-exponential transient, which represents the dissociation of bound HMM from actin, was plotted vs the HMM concentration. The data were fitted to a binding curve described by a hyperbola. The following binding constants were determined: wild-type HMM, 1.45×10^7 M^{-1} (●, —); D399K HMM, 1.27×10^7 M^{-1} (○, •••); and R393Q HMM, 2.5×10^7 M^{-1} (▼, - - -). Two data points for the D399A HMM (▽) are shown. Conditions: 0.5 M KCl, 25 mM MOPS (pH 7.0), 5 mM $MgCl_2$, and 0.1 mM EGTA at 20 °C.

of R393 of human nonmuscle myosin IIA to glutamine had virtually no effect on the actin-activated MgATPase activity or the in vitro motility activity of this HMM-like fragment (Table 1 and Figure 4). Similarly, the MgATPase activity in the absence of actin remained less than 0.02 s^{-1}.

Other mutations of this loop were made to test the importance of the negatively charged aspartate at residue 399. Mutation of D399 of human nonmuscle myosin IIA to either an alanine or a lysine had profound effects upon the enzymatic and mechanical properties (Table 1 and Figure 4). The V_{max} of the actin-activated MgATPase activity of both mutants was reduced approximately 8-fold while the K_{ATPase} was not as dramatically affected. The rate of in vitro motility was also reduced to 0.15 ± 0.03 μm/s for the lysine mutation and 0.10 ± 0.014 μm/s for the alanine mutation. The MgATPase activities in the absence of actin of both mutants were less than 0.02 s^{-1}.

A fourth mutation in this region was made in which amino acids 393−402 were deleted. This deletion greatly shortens the loop and eliminates both the positive charge at position 393 and the negative charge at position 399. The V_{max} of the actin-activated MgATPase activity of this mutant was reduced approximately 8-fold compared to wild type with little effect on the K_{ATPase} (Table 1). The in vitro motility rate of the mutant was 0.11 ± 0.011 μm/s (Figure 4). The MgATPase activity in the absence of actin was less than 0.02 s^{-1}.

During the kinetic cycle for hydrolysis of ATP, myosin cycles between transient states in which the affinity for actin is alternately high and low. The high-affinity states have been termed "strongly bound" states and the low-affinity ones "weakly bound" states. In the absence of nucleotide or when containing a bound ADP, myosin binds to actin with a high affinity. Binding of ATP or its hydrolysis products, ADP·P_i, to myosin dramatically weakens the binding affinity for actin. The small effect on the K_{ATPase} suggested that the mutations had little effect on the affinity of HMM for actin in the presence of ATP. Under the K_{ATPase} conditions HMM is largely in the weakly bound state. We measured the binding of the wild-type and three mutant HMM molecules to actin in the presence of ADP by the method of Kurzawa and Geeves (*22*) in order to test whether the loop mutations affected the affinity of HMM for actin in a strongly bound state. There was little effect of the mutations at residue 399 or 393 on the binding to actin in the presence of ADP (Figure 5). Fitting the data for the wild type and the D399K and R393Q mutations showed less than a factor of 2 effect on the binding constants. A few data points obtained with the D399A mutation fell within the same range. Insufficient amounts of the Δ393−402 mutant were obtained to perform this experiment. It was necessary to increase the ionic strength in this experiment and to add ADP, since at lower ionic strengths and in the absence of nucleotide the HMM molecules bound too tightly to actin to accurately determine a binding constant.

DISCUSSION

The core structure of myosins is probably highly conserved in terms of major structural elements such as helices and β-sheets, with much of the variability occurring at various surface loops (*24*). Some of these surface loops have been shown to be important in determining the kinetic and mechanical properties of myosins (*25*−*29*). Loop I, located near the nucleotide binding pocket, is highly variable in sequence among different myosins (*1*, *30*). It is the site of alternative splicing in several myosin II isoforms that has been shown to modulate enzymatic activity (*19*, *27*, *28*, *31*). Loop II, located at the tip end of myosin, has been implicated in actin binding. This loop is also variable in sequence and length. Chimeric recombinant vertebrate smooth muscle and *Dictyostelium* myosin molecules with substitutions at the loop have altered enzymatic properties (*26*, *29*).

We have focused on a surface loop near the tip of the myosin head that is remarkable in two features. First, in

human β-cardiac myosin, this loop contains an arginine residue at position 403 that, when mutated to a glutamine, leucine, or tryptophan, gives rise to familial hypertrophic cardiomyopathy (HCM), a disease characterized by asymmetric hypertrophy of the heart, which leads to often fatal arrhythmias (*7*, *8*, *32*). An arginine or lysine at this positions is found in all published myosin II sequences ($n = 44$). Second, the enzymatic properties of myosin I isoforms from lower eukaryotes such as *Acanthamoeba* and *Dictyostelium* are regulated by phosphorylation of a serine or threonine residue located in this loop (*14*). Myosin heavy-chain kinases have been isolated from these species that efficiently phosphorylate the residue (*33*, *34*). The function of this residue has recently been studied in vitro by use of recombinant *Acanthamoeba* myosin I expressed in Sf9 cells. Mutation of S329 of *Acanthamoeba* myosin IC to alanine or asparagine eliminates enzymatic activity in vitro, whereas mutation to a glutamic acid residue gives a constitutively active myosin with wild-type levels of activity (*35*). Mutation to an aspartic acid residues results in a constitutively active myosin with about 50% the activity of wild-type myosin I. Mutation of this residue in *Aspergillus* myosin I to an alanine results in slower growth of the cells but no change in endocytosis, whereas mutation to a glutamic acid results in an increase in the endocytotic rate over that of wild type (*36*). Several other myosins have a serine or threonine residue at this location, such as myosin VI molecules and a myosin recently cloned and sequenced from *Tetrahymena*, but none of these molecules has been purified, studied enzymatically, or shown to require phosphorylation for activity (*15*, *16*, *36*). Virtually all other myosins (100 out of 105 sequences examined), including all myosin II molecules sequenced to date, have either an aspartic acid or glutamic acid residue at this site, suggesting that a negative charge is important at this site (*1*, *17*).

Mutation of D399 of human nonmuscle myosin IIA to an arginine or an alanine results in considerable loss of enzymatic activity and slower in vitro motility. Truncation of the loop by deletion of amino acids 393−402 results in even more severe loss of activity. However, the mutant myosins are still functional, which indicates that a negative charge at this residue is not absolutely essential for enzymatic activity. There was little effect on the K_{ATPase}, suggesting that the binding constant in the presence of ATP (weakly bound state) is not likely to be greatly affected. Similarly, there was little effect on the affinity of three of the mutations (R393K, D399K, and D399A) for actin in the presence of ADP, demonstrating that these amino acids do not contribute to the binding of myosin to actin in the strongly bound state. Rayment et al. (*6*) suggested that this loop probably interacts directly with actin from structural modeling studies in which the atomic structure of chicken skeletal muscle S1 was visually docked with an atomic model of the F-actin filament. However, Mendelson and Morris (*37*), using a different modeling approach, came to the opposite conclusion. They felt that R403 (equivalent to R393 in the present study) was not sufficiently close to make electrostatic interactions with residues on actin. It is of interest that the length of this loop in *Acanthamoeba* and *Dictyostelium* myosin I molecules is 5−7 amino acids longer than in most other myosins (*1*). This extra length may enable the phosphorylated residue of these myosins to interact with regions of actin or of the myosin backbone that are inaccessible in other myosins.

We found that mutation R393Q (equivalent to the HCM-associated R403Q of β-cardiac myosin) had almost no effect on the enzymatic properties of human nonmuscle myosin IIA. The comparable mutation in a recombinant HMM-like fragment of human β-cardiac myosin caused a 3-fold reduction in V_{max}, a 3-fold increase in K_{ATPase}, and a 4-fold decrease in in vitro motility compared to wild type (*12*). Similar effects were observed on the in vitro motility of myosin isolated from individuals heterozygous for the R403Q mutation (*9*). A homologous mutation has been made in recombinant α-cardiac myosin fragments (*10*, *11*) and in *Dictyostelium* myosin (*38*). When the mutations were bound in a cardiac myosin backbone, both the actin-activated MgATPase activity and in vitro motility activity were decreased. In contrast, the effect of the mutation on the in vitro motility activity of *Dictyostelium* myosin is not as great as was found in the cardiac systems. Our study shows that the corresponding R393Q mutation has little effect on the binding to actin in the strongly bound states or on the enzymatic and motile properties of the nonmuscle myosin fragment, suggesting that the choice of myosin heavy-chain backbone plays a role in determining the effect of this mutation. The sequence of this loop varies to some extent among different myosins (*1*). Thus, we believe it is best to use the human β-cardiac myosin or subfragments of this myosin when evaluating the effect of the HCM-associated mutations.

ACKNOWLEDGMENT

We thank Robert S. Adelstein and Edward Korn for discussions and for critically reading the manuscript.

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BI000133X