Functional Consequences of Mutations in the Smooth Muscle Myosin Heavy Chain at Sites Implicated in Familial Hypertrophic Cardiomyopathy*

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Familial hypertrophic cardiomyopathy (FHC) is frequently associated with mutations in the β-cardiac myosin heavy chain. Many of the implicated residues are located in highly conserved regions of the myosin II class, suggesting that these mutations may impair the basic functions of the molecular motor. To test this hypothesis, we have prepared recombinant smooth muscle heavy meromyosin with mutations at sites homologous to those associated with FHC by using a baculovirus/insect cell expression system. Several of the heavy meromyosin mutants, in particular R403Q, showed an increase in actin filament velocity in a motility assay and an enhanced actin-activated ATPase activity. Single molecule mechanics, using a laser trap, gave unitary displacements and forces for the mutants that were similar to wild type, but the attachment times to actin following a unitary displacement were markedly reduced. These results suggest that the increases in activity are due to a change in kinetics and not due to a change in the intrinsic mechanical properties of the motor. In contrast to earlier reports, we find that mutations in residues implicated in FHC affect motor function by enhancing myosin activity rather than by a loss of function.

2 (4). Many of these residues are conserved in a wide variety of myosin II isoforms, suggesting that they may be important for the normal function of the molecule. For example, Arg403, whose mutation to Gln (R403Q) causes one of the most severe phenotypes of the disease, is located at the base of a surface loop believed to interact with actin (5, 6). Earlier studies showed that cardiac isoforms with this mutation were impaired in both enzymatic (lower $V_{max}$ and higher $K_m$ of actin-activated ATPase activity) and motile activities (lower actin translocation velocity in the in vitro motility assay) (7–10). A number of mutations have also been located in the C-terminal region of subfragment 1 that is associated with the light chains. Several lines of evidence suggest that the light chain-binding domain acts as a “lever arm” to amplify and transmit conformational changes that originate in the motor domain (5, 11–14). Mutations in this region often occur in close proximity to the essential light chain (ELC), suggesting that the interface between the motor domain and the ELC may be important for coupling ATP hydrolysis with mechanical movement (4).

The primary aim of the present study is to investigate the functional consequences of introducing missense mutations into the “myopathy loop” (Arg403), and into the “lever arm” region (Arg719 and Asp775) of a vertebrate muscle myosin. A smooth muscle myosin fragment, similar to heavy meromyosin (HMM) prepared by chymotryptic digestion, was expressed in the baculovirus/insect cell system and isolated in sufficient yield for extensive biochemical and mechanical studies. Although human β-cardiac myosin would clearly be the isoform of choice for such mutational studies, expression levels of human β-cardiac heavy chain (9) and of rat α-cardiac myosin (8) have been very low, thus limiting the extent to which the properties of the protein can be analyzed. Transgenic mice carrying the equivalent FHC mutations in their heart muscles are a valuable model system for studying FHC, but the functional consequences of mutations in a fast myosin isoform, such as mouse α-cardiac myosin, may well be different from those in β-cardiac myosin, a slow myosin isoform. Despite sequence differences, smooth muscle myosin is in many respects functionally similar to a slow β-cardiac myosin, insofar as they both share low values for actin-activated ATPase activity and actin translocation velocity (15, 16).

An important advantage in studying point mutations in the smooth muscle heavy chain is that the crystal structure of the expressed motor domain with its associated ELC has been solved (17) and thus provides a framework for considering the effect of point mutations on the functional properties of myosin. The three-dimensional structure of the smooth muscle myosin motor domain is virtually identical to that of the chicken skel-
et al isoform (5), and therefore the cardiac myosin motor domain will also have a similar fold. The sequences flanking the mutated residues are highly conserved between cardiac and smooth muscle isoforms, and therefore it was expected that smooth muscle HMM containing FHC mutations would show the same loss of function as reported for cardiac myosin. Instead, we find an increase in actin-binding affinity, actin-activated ATPase activity, and actin translocation velocity for several of the mutants compared with wild-type smooth HMM, while preserving the intrinsic force- and motion-generating capacities of the individual motors. The possibility exists that some of these functional differences between smooth and cardiac mutants may be isoform-specific, but this explanation cannot account for all of the discrepancies in the literature. Instead, we favor the view that these mutations affect cardiac function by a gain in function, presumably leading to an increase in power output beyond that normally tolerated. Furthermore, since patients are heterozygous for FHC mutations, their thick filaments will probably contain a heterogeneous mixture of myosin heads with different cycling kinetics, a condition that might alter ventricular performance, and thereby contribute to a compensatory hypertrophy in cardiac muscle tissue.

MATERIALS AND METHODS

In Vitro Mutagenesis—Chicken gizzard myosin heavy chain cDNA (18) was used as a template for site-directed mutagenesis. Five distinct point mutations were introduced at three different residues (R406Q, R406W, R731Q, R731W, and D787G) in the smooth muscle myosin sequence, equivalent to R403Q, R403W, R719Q, R719W, and D775G in FHC, respectively, by the method of Kunkel (19). Thereafter, each mutant is designated by the human β-cardiac sequence. To study the functional differences between equivalent “myopathy loops” (i.e. sequences flanking the Arg residue) of cardiac and smooth muscle isoforms, we created cardiac/smooth muscle chimeras by replacing the native smooth muscle myosin loop with the wild-type human β-cardiac sequence or with the human β-cardiac sequence containing the R403Q mutation (see Table I). These chimeric mutants have been named the cardiac myopathy loop (CML) and CML/R403Q, respectively. All mutations were confirmed by DNA sequencing.

Expression and Purification of the Protein—Recombinant baculovirus was isolated by conventional protocols (20). Sf9 cells were infected with recombinant virus coding for a truncated heavy chain fragment similar to chymotryptic HMM (intermediate HMM, 1175 amino acids) and another virus coding for both the smooth muscle regulatory light chain (21) and ELC (22). The expressed protein was isolated by actin binding and release with MgATP (23) or by chromatography of a fusion-tagged protein on an anti-FLAG affinity column (24). The yields of wild type and mutant HMMs were measured by mean variance analysis ranged from 28046 to 28054.

RESULTS

Biochemical Characterization of HMM with Point Mutations in the “Myopathy” Loop—A basic residue, Arg403, located at the base of a surface loop that is assumed to interact with actin, is conserved virtually in the entire myosin II class, see Fig. 1. To test the hypothesis that this residue is important for the optimal function of any myosin motor, we engineered two point mutations (R403Q and R403W) at the equivalent site in the smooth muscle HMM backbone and expressed these mutants using the baculovirus/insect cell system. The sequences of the cardiac and smooth muscle myosin loops are compared in Table I. The most notable difference between the smooth and cardiac “myopathy” loops is one additional positive charge in the cardiac myosin motor head sequence (Arg411 in smooth versus Asn408 in cardiac).

The phosphorylated R403Q and R403W mutants showed 30–40% higher velocities than wild type HMM in the motility assay at both 25 and 60 mM KCl (Fig. 2 and Table II). The dephosphorylated R403Q and R403W mutants showed approximately 10% of the velocities of the phosphorylated species at

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Mutations in the actin-binding region of HMM

Sequences in the smooth muscle myosin surface loop equivalent to the "myopathy loop" in human β-cardiac myosin are shown with the corresponding point mutations. Chimeras consisting of the "cardiac myopathy loop" introduced into the smooth muscle myosin backbone (CML), and the CML mutant with the Arg to Gln mutation in residue 403 (CML/R403Q) are indicated.

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<tr>
<td>Cardiac</td>
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</tr>
<tr>
<td>Smooth</td>
<td>R V K V G R D V V Q K</td>
</tr>
<tr>
<td>Points mutants</td>
<td></td>
</tr>
<tr>
<td>R403Q</td>
<td>R V K V G R D V V Q K</td>
</tr>
<tr>
<td>R403W</td>
<td>R V K V G R D V V Q K</td>
</tr>
<tr>
<td>CML</td>
<td>R V K V G N E Y V T K</td>
</tr>
<tr>
<td>CML/403Q</td>
<td>Q V K V G N E Y V T K</td>
</tr>
</tbody>
</table>

FIG. 1. Crystal structure of the smooth muscle myosin motor domain complexed with the essential light chain (MDE), and the location of three point mutations indicated in space-filling models. The heavy chain (residues 1–819) is colored yellow; the converter region (721–778) is shown in green, and the essential light chain is shown in gray. The mutated residues in the smooth heavy chain sequence are Arg⁴⁰⁶ (cardiac Arg⁴⁰³), Arg⁷³³ (cardiac Arg⁷¹⁹), and Asp⁷⁸⁹ (cardiac Asp⁷⁷⁸).

FIG. 2. Rate of actin movement by phosphorylated wild-type HMM and HMMs with mutations in the "myopathy loop." Values are for mean velocities ± S.D. for four or more independent preparations at two ionic strengths. For each preparation, 30–50 actin filaments were measured to obtain an average velocity at each condition. *, p < 0.05 versus WT; **, p < 0.01 versus WT.

Both 25 and 60 mM KCl (values for deP ranged from 0.16–0.2 μm/s; see Table II), indicating that attainment of a completely "off" state is compromised as a result of these mutations.

The actin-activated ATPase activity of the phosphorylated R403Q and R403W mutants differed from wild type HMM in both $V_{max}$ and $K_m$. Both mutants showed a 2-fold higher $V_{max}$ as well as a 40% lower $K_m$ for actin (Fig. 3A and Table II). Since the $K_m$ for actin is a function of the rate constants of the actin binding step as well as other kinetic transitions in the ATPase cycle (32), a sedimentation assay was performed to directly evaluate the strength of actin binding in the presence of MgATP (Fig. 3B). The binding constant of phosphorylated wild type HMM was $1.7 \times 10^4 \text{ M}^{-1}$, consistent with earlier measurements on chymotryptic smooth muscle HMM (33). In contrast, the affinity of both phosphorylated mutants was 5-fold higher than wild type HMM (Fig. 3B and Table II).

Based on the higher rate of motility, the higher $V_{max}$, the lower $K_m$, and the lower $K_d$ for actin, both R403Q and R403W mutations in the context of the smooth muscle backbone enhanced rather than impaired the enzymatic and mechanical properties of the molecule.

TABLE 1
Mutations in the actin-binding region of HMM

Sequences in the smooth muscle myosin surface loop equivalent to the "myopathy loop" in human β-cardiac myosin are shown with the corresponding point mutations. Chimeras consisting of the "cardiac myopathy loop" introduced into the smooth muscle myosin backbone (CML), and the CML mutant with the Arg to Gln mutation in residue 403 (CML/R403Q) are indicated.

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<tr>
<td>Points mutants</td>
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<tr>
<td>R403Q</td>
<td>R V K V G R D V V Q K</td>
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<tr>
<td>R403W</td>
<td>R V K V G R D V V Q K</td>
</tr>
<tr>
<td>CML</td>
<td>R V K V G N E Y V T K</td>
</tr>
<tr>
<td>CML/403Q</td>
<td>Q V K V G N E Y V T K</td>
</tr>
</tbody>
</table>

Analysis of the event durations of the displacement records based on mean variance analysis (29) showed a difference in $t_{on}$ between wild type and the mutants. The duration of the events was ~2-fold shorter for R403Q than for wild type HMM under the lightly loaded and subsaturating 10 μM MgATP conditions of the optical trap (Fig. 4A and Table III). The shorter event duration is consistent with the 30–40% increase in velocity in the motility assay. This mutation, therefore, affects the rate at which transitions occur in the cross-bridge cycle but does not alter the intrinsic displacement or the unitary force of the power stroke.
The motility values for dephosphorylated HMM mutants are in parentheses. The absence of such values indicates that no movement was observed in the dephosphorylated state, as is the case for completely regulated smooth HMM. The given values represent the mean ± S.D. for four or more independent preparations.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Motility (25 mM KCl)</th>
<th>Motility (60 mM KCl)</th>
<th>ATPase $V_{max}$</th>
<th>ATPase $K_m$</th>
<th>Actin binding $K_d$</th>
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<tr>
<td>WT</td>
<td>1.1 ± 0.10</td>
<td>1.56 ± 0.17</td>
<td>1.8 ± 0.4</td>
<td>28 ± 7</td>
<td>58</td>
</tr>
<tr>
<td>R403Q (deP)</td>
<td>1.56 ± 0.26</td>
<td>2.02 ± 0.42</td>
<td>3.4 ± 0.5</td>
<td>18 ± 5</td>
<td>11</td>
</tr>
<tr>
<td>R403W (deP)</td>
<td>1.45 ± 0.05</td>
<td>2.07 ± 0.08</td>
<td>3.8 ± 0.1</td>
<td>16 ± 6</td>
<td>13</td>
</tr>
<tr>
<td>CML</td>
<td>1.11 ± 0.04</td>
<td>1.29 ± 0.16</td>
<td>1.4 ± 0.2</td>
<td>25 ± 4</td>
<td>95</td>
</tr>
<tr>
<td>CML/R403Q</td>
<td>1.19 ± 0.05</td>
<td>1.55 ± 0.07</td>
<td>2.3 ± 0.3</td>
<td>61 ± 16^c</td>
<td>118</td>
</tr>
</tbody>
</table>

^a p < 0.01 versus WT.
^b p < 0.05 versus WT.
^c p < 0.01 versus CML.

**Fig. 3.** Actin-activated ATPase activity (A and C) and binding to actin in a sedimentation assay (B and D) of phosphorylated wild-type HMM and HMMs with mutations in the “myopathy loop.” Results from one representative preparation are shown for wild-type and each mutant. A, WT (open circles), R403Q (closed circles), and R403W (closed triangles). Data were fitted by using Michaelis-Menten kinetics. $V_{max}$ and $K_m$ values, respectively, are as follows: 1.8 s$^{-1}$ and 33 $\mu$m for WT; 3.4 s$^{-1}$ and 24 $\mu$m for R403Q; 3.7 s$^{-1}$ and 24 $\mu$m for R403W. B, percentage of mutant and wild-type HMM bound to actin in the presence of MgATP as a function of actin concentration. Symbols are the same as in A. Each point represents an average of two independent preparations. Data were fitted using the following equation: percentage bound = 100 x (actin)/(Kd + [actin]). Kd values are 58 $\mu$m for WT, 11 $\mu$m for R403Q, and 13 $\mu$m for R403W. C, CML (open squares) and CML/R403Q (closed squares). $V_{max}$ and $K_m$ values, respectively, are as follows: 1.2 s$^{-1}$ and 23 $\mu$m for CML; 2.2 s$^{-1}$ and 62 $\mu$m for CML/R403Q. D, symbols are the same as in C. Kd values are 95 $\mu$m for CML and 115 $\mu$m for CML/R403Q.

**Fig. 4.** A, representative unitary displacement traces from phosphorylated WT, R403Q, R719Q, and D778G. All records were filtered at 2 kHz and sampled at 4 kHz. Note that the event duration ($t_{max}$) is shorter in all mutants compared with WT. B, representative unitary isometric force traces from phosphorylated HMMs. Force records were filtered and sampled in a manner identical to displacement data. C, scatter plot representing the distribution of the unitary displacements produced by wild-type and mutant HMMs. Each open circle represents the fit from a single mean variance histogram. The mean and S.D. are indicated for each distribution.

**Mutations of the “Myopathy Loop” in Smooth HMM to the Cardiac Muscle Sequence—**Since the available literature on the R403Q mutation in cardiac myosin predicted a loss of functional properties, we tried to determine if the increase in activity arising from the R403Q mutation in smooth muscle myosin could be accounted for by the different sequence of the myopathy loop itself. We therefore generated a chimeric HMM in which the 11-residue myopathy loop was composed of the cardiac sequence, hence referred to as the CML, or “cardiac myopathy loop.” The R403Q mutation was also engineered into this chimera (CML/R403Q) (see Tables I and II).

The introduction of the cardiac sequence into this surface loop had relatively little effect on the properties of the molecule. The in vitro motility rates (Fig. 2) and actin-activated ATPase activity ($V_{max}$ and $K_m$) (Fig. 3C) for CML were similar to wild type HMM. The dissociation constant ($K_d$) for actin binding in the presence of MgATP, as determined by a sedimentation assay, increased, from 58 $\mu$m for wild type to 95 $\mu$m for CML, indicating that the affinity for actin was somewhat weakened by the introduction of the cardiac sequence (Fig. 3D and Table II).

Further introduction of the R403Q mutation into the CML chimera did not cause a significant increase in motility (Fig. 2 and Table II) but increased both $K_m$ for actin (61 versus 25 $\mu$m) and $K_d$ (118 versus 95 $\mu$m), in contrast to the results obtained...
TABLE III

Unitary force and displacement of mutant HMMs

All values are the mean ± S.E. The quantity in parentheses represents the number of mean variance histograms used to calculate the mean.

<table>
<thead>
<tr>
<th></th>
<th>Duni</th>
<th>Funi</th>
<th>ton</th>
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<tbody>
<tr>
<td></td>
<td>nm</td>
<td>pN</td>
<td>ms</td>
</tr>
<tr>
<td>WT</td>
<td>10.9 ± 0.4 (11)</td>
<td>1.3 ± 0.1 (9)</td>
<td>222 ± 34 (11)</td>
</tr>
<tr>
<td>R403Q</td>
<td>10.2 ± 0.5 (13)</td>
<td>1.3 ± 0.1 (15)</td>
<td>90 ± 10³ (13)</td>
</tr>
<tr>
<td>R719Q</td>
<td>10.2 ± 0.4 (16)</td>
<td>1.4 ± 0.1 (14)</td>
<td>83 ± 5³ (16)</td>
</tr>
<tr>
<td>D778G</td>
<td>10.1 ± 0.3 (16)</td>
<td>1.3 ± 0.2 (3)</td>
<td>87 ± 8³ (16)</td>
</tr>
</tbody>
</table>

*p < 0.05 versus WT.

for the R403Q mutation in smooth HMM. However, the ATPase activity (V_max) of CML/R403Q was significantly higher than for CML, mimicking the higher V_max of the R403Q mutant compared with wild type HMM.

Mutations in the Converter/Lever Arm Region—Two sites were targeted for mutation in the motor domain/lever arm interface. One site is in the so-called converter region, which couples the motor domain to the lever arm (Arg731 in cardiac or Arg734 in smooth), and the other site is at the amino terminus of the long α-helix that binds the regulatory light chain and ELC (Asp778 in the cardiac sequence or Asp 789 in smooth) (see Fig. 1).

The D778G mutant showed one of the highest velocities of any of the mutants that were characterized here, as much as 50% higher than that of wild type HMM (2.37 μm/s versus 1.56 μm/s at 60 mM KCl; Fig. 5 and Table IV). As with the R403Q mutation, the increase in velocity may be accounted for by an alteration to the kinetics of the attached state (i.e. decreased t_on compared with wild type, 87 versus 222 ms; Table III) rather than an alteration to d, which is similar to wild type (Table III). The lack of any effect of the mutation on the motor’s intrinsic mechanics is also reflected in similar unitary forces compared with wild type (Table III). The D778G mutant also showed a high actin-activated ATPase activity (3.0 s⁻¹ versus 1.8 s⁻¹) with a K_m value comparable with that of wild type HMM (Table IV).

In contrast to the other mutants, R719Q showed a somewhat reduced actin filament velocity relative to wild type (Fig. 5A, Table IV), despite a decrease in attachment time (t_on) (Table III), which in the absence of other factors would have been expected to increase the velocity. The R719Q mutant showed an extremely low K_m for actin compared with wild type (6 versus 28 μM) (Fig. 5B and Table IV). This 5-fold difference in K_m was maintained when the dissociation constant for actin (K_d) was measured directly in a pelleting assay in the presence of MgATP (Fig. 6A and Table IV). The R719Q mutant also showed a large effect on phosphorylation-dependent regulation of smooth muscle myosin; whereas wild type HMM shows no motility in the dephosphorylated state, the velocity of dephosphorylated R719Q was as high as 60% of the value for phosphorylated HMM at both ionic strengths (0.49 μm/s at 25 mM KCl and 0.75 μm/s at 60 mM KCl; Table IV). The R719W mutant showed a closer resemblance to wild type in terms of phosphorylation and, in particular, was completely regulated by phosphorylation. It appears that the hydrophobicity of the side chain of the mutated residue is of least importance as the charge in determining the native properties of the molecule.

Stringent Assay Conditions—In order to determine whether some mutants would show a larger phenotype under more stringent assay conditions, in vitro motility experiments were done under conditions where the methylcellulose concentration was reduced in stepwise increments to zero from the standard conditions, within the same flow cell (0.5% at 25 mM KCl and 0.7% at 60 mM KCl). Methylcellulose is a viscosity-enhancing agent that enhances actin filament binding to the HMM surface by limiting diffusion of the filaments in the presence of MgATP. At 25 mM KCl, wild type and all of the mutants examined (R403Q, R403W, CML, CML/R403Q, and R719Q) supported directional movement of actin filaments with similar velocity to that of the standard condition, independent of the methylcellulose concentrations.

In contrast, the motility at 60 mM KCl was dependent on the methylcellulose concentration for wild type and the CML and CML/R403Q mutants. Directional movement of actin filaments was observed only above 0.14% methylcellulose for wild type and CML/R403Q and above 0.2% methylcellulose for CML, and then their velocities were similar to those of the standard condition. At lower methylcellulose concentrations, actin filaments showed only Brownian motion or otherwise were detached from the HMM surface. However, even at 60 mM KCl, the velocities of the R403Q, R403W, and R719Q mutants were unaffected by the absence of methylcellulose. The common feature of these three mutants is that they showed lower K_m as well as lower K_d for actin binding in the presence of MgATP. Thus, this assay is sensitive to the relative affinity of actin for HMM and is independent confirmation of the fact that these mutations have enhanced the affinity for actin.

DISCUSSION

Molecular genetic studies have defined FHC as a disease of the muscle sarcomere, based on the finding that at least six different genes coding for contractile proteins contain mutations (reviewed in Ref. 3). Many of these mutations, particularly those found in the cardiac myosin heavy chain, have been reported to severely diminish the functional properties of the myosin, either by lowering the actin filament velocity in a motility assay and/or by reducing the actin-activated ATPase activity (3). Contrary to these studies, we find an enhancement in the steady-state enzymatic activity for all of our heavy chain mutants, and in the case of R403Q, R403W, and D778G, the
its ELC light chain (named MDE (17)) (Fig. 1). As first observed, the structure of the smooth muscle motor domain complexed with actin upon nucleotide release.2 These structural findings are consistent with intrinsic fluorescence studies that showed a more polar environment surrounding an engineered Trp413 in the loop of R403Q mutation in the absence of MgADP (36). Thus, the loop appears to be intimately involved in actomyosin interactions, and mutations at the interface may be expected to affect the enzymatic activity and mechanical properties of myosin.

Mutation of Arg403, a residue that is highly conserved in the myosin II family, has consistently been reported to result in lower functional activity, whether it be in expressed human β-cardiac myosin (9), in expressed rat α-cardiac myosin (8), or even in Dictyostelium discoideum myosin (37). Similarly, myosin extracted from cardiac muscle biopsies of patients with the R403Q mutation have shown consistently slower actin filament velocities than myosin from normal control tissues (7, 38). Given that our results for smooth muscle myosin Arg403 are different from those in the literature, the question naturally arises as to whether this is due to the use of smooth muscle myosin as opposed to cardiac muscle myosin. Although the sequences flanking Arg403, the so-called cardiomyopathy loop, are fairly well conserved among myosin IIs, there are, nevertheless, differences in this region. An attempt to address this question was made by engineering the cardiac cardiomyopathy loop into the smooth muscle background and creating the CML chimeric HMM; this molecule had similar functional properties to wild type HMM, but the introduction of the R403Q mutation resulted in a smaller increase in motility and enzymatic activity for CML/R403Q than for the smooth HMM mutant. Although these chimeric constructs obviously do not mimic cardiac myosin, they do show that, independent of the source of the cardiomyopathy loop, alterations in this surface loop do not result in a significant loss of function.

So far we have only discussed measurements made on a population of HMM molecules. The enhanced actin filament velocity ($v_{actin}$) for the R403Q mutant could, in principle, be caused by a larger unitary displacement ($d$) or by a shorter time ($t_{on}$) that the cross-bridge is attached to actin following the power stroke. By making unitary mechanical measurements in an optical trap, we showed that the single molecule mechanical properties were identical for R403Q and wild type HMM; each gave a displacement of 10 nm and a unitary force of 1.3 pN. However, $t_{on}$ values were about 2-fold less for the mutants, which is consistent with the increased motility, if we assume that $v_{actin}$ can be approximated by $d/t_{on}$. Thus, the results obtained at the single molecule level are consistent with the ensemble motility assays and imply that the 403 mutation in smooth muscle HMM affects the cycling kinetics of the molecule.

But do these results apply to cardiac myosin or are they dependent on the type of myosin isoform that is mutated? Recent in vitro motility data for mouse α-cardiac myosin, isolated from homozygous transgenic mice expressing the R403Q mutation, showed that the mutant myosin moved actin filaments at 60% higher velocity and had a higher actin-activated ATPase activity than the control myosin (39). These results are in direct conflict with the 4–5-fold reduction in actin filament velocity and ATPase activity found for rat α-cardiac myosin with the R403Q mutation isolated from a baculovirus/insect cell expression system (8). Since no one expects mouse α-cardiac myosin to be different from rat α-cardiac myosin, these results suggest an important difference between the behavior of the two.

**TABLE IV**

Properties of mutants at motor domain/lever arm interface

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<th>Construct</th>
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<td>28 ± 7</td>
<td>10</td>
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<tr>
<td>R719Q</td>
<td>0.86 ± 0.1a (deP, 0.49 ± 0.08)</td>
<td>1.45 ± 0.08</td>
<td>2.4 ± 0.2b</td>
<td>6 ± 0.7a</td>
<td>10</td>
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<tr>
<td>R719W</td>
<td>1.02 ± 0.2</td>
<td>1.45 ± 0.37</td>
<td>2.5 ± 0.3a</td>
<td>22 ± 4</td>
<td>ND</td>
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<tr>
<td>D778G</td>
<td>1.45 ± 0.07b</td>
<td>2.37 ± 0.11b</td>
<td>3.0 ± 0.5a</td>
<td>25 ± 7</td>
<td>ND</td>
</tr>
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</table>

* $p < 0.01$ versus WT.
* $p < 0.05$ versus WT.
* ND, not determined.

**FIG. 6.** Actin-activated ATPase activity (A) and binding to actin in a sedimentation assay (B) of phosphorylated wild-type HMM and HMMs with mutations at the motor domain/lever arm interface. Results from one representative preparation are shown for wild type and each mutant. A, WT (open circles), R719Q (closed triangles), and D778G (open squares). $V_{max}$ and $K_{m}$ values, respectively, are as follows: 1.8 s$^{-1}$ and 33 μM for WT; 2.7 s$^{-1}$ and 5.9 μM for R719Q; 3.1 s$^{-1}$ and 19 μM for D778G. B, percentage of R719Q and WT bound to actin in the presence of MgATP as a function of actin concentrations. Symbols are the same as in A. $K_v$ values are 58 μM for WT and 10 μM for R719Q.

Sliding velocity also showed a significant increase. It may seem counterintuitive to have increased activity in cardiac myosin lead to a diseased condition; however, enhanced activity, potentially in the form of a higher power output could be just as detrimental as reduced output for a heart that must be designed to operate within narrow specifications for optimum performance.

**Mutations in the Cardiomyopathy Loop**—The FHC mutations made in smooth muscle HMM can be located in the atomic structure of the smooth muscle motor domain complexed with its ELC light chain (named MDE (17)) (Fig. 1). As first observed for striated subfragment 1, the Arg403 residue lies at the base of a surface loop in the upper 50-kDa region of the molecule (5, 17). Recently, the crystal structure of smooth MDE has been docked by a computer-based modular fitting procedure into a three-dimensional reconstruction of actin decorated with smooth subfragment 1 in the presence and absence of MgADP.2 This objective docking method confirmed that the cardiomyopathy loop interacts with actin in the region near the nucleotide binding pocket of actin. But even more important, a comparison of the actomyosin structure in the presence and in the absence of MgADP showed a significant change in binding of the loop to actin upon nucleotide release. These structural findings are consistent with intrinsic fluorescence studies that showed a more polar environment surrounding an engineered Trp113 in rigor than in MgADP (36). Thus, the loop appears to be intimately involved in actomyosin interactions, and mutations at the interface may be expected to affect the enzymatic activity and mechanical properties of myosin.

Mutation of Arg403, a residue that is highly conserved in the...
disparate findings need to be reconciled. Although rat cardiac myosin was the first active recombinant vertebrate muscle myosin to be isolated from any expression system (8), a remarkable achievement at the time, the amount of purified cardiac myosin obtained by this procedure was extremely small. Subsequent work showed that smooth and nonmuscle myosins are expressed at much higher protein levels, for reasons that are still poorly understood. Muscle biochemists have long known that myosin is a relatively unstable molecule that rapidly loses activity at low protein concentrations. Therefore it is not surprising that recombinant myosin, and in particular myosins with mutations in highly conserved regions, would become unstable in dilute solutions with accompanying loss of function. It is also well known that some denatured forms of myosin can form “rigor-like” linkages with actin that do not cycle but exert a load on the actin filament that retards its ability to be moved by the cycling cross-bridges (40). Even if only a small fraction of the myosin forms such rigor linkages, it could readily account for many of the reduced actin velocity values reported for myosins with FHC mutations (9, 38).

Finally, one needs to address the question of whether FHC mutations in the β-cardiac myosin heavy chain would have a different effect on the functional properties of myosin than in the α-cardiac heavy chain isoform. Once again, a recent study on human β-cardiac myosin isolated from biopsies of patients with FHC mutations showed that the actin filament velocity in an in vitro motility assay was about 30% higher for the R403Q myosin (heterozygous for the mutation) than for the control myosin (41). The enhanced function could be accounted for by an increased rate of detachment of myosin from actin in a laser trap assay (i.e. t_on was reduced) (41) as described above. One possible explanation for the discrepancy between these recent measurements and the much lower motility values published previously for myosin isolated from similar biopsies (38) may lie in the extent to which rigor linkages retard the actin velocity. The quality of the myosin isolated from small amounts of frozen tissue will vary considerably, depending on how successfully the myosin preparation is treated to minimize detrimental effects from denatured, rigor-like molecules. Ultimately, a higher actin velocity is more reliable than a lower value, since experimental problems encountered in handling myosin (and actin) all tend to reduce activity.

Mutations in the Lever Arm Region—The light chain binding domain and converter region are closely coupled in the myosin head, insofar that any change in the position of the converter is accompanied by a simultaneous change in orientation of the lever arm (17). Moreover, these domain movements must be closely coupled to nucleotide-induced conformational changes in the active site (42). Therefore, it is not surprising that mutations in the converter would affect the enzymatic activity of the molecule, as we have found for R719Q and R719W, both of which showed a marked increase in ATPase activity. The converter appears to be remarkably sensitive to the type of side chain introduced at this position in the sequence, since the Arg to Gln replacement largely abolished the phosphorylation-dependent regulation of HMM (Table IV), whereas Arg to Trp had no effect on regulation.

The actin filament velocity of R719Q was similar to that of wild type HMM, although t_on was reduced. It is assumed that V_act, in the motility assay, which is done at millimolar MgATP concentrations, is limited by the time to release MgADP from the active site (43). However, the t_on measured in the laser trap at 10 μM MgATP is dominated by the time waiting for the next ATP to bind to the active site (30, 44). Only if the difference in MgADP off rates between two HMMs is correlated with a similar change in the rate of MgATP binding will differences in t_on at low MgATP correctly predict the difference in motility of two HMMs at saturating MgATP concentrations. Such is the case with two isoforms of smooth muscle myosin that differ in a 7-amino acid insert near the nucleotide binding site. The noninserted form is 2-fold lower than the inserted form in all of the following parameters: motility, rate of MgADP release and MgATP binding, and t_on measured at 10 μM MgATP (30). In contrast, a plausible explanation of why R719Q has the same motility but a shorter t_on compared with wild type HMM is that the MgADP off rate is the same as wild type HMM, but the second order rate constant for ATP binding to the mutant is 3–4-fold higher than to wild type HMM, resulting in a shorter dwell time at low MgATP concentrations.

Recent structural studies on two-dimensional crystals of dephosphorylated smooth muscle HMM have suggested a provocative mechanism for how dephosphorylated myosin is maintained in an “off” state; one myosin head was observed to bind intramolecularly to the converter region of the neighboring head (45). Such an interaction would block the actin-binding region of one head and lock the converter into an inactive state on the second head. Introduction of a specific point mutation, such as R719Q, into the converter might well perturb this head-head interaction and lead to a constitutively “on” state. Similarly, the Arg403 mutation to Gln or Trp, which also reduced regulation by phosphorylation, although to a lesser extent, may be explained by a weaker interaction between the cardiomyopathy loop of one head with the converter region of the neighboring head.

The Asp778 residue is invariant in the myosin II class and is located in the long α-helix close to the ELC. Despite being located in such a different region of the myosin molecule, the Asp778 to Gly mutant had very similar functional properties to those of the Arg403 mutation (i.e. enhanced ATPase activity and actin filament velocity). The fact that point mutations so distant from the active site can have such similar effects on kinetic and mechanical properties again emphasizes how intimately linked the different domains are in the myosin molecule. Unlike the cardiomyopathy loop mutations, our motility results for R719Q and D778G (relative to controls) are not that different from the values reported for FHC patients with mutations in the lever arm region (35). A mutation in the ELC (M149V) gave a significant increase in actin filament velocity, whereas the R719Q mutation had a negligible effect on motility (35). Conceivably, the protein fold in this region of myosin is more stable to point mutations than elsewhere in the molecule.

Conclusions—Several point mutations associated with FHC have been shown to increase the enzymatic activity and mechanical properties of smooth muscle myosin. This enhanced activity is most likely independent of the type of myosin isoform. The large reduction in activity observed in earlier studies with cardiac myosins may reflect an increased instability caused by the mutations rather than an inherent property of the myosin. It should be stressed that our discussion of the literature is limited to assays involving pure myosin and actin; the effect of regulated thin filaments, such as would be present in muscle fibers or whole hearts, on the interactions with myosin mutants is not considered here. Despite this limitation, the gain of function found for the mutant myosins may well contribute to the mechanism by which a compensatory hypertrophy is developed as an adaptive response to greater ATP consumption.

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Functional Consequences of Mutations in the Smooth Muscle Myosin Heavy Chain at Sites Implicated in Familial Hypertrophic Cardiomyopathy

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