The cardiac myosin heavy chain Arg-403 → Gln mutation that causes hypertrophic cardiomyopathy does not affect the actin- or ATP-binding capacities of two size-limited recombinant myosin heavy chain fragments

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Our aim was to investigate the potential functional consequences of myosin heavy chain (MHC) mutations identified in patients with familial hypertrophic cardiomyopathy. We observed the presence of a mutated β -MHC mRNA in a formalin-fixed paraffin-embedded myocardial tissue of a proband from family A, which Geisterfer-Lowrance et al. [Geisterfer-Lowrance, Kass, Tanigawa, Vosberg, McKenna, Seidman and Seidman (1990) *Cell* 62, 999–1006] identified as carrying the Arg-403 to Gln mutation. Recombinant DNA methods were then used to obtain size-limited, soluble and undenatured fragments of mutated

myosin subfragment 1 focused around the 403 mutation. The present analysis indicated that the 403 mutation did not quantitatively alter the actin- or ATP-binding capacities of two 246-residue or 524-residue-long recombinant MHC fragments containing this mutation. The absence of any apparent impact of the 403 mutation in the recombinant MHC fragments on interactions between actin and ATP is discussed in relation to numerous biochemical and structural reports which demonstrate the crucial role of the central MHC segment, where the 403 mutation occurs, in myosin functions.

INTRODUCTION

Familial hypertrophic cardiomyopathy (FHC) is a primary cardiac disease inherited in an autosomal dominant pattern [1]. This disease is an important cause of sudden death in young people. It is characterized by increased cardiac mass, with localized and variable myocyte and myofibrillar disarray [2,3]. Molecular genetic analyses at the DNA level revealed 24 different mutations in the β -myosin heavy chain (β -MHC) gene [4]. The most commonly reported mutation for FHC in the β -MHC gene (presently in eight unrelated families) is a mis-sense mutation in exon 13, whereby an adenine is substituted for a guanine in position 1294 of the coding sequence [4–9]. This induces an Arg to Gln substitution in the corresponding protein at amino acid 403, which has been correlated with a high incidence of sudden death [4]. Different mutations in three other families have also been described in the same position (residue 403), suggesting that it is a potent mutation hotspot [10,11]. Ectopic transcripts of β -MHC mRNAs with the G to A mutation were detected in blood lymphocytes of a patient with FHC [12]. Mutated mRNAs have been directly observed in cardiac and slow skeletal muscles of patients with FHC [6,13,14]. Moreover, the Arg-403 to Gln mutant β -MHC was shown to be expressed in the soleus muscle of a patient with FHC by Western-blot analysis [3].

The recently reported crystalline three-dimensional structure of the myosin head [15,16] revealed that the myosin head comprises two domains, the 'motor domain', consisting of an N-terminal segment of the heavy chain (up to about residue 780), and the 'transmission domain', consisting of a long α -helix of the heavy chain and two light chains (Figure 1) [17]. The motor domain itself is composed of specific actin- and nucleotide-binding sites. The myosin segment around residue 403 is on the upper part of the cleft, which is postulated to be involved in the opening and closing mechanism for communication between the nucleotide-binding site and the actin-binding site [16]. The MHC mutation at residue 403, like other mutations in the same myosin

area [6,8], was thus postulated to be localized at the actin interface. It could potentially modify either myosin subfragment 1 (S-1)–actin contacts or communication between the main ATP- and actin-binding sites within the motor domain.

Evidence on the functional impact of the 403 mutation was obtained in studies using native slow mutated myosin [13], myosin heavy and light chains co-expressed in baculovirus [18], and full-length mutant MHC expressed in COS cells [19]. Mutant β -MHC-translocated actin filaments were found to be three to five times slower than in normal controls in an *in vitro* motility assay [13,18]. The Arg-403 to Gln mutation resulted in a cardiac

Figure 1 Schematic representation of the S-1 head structure and of the S-1 recombinant subfragments prepared from *E. coli*

The drawings are based on the resolved structure [15], but only areas relative to S-1, proteolytic S-1 heavy chain fragments (23-, 50-, and 20-kDa) and to myosin light chains [essential light chain (ELC) and regulatory light chain (RLC)] are represented. The 403 mutation is located on top of the upper part (relative to its cleft) of the 50 kDa fragment near the actin interface.

S-1 with normal ATPase activity in the absence of actin, but with a near 5-fold reduction in actin-activated ATPase [18]. A significant amount of COS cells transfected with Arg-403 to Gln MHC mutants failed to form filamentous structures [19]. These studies demonstrated that the 403 mutation influences the rate of transition within the acto-myosin cross-bridge cycle and, more unexpectedly, thick filament formation. We recently used recombinant DNA methods to obtain soluble, undenatured tailored contiguous β -MHC head fragments and determine their *in vitro* ATP- and F-actin-binding capacities [20]. Residue 403 is located in the middle of one such active actin-binding fragment (residues 249–524) and within the MHC fragment (residue 1–524) capable of interacting with ATP [20].

In this study, we checked for the presence of mutated β -MHC mRNA in formalin-fixed paraffin-embedded (FFPE) myocardial tissue of a proband from family A, which Geisterfer-Lowrance et al. [5] identified as carrying the Arg-403 to Gln mutation. Two β -MHC head fragments with the 403 mutation were then produced in *Escherichia coli* and compared with the corresponding normal MHC fragments for their interactive capacities with actin and ATP.

MATERIALS AND METHODS

Reagents

Taq DNA polymerase was supplied by Bioprobe Systems (France). Oligonucleotide primers were synthesized by Eurogentec (Belgium). Restriction endonucleases, *E. coli* DNA polymerase I large Klenow fragment, expression vector pIH 902, bacterial strain TB1 and amylose resin were obtained from New England Biolabs. The DNA ligation kit was from Amersham International and isopropyl β -D-thiogalactose (IPTG) was from Eurogentec. Tosyl-lysylchloromethane (TLCK)-treated α -chymotrypsin, ATP and ϵ -ATP were purchased from Sigma. The type-2 agarose-ATP column (ATP- N^6 -hexane-agarose) was purchased from Pharmacia. All other reagents used in the experiments were of analytical grade.

Proteins

Myosin was prepared from bovine left ventricular myocardium [21] and proteolysed with chymotrypsin to produce S-1 and rod subfragments. S-1 was then isolated by centrifugation at low salt concentrations [22]. F-actin was prepared from rabbit skeletal muscle as described by Mornet and Ue [23].

RNA extraction

RNA was extracted, by a simple extraction method [24] with slight modifications, from FFPE left-ventricle cardiac tissue from a proband of family A (a generous gift of C. Seidman, Boston, U.S.A.) carrying the 403 β -MHC mutation. FFPE tissue sections were cut (10 μ m thick) with a microtome and excess paraffin was removed with a razor blade. The paraffin was extracted twice with xylene for 5 min at 55 °C followed by two ethanol washes to remove xylene. The material was incubated for 6 h at 60 °C in 100 μ l of a lysis buffer containing 20 mM EDTA, 2% (w/v) sodium dodecyl sulphate, 500 μ g/ml proteinase K and 20 mM Tris/HCl (pH 8.0). Nucleic acids were then purified by two phenol/chloroform and two chloroform/isoamyl alcohol extractions; ethanol precipitation was with 200 μ g/ml glycogen as carrier. Following precipitation, samples were centrifuged (15000 g) for 30 min at 4 °C, the supernatant was carefully removed and the pellet air-dried. Control RNA was extracted from frozen human normal ventricle cryostat sections and treated as above.

Reverse transcription (RT)-PCR amplification and analysis

Nucleic acids were dissolved in 5 μ l of RNAase-free water. We took advantage of the RNA-dependent polymerase activity of *Taq* polymerase [25,26] to perform reverse transcription and PCR amplification with the same enzyme in the same tube. Amplifications were carried out in a 50 μ l final volume containing 5 μ l of 5 $\times$ *Taq* buffer [83 mM (NH₄)₂SO₄, 33.5 mM MgCl₂, 50 mM 2-mercaptoethanol, 850 μ g/ml BSA and 335 mM Tris/HCl, (pH 8.8)] and 2.5 μ l of dimethyl sulphoxide, 0.5 mM dNTPs, 200 ng of forward and reverse oligonucleotide primers (see sequences in Figure 1), 1 μ l of RNA sample and 2.5 units of *Taq* polymerase. After heating for 6 min at 92 °C and cooling for 1 min at 47 °C, 35 cycles were performed with 4 min extension at 65 °C, 30 s denaturation at 92 °C and 30 s annealing at 47 °C. No mineral oil was added since thermal cycling was performed with a computer-controlled robot (a small portal crane) which transports samples (400 μ l microtubes) through three preheated glycerol baths (Bühler GmbH, Tübingen/D) with complete submersion of the tubes. PCR products previously purified on Qiagen cartridges, which remove proteins and oligonucleotides, were digested by *Dde*I and analysed by NuSieve-GTG agarose gel electrophoresis.

Plasmid construction for β -MHC fragment expression

A human β -MHC cDNA fragment coding for amino acid residues 1–524 was excised from the p β HC13 plasmid [27] by *Nco*I and *Hind*III cleavage. This 1585 bp fragment, after filling the 3' recessed ends, was ligated to the blunt-ended pIH 902 vector linearized by *Eco*RI. Expression in *E. coli* strain TB1 led to a soluble 100 kDa maltose-binding protein (MBP) fusion protein (referred to as NM). The shorter construct was obtained from the same 1585 bp fragment using single-cut restriction enzymes: *Eco*RI digestion of this insert gave two 743 bp and 842 bp fragments. The latter was polished and then ligated to the blunt-ended pIH 902 vector linearized by *Eco*RI. It led to expression of a 73 kDa fusion protein (named M) spanning residues 249–524. The same procedure was applied to the p β HC13-PM403 plasmid upon which the mutation was previously introduced by site-directed mutagenesis following standard procedures and controlled by DNA sequencing. NM403 and M403 were thereby obtained and expressed fused to MBP.

Expression of recombinant β S-1-HC fragments

A volume of 150 ml of Luria-Bertani medium containing 2 g/l glucose and 50 μ g/ml ampicillin was inoculated with 3 ml of bacterial culture grown overnight in the same medium. Bacteria were grown in an incubator shaker at 37 °C to an absorbance at 600 nm of 0.6. After lowering the temperature to 30 °C, IPTG was added to a final concentration of 0.05 mM and cells were incubated for an additional 4 h. The bacterial culture was harvested by centrifugation at 4000 g for 20 min. The bacterial pellet was suspended at 4 °C in 10 ml of buffer A [0.2 M NaCl, 1 mM EDTA, 1 mM NaN₃, 10 mM 2-mercaptoethanol and 10 mM Tris/HCl (pH 7.5)] with 0.25% Tween 20. Cell lysis was performed by sonication and a soluble extract was obtained by centrifugation of the crude lysate at 12000 g.

Purification of recombinant β S-1-HC fragments

Soluble extracts were diluted with buffer A to reduce the protein concentration below 2.5 mg/ml (approx. 3-fold dilution), and then loaded on to an amylose resin column (5 ml) equilibrated with the same buffer at a flow rate of 0.5 ml/min. The column was then washed with five column volumes of buffer A containing

0.25% Tween 20, and five volumes of buffer A without Tween. Elution of bound proteins was performed with 10 mM maltose in buffer A. Purified proteins were quantified in a Bradford protein assay. They were occasionally concentrated on Centricon 10, and stored at 4 °C until use. By this procedure, 1.0–5.0 mg of purified proteins was obtained from 150 ml of bacterial culture.

ATP-binding assays

β S-1-HC recombinant fragments NM or NM403 (1 μ M) in 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol and 15 mM Tris/HCl (pH 7.5) were loaded on to an ATP-agarose column (gel volume: $\sim$ 500 μ l) equilibrated with the same buffer. After washing, bound proteins were eluted with 10 mM Mg-ATP in the same buffer. The elution profile was analysed by Western blotting with an anti-S-1 monoclonal antibody (11E10) after SDS/PAGE, as previously described [28], on each chromatographic fraction (250 μ l).

Labelling with [α -³²P]ATP

β S-1-HC recombinant NM fragments were photoaffinity labelled at the ATP-binding site with [α -³²P]ATP using the method described by Maruta and Korn [29]. Proteins (1 μ M) in 0.1 M KCl, 1 mM MgCl₂, 5 mM dithiothreitol, 50% glycerol and 15 mM Tris/HCl (pH 7.5) were incubated with 0.5 μ M [α -³²P]ATP (1000–3000 Ci/mmol; 1 Ci = 37 GBq) for 30 min at room temperature with or without u.v. (254 nm) irradiation in the absence or presence of 1 or 1000 μ M of unlabelled ATP. The cross-linked products were analysed by autoradiography after SDS/PAGE fractionation by overnight exposure to X-ray film at –80 °C with an intensifying screen.

Binding of a fluorescent ATP analogue

The interaction of ϵ -ATP with the myosin NM subfragments was studied by quenching the free nucleotide with acrylamide, as described by Ando et al. [30] and Chaussepied et al. [31] in a Kontron spectrofluorimeter. Titration was monitored by adding increasing concentrations of ϵ -ATP to a solution containing 6 μ M of β S-1, 6 or 5 μ M of NM and NM403, 100 mM acrylamide, 2 mM MgCl₂, 5 mM KCl and 40 mM Tris/HCl (pH 7.5) at 25 °C. Nucleotide fluorescence was measured at 410 nm with excitation at 320 nm.

Actin-binding assay

Co-sedimentation assay

Myosin fragments (2 μ M) were incubated with F-actin at various concentrations (0.25, 0.5, 1.0, 2.0 and 4.0 μ M) for 10 min at 25 °C in a buffer containing 50 mM KCl, 0.1 mM CaCl₂ and 20 mM Tris/HCl (pH 7.5), and then centrifuged in a Beckman Airfuge at 100 000 *g* for 15 min at 25 °C. Pellets and supernatants were analysed by SDS/PAGE. Quantification was obtained by densitometric scanning of the bound (in the pellet) and the free (in the supernatant) proteins. Affinity constants were deduced from Scatchard plots calculated from the scanning values (the slope corresponds to $-K_A$ M⁻¹).

Miscellaneous

The protein concentration was determined by the Bio-Rad protein assay. Proteins were separated by SDS/PAGE and visualized by Coomassie Blue staining. Densitometric scanning was performed with an Apple OneScanner coupled to an image processing and analysis program (IMAGE version 1.41, National Institute of Health). Agarose gel electrophoresis was carried out

with NuSieve-GTG agarose from FMC products and DNA was visualized by ethidium bromide staining. All DNA manipulations were performed according to standard procedures [32].

RESULTS

Expression of the mutant β -MHC mRNA

Cardiac FFPE tissue was obtained from the left ventricle of a proband of family A [5]. Total RNA extracts were used as templates for one-step RT-PCR amplifications with specific primers flanking the mutated MHC area. *Dde*I restriction maps were then obtained revealing an additional *Dde*I restriction site introduced by the G to A nucleotide mutation in the β -MHC gene of the diseased heart. The amplification strategy is shown in Figure 2. Two different upstream oligonucleotide primers, corresponding to myosin sequences in exons 9 and 12, were designed since they were upstream from the putative mutation in exon 13. Each primer was paired with one downstream oligonucleotide

Figure 2 Amplification strategy and PCR controls

(a) The point mutation is indicated under the trypsin map of S-1 [27] and above the exon map of the β -MHC mRNA [37]. Arrows show the PCR primer positions. Primers A (5'-GGAGTTCAAGCTGAAGCAGCGG-3') and B (5'-GTTAGATGAGGTCAATGCAGGCCT-3') directed amplification of a 508 bp AB PCR product (coding for amino acids 362 to 518), primers C (5'-TCGGGAAATTCATTCAAATTCATTTGGGGC-3') and D (5'-GTGAAGTTGATGCAGAGC-3') gave a 712 bp CD PCR product (coding for amino acids 245 to 481). Their respective *Dde*I restriction maps are presented. The asterisk corresponds to the additional *Dde*I site generated by the mutation. (b) Correlation plot between the number of FFPE tissue sections used to prepare a 5 μ l nucleic acid extract, as outlined in the Materials and methods section, and the amplification yields with primer pairs A/B and C/D deduced by densitometric scanning of ethidium bromide-stained analytical agarose gels.

Figure 3 *DdeI* mapping of PCR products

After PCR amplification, products obtained with both primer pairs (AB and CD see Figure 2) were digested by *DdeI* and analysed by agarose gel electrophoresis. Bands resulting from additional *DdeI* cleavage (indicated by an asterisk on the bottom map) are pointed out on the gels by arrows with their respective sizes printed in bold type.

primer coding for different sequences in exon 15 (A/B and C/D pairs in Figure 2a). The amounts of the two corresponding amplified fragments were closely proportional to the number of microtome heart sections (Figure 2b). These products, amplified from the diseased heart and from one normal human heart used as control, were the expected sizes according to the known spliced human β -MHC sequence (Figures 3a and 3b). *DdeI* digestion of the two amplified products revealed that additional bands were present in the proband samples and absent in the normal ventricle samples (Figures 3a and 3b). The bands were the expected sizes, in accordance with the mutation positions along the MHC sequence and the respective sequences of the two amplified MHC fragments used for the restriction analysis. This demonstrated that some mRNA with the mis-sense mutation of the β -MHC gene, in addition to the normal β -MHC mRNA, was expressed in the diseased heart. No attempt was made to quantify the respective initial amounts of normal versus mutated mRNAs after 35 amplification cycles.

Functional impact of the Arg-403 to Gln β -MHC mutation

To examine the functional effects of the Arg-403 to Gln change in the β -MHC, we compared the capacities of two different recombinant MHC fragments focused around normal or mutated residue 403 to interact with actin and ATP [20]. The actin-binding S-1-HC fragments, spanning from residues 249 to 524, centrally located in the myosin head [15], were referred to as M or M403, normal and mutated forms respectively. The long N-terminal ATP-binding S-1 fragments, spanning residues 1 to 524, were referred to as NM or NM403, normal and mutated forms respectively (Figures 1 and 4). These MHC fragments were easily obtained as soluble and undenatured proteins by recombinant DNA techniques using a 1585 bp cDNA fragment coding for the normal MHC fragment NM (and M)[20]. The cDNA fragment was mutagenized to produce mutated MHC fragments (see the

Figure 4 Functional domains of the S-1 heavy chain

The recombinant β -MHC fragments used in this study were previously shown to have functional capacities, they are represented under the trypsin S-1 map by rectangles with their respective names and positions according to the β -MHC numbering scheme [37]. Fragment NM binds ATP, and fragment M interacts with F-actin [20].

Materials and methods section). This procedure was used instead of that with mutated MHC cDNA fragments amplified from the diseased heart (see above). It minimized the possible risk of additional mutations due to long mRNA preservation in FFPE heart and extensive PCR amplification. MHC fragments, obtained by insertion of the corresponding MHC cDNA fragments in pIH 902 expression plasmid, were produced in *E. coli* as

Figure 5 Comparative ATP binding between normal and 403 mutated recombinant β -S-1 heavy chain fragments

(a) 1 μ M of NM and NM403 were treated as outlined in the Materials and methods section, with or without u.v. irradiation at 254 nm and in the absence or presence of 1 μ M or 1000 μ M of free ATP. Cross-linked products were analysed by autoradiography after SDS/PAGE fractionation. (b) Titration of the binding of a fluorescent nucleotide analogue was monitored by adding increasing concentrations of ϵ -ATP to a solution containing 6 μ M of β -S-1, 6 or 5 μ M of NM and NM403, 100 mM acrylamide, 2 mM MgCl_2 , 5 mM KCl and 40 mM Tris/HCl (pH 7.5) at 25 $^{\circ}\text{C}$. Nucleotide fluorescence was measured at 410 nm with excitation at 320 nm.

MBP fusion polypeptides and purified by amylose column chromatography.

ATP binding

Amylose column-purified normal and mutated NM MHC fragments were used to examine the influence of the 403 β -MHC mutation on ATP binding. Both MHC fragments bound to agarose-ATP resin columns. The respective overall amounts of bound MHC fragments were densitometrically measured (results not shown). There were no significant differences between ATP-binding levels of fragments NM and NM403. U.v. cross-linking of radioactive ATP to each purified NM fragment was quantitatively measured in the absence of ATP and following competition with two different concentrations of unlabelled ATP. There were no observed differences in the cross-linking capacities of NM and NM403 fragments with ATP in any conditions used (Figure 5a). Titration of ATP binding by each NM MHC fragment was also done by the fluorescent ATP-quenching method. NM and NM403 fragments bound ϵ -ATP with the same kinetics and affinity parameters as observed with the native chymotryptic S-1 head (referred to as β S-1) (Figure 5b). These different experimental approaches clearly demonstrated that the 403 mutation did not modify the capacities of the studied MHC fragments to bind ATP. Note that neither recombinant NM [20] nor NM403 hydrolysed ATP.

F-actin interaction

The effect of the 403 mutation on F-actin affinity was examined via the F-actin-binding properties of amylose-column-purified, 246-residue-long M fragments, expressed with and without the mutation. Co-sedimentation assays with increasing amounts of F-actin permitted quantitative comparisons of both F-actin affinities (Figure 6a). Both M and M403 β -MHC fragments had the same affinities (K_d of approx. 4×10^{-7} M) for F-actin (Figure 6b). The addition of 5 mM ATP or 2 mM polyglutamic acid did not influence co-sedimentation of either M or M403 fragments with F-actin. We previously determined that the native β S-1 affinity was approx. 1.5×10^{-7} M in the same experimental conditions but in an ATP-dependent manner [20]. Increasing amounts of native S-1 inhibited actin binding in an equimolar way to M or M403 β -MHC fragments (data not shown).

DISCUSSION

We used RT-PCR to examine β -MHC gene mRNA transcripts from an FFPE ventricular autopsy from a proband of the large French-Canadian family A with hypertrophic cardiomyopathy [5]. We found that both normal and mutant β -MHC alleles were transcribed in the diseased heart of one patient who died many years ago. This is the first retrospective demonstration of transcription of a mutant β -MHC gene in the myocardium of a hypertrophic cardiomyopathy patient. It confirms previous results on the presence of a β -MHC mutation in exon 9 in mRNA from blood lymphocytes of a patient with hypertrophic cardiomyopathy [12]. It also confirms detection of the mis-sense mutation in exon 13 of the β -MHC gene in mRNAs isolated from ventricular endomyocardial or skeletal muscle biopsies obtained from two different families with FHC [6,13,14]. As normally observed in myocardial biopsies, we detected the β -MHC mutant mRNA in the fixed autopsy myocardium after only 35 amplification cycles, in contrast to the 80 amplification cycles required to detect mutant mRNA in lymphocytes. There thus seemed to be enough mutated mRNA in the myocardium (Figures 2a and 2b) to produce substantial amounts of mutated β -MHC [13]. Quantitative RT-PCR experiments from well-defined areas of the diseased myocardium should now be conducted to determine the ratios of wild-type to mutant transcripts in local myocardial areas. This could reveal whether mutated mRNA is preferentially expressed where myofibrillar disarrays have been detected [2]. This approach, which could provide information on local accumulation and assembling of mutated MHC [3], can be performed with minute quantities of myocardial tissue, as demonstrated in the present study.

The present analysis indicated that the 403 mutation did not quantitatively alter the actin- or ATP-binding capacities of two 246-residue or 524-residue recombinant MHC fragments containing this mutation. The essential question that arises is whether the soluble recombinant MHC fragments used were structurally similar to the corresponding MHC segments within the entire myosin head. The fact that the analysed recombinant MHC fragments had high actin- and ATP-binding affinities (Figures 5 and 6), comparable with those of native myosin S-1, indicates that folding of the recombinant MHC forms was at least partially achieved. The 1:1 competition observed between the recombinant MHC fragments and the native S-1 for F-actin also suggests that the measured binding capacities were not due to a sticky conformational state. For these reasons, we consider that the MHC fragments used in this study could mimic a few interactions that occurred within the native myosin molecule during the contractile process. It is clear that our size-limited MHC frag-

Figure 6 Comparative F-actin binding between normal and 403 mutated recombinant β S-1 heavy chain fragments

(a) SDS/PAGE analysis of co-sedimentation assays carried out with 2 μ M of each β S-1 fragment and increasing amounts of F-actin (0.25, 0.5, 1, 2 and 4 μ M). Only the M profile is presented, M403 gave the same result (not shown). (b) Quantification was deduced by densitometric scanning of the bound (in the pellet) and the free (in the supernatant) proteins. Affinity constants were deduced from Scatchard plots calculated from the scanning values (the slope corresponds to $-K_A$ M $^{-1}$).

ments possessed only part of the ATP- and actin-binding capacities of intact native S-1. It is known that other MHC fragments also interact with F-actin, that contiguous MHC fragments influence their interaction capacities [20], and that only intact S-1 hydrolyses ATP. Recent work [33] extends the conclusions drawn from the crystal structure, showing the importance of the MHC loop around residues 613–621 in the actin-binding mechanism and the multiplicity of the actin-binding interface.

Numerous biochemical and structural reports demonstrate the crucial role of the central MHC segment, where the 403 mutation occurs. Dramatic modifications induced by the 403 mutation have been documented through analysis of highly integrated functions such as actin-activated ATPase activity, the ability of myosin to move actin filaments *in vitro* [13,18] and the assembly of mutant MHC in COS cells [19]. Quite drastic chemical, proteolytic or thermal treatment of the 50 kDa fragment led to complete ATPase inhibition of the myosin head [34,35]. The working hypothesis of a cross-bridge cycle deduced from the S-1 structure [15,16], and our recent observations on actin–myosin interfaces [20], clearly indicate that the large 50 kDa MHC fragment (and its sub-fragment M) is the central part of the myosin motor domain. The 50 kDa fragment contributes to the structure of the central core of the myosin motor domain and is also postulated as being the main initial actin–myosin interface [15,16]. This major contact area between actin and the myosin head occurs thanks to a cleft modulated by an opening/closing mechanism under ATP hydrolysis (Figure 1). Residue 403 is located near the putative main actin-binding site, in a loop on the upper part of the cleft. The central core of the myosin motor domain is composed of a seven-stranded β -sheet. Three β -sheets (residues 247 to 254, 260 to 268 and 457 to 465) are only issued from the MHC fragment M; the four other β strands arise from both distal segments of the myosin head. It has been suggested that the core contributes in communications between the nucleotide and the main actin-binding sites [15,16]. In the light of these results and despite limited significance of the binding measurements deduced from size-limited recombinant MHC fragments, the absence of any apparent impact of the 403 mutation on interactions between actin and recombinant MHC fragment M was quite unexpected. Because of its location outside of the core, the Arg to Gln mutation of residue 403 probably does not modify this crucial structure. It could influence the position of the central core with respect to the second part of the central motor domain. The present results suggest that the mutation, which impairs some complex myosin functions, modulates the communicating capacity of the central region of the myosin head without affecting its actin affinity. The absence of impact of the 403 mutation on ATP binding could be predicted. The relatively long distance between the ATP-binding site and the mutation location on the actin-binding site could explain these negative results. It should be stressed that the mutation could *a priori* have a long-distance effect since only the long recombinant MHC NM fragment, and not shorter fragments, was able to bind ATP in the same range as native S-1 [20].

In conclusion, the present results suggest that a MHC mutation could subtly modulate some integrated functions of the myosin motor with or without modifying some elementary functions. More extended and sophisticated myosin constructions should now be tailored to investigate the effects of each mutation class on relatively integrated functions, such as F-actin-activated ATPase activity and/or structural stability induced by myosin light chains and motility. Some recent reports on the roles of the different myosin subunits [36] and of the different myosin regions [17,37], in addition to recent data on the myosin structure,

should help in drawing up suitable guidelines for studying the functional impact of many other MHC mutations identified in cardiomyopathic patients.

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