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ARTICLE

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Functional and spectroscopic studies of a familial hypertrophic cardiomyopathy mutation in Motif X of cardiac myosin binding protein-C

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Abstract Familial hypertrophic cardiomyopathy is an autosomal dominant genetic disorder caused by mutations in cardiac sarcomeric proteins. One such mutation is a six amino acid duplication of residues 1248–1253 in the C-terminal immunoglobulin domain of cardiac myosin binding protein-C, referred to as Motif X. Motif X binds the myosin rod and titin. Here we investigate the structural and functional alteration in the mutant Motif X protein to understand how sarcomeric dysfunction may occur. The cDNA encoding Motif X was cloned, mutated and expressed as wild-type and mutant proteins in a bacterial expression system. Circular dichroism spectroscopy confirmed that the normal and mutant Motif X exhibited a high β-content, as predicted for immunoglobulin domains. Thermal denaturation curves showed that Motif X unfolded with at least two structural transitions, with the first transition occurring at 63 °C in the wild-type but at 40 °C in the mutant, consistent with the mutant being structurally less stable. Sedimentation binding studies with synthetic myosin filaments revealed no significant difference in binding to myosin between the wild-type and the mutant Motif X. Molecular modeling of this duplication mutation onto an homologous IgI structure (telokin) revealed that the duplicated residues lie within the F strand of the immunoglobulin fold, on a surface of Motif X distant from residues previously implicated in myosin binding. Taken together, these data suggest that the Motif X mutation may interfere with other, as yet unidentified, functional interactions.

Keywords Myosin binding protein-C · Familial hypertrophic cardiomyopathy · Cardiac muscle

Abbreviations *CD*: circular dichroism · *FHC*: familial hypertrophic cardiomyopathy · *FnIII*: fibronectin type III · *IgI*: immunoglobulin I · *IPTG*: isopropyl β-D-thiogalactoside · *LB*: Luria-Bertani · *LMM*: light meromyosin region of myosin · *MyBP-C*: myosin binding protein-C · *NTA*: nitrilotriacetic acid · *S-2*: subfragment-2 of myosin · *SCR*: structurally conserved region

Introduction

Familial hypertrophic cardiomyopathy (FHC) is an autosomal dominant genetic disorder that is one of the most common forms of inherited heart disease and is associated with sudden death in adolescents and young adults. It is characterized macroscopically by increased left ventricular mass in the absence of a secondary cause and histologically by myofibrillar and myocyte disarray. FHC has been linked to mutations in muscle sarcomeric proteins, including the two most common, the cardiac β-myosin heavy chain gene and the cardiac myosin binding protein-C (MyBP-C) gene (reviewed in Redwood et al. 1999). Therefore, FHC has been described as a disease of the sarcomere.

MyBP-C is located in the crossbridge-containing A band of the sarcomere as a series of 7–9 transverse stripes, spaced 43 nm apart (Craig and Offer 1976; Dennis et al. 1984). Skeletal MyBP-C is a 130 kDa rod-shaped protein predicted to contain a series of 10 globular domains,

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based on repetitive motifs, each 90–100 amino acids in length. These modular domains are predicted to resemble either the immunoglobulin I (IgI) or the fibronectin type III (FnIII) motif (Einheber and Fischman 1990). There are seven IgI domains and three FnIII domains that are numbered I to X from the N-terminus. Based on its overall structure, MyBP-C is classified as belonging to the intracellular immunoglobulin superfamily. The immunoglobulin superfamily is one of the most divergent superfamilies, both in terms of sequence and function (Chothia and Lesk 1982). IgI domains are assembled as two β-sheets that pack tightly to form a β-sandwich and provide a stable scaffold that often hosts a highly specific binding site (Bork et al. 1994). Small variations in the fold may directly affect the flexibility and specificity in protein-protein interactions. The human cardiac isoform has 1274 amino acids, possesses an additional IgI module at its N-terminus (Motif 0), contains a 27-residue loop insert in Motif V and can be phosphorylated near its N-terminus (Gautel et al. 1995).

Although the exact function and structure of MyBP-C is uncertain, evidence suggests that it has both structural and regulatory roles, including sarcomere assembly and cross-bridge regulation (Freiburg and Gautel 1996; Moos and Feng 1980; Okagaki et al. 1993). It can bind myosin at both subfragment-2 (S-2) and light meromyosin (LMM) (Moos et al. 1975; Starr and Offer 1978), as well as titin (Freiburg and Gautel 1996; Furst et al. 1992; Koretz et al. 1993) and actin filaments (Moos et al. 1978). Both the N- and C-terminal regions of MyBP-C bind to the myosin thick filament. The S-2 binding region is located in the "regulatory domain" of MyBP-C, between motif domains I and II (Gruen and Gautel 1999). Phosphorylation results, in part, from adrenergic stimulation and causes an increase in systolic tension (Gautel et al. 1995). The LMM and titin binding regions are located in the last three C-terminal domains (Freiburg and Gautel 1996; Gilbert et al. 1996; Okagaki et al. 1993) and are the minimal requirement for incorporation into the C-zone of the sarcomere (Gilbert et al. 1996).

A feature of importance for all isoforms of MyBP-C with respect to their function is the last 102 residues in the C-terminal IgI domain (Motif X), which alone can bind to the LMM fragment (Okagaki et al. 1993). A recent study has identified key residues in skeletal Motif X involved in LMM binding (Miyamoto et al. 1999). Several FHC mutations have been identified in MyBP-C which account for 15–20% of FHC families (see FHC mutation database: http://www.angis.org.au/Databases/Heart). The majority of these mutations are truncations, which result in the premature termination of translation of the C-terminus of MyBP-C, eliminating the myosin and/or titin binding sites (Redwood et al. 1999). In addition to several mis-sense mutations that result in single amino acid substitutions, an in-frame six amino acid duplication in the Motif X C-terminal domain has been reported (Watkins et al. 1995).

The aim of this communication is to examine the structural and functional deficits that result from the mutant duplication of six amino acid residues in Motif X of cardiac MyBP-C protein that causes FHC (Watkins et al. 1995). Specifically, cDNA coding for the human cardiac wild-type and mutant Motif X domains were expressed in *Escherichia coli* and their structure, stability and function were studied using circular dichroism (CD) spectroscopy, computer modeling and a sedimentation binding assay with myosin filaments. Our results demonstrate that the mutant duplication minimally perturbs the folding and structural stability of Motif X, and does not interfere with myosin binding, one of the two major functions of this domain.

Materials and methods

Proteins

Motif X cDNA of cardiac MyBP-C was isolated from a human heart cDNA library (Matchmaker cDNA, lot 5134, Clonetech Laboratories, Palo Alto, Calif., USA). The module boundary was selected on the basis of a sequence alignment with conserved immunoglobulin residues from the titin m5 and chicken gizzard telokin module, in which atomic resolution structures have been established (Holden et al. 1992; Pfuhl and Pastore 1995). PCR site-directed mutagenesis was employed to construct the mutant Motif X cDNA and involved the insertion of an 18 base pair duplication (GGGGGCATCTATGTCTGC). The DNA fragments ($\pm$18 base pair insertion) corresponding to nucleotides 3576 to 3854 of MyBP-C [AC X84075 (Gautel et al. 1995)] were subcloned into the pQE-60 QIAexpress type ATG vector and transformed into the *E. coli* SG13009 cell line (Qiagen, Valencia, Calif., USA). The expressed protein product included a C-terminal 6-His purification tag. Two additional residues were included at the N-terminus (Met-Ser) and three additional residues (Gly-Arg-Ser) were included as a linker prior to the 6-His purification tag, as a result of insertion of the PCR fragments at the *Nco*I and *Bgl*II sites, respectively. Additionally, human cardiac MyBP-C Motif V (nucleotides 1968–2342) was cloned using the same methods as used for Motif X for use as a control IgI domain.

Transformed cell lines were grown at 37 °C in Luria-Bertani (LB) broth (100 μg mL^{-1} ampicillin and 50 μg mL^{-1} kanamycin) to an optical density of 0.5 units at 595 nm. Protein expression was induced by the addition of 1 mM isopropyl β-D-thiogalactoside (IPTG) and cells grown for an additional 4–5 h. The cells were harvested by centrifugation and resuspended in 10 mL g^{-1} wet pellet weight of binding buffer (300 mM NaCl, 10 mM imidazole, 20 mM Tris-HCl, pH 7.0), sonicated and treated with lysozyme at 0.5 mg mL^{-1} for 30 min. After centrifugation the soluble Motif X wild-type supernatant was fractionated by Ni^{2+}-nitrilotriacetic acid (NTA) metal chelate affinity chromatography. A significant proportion of mutant Motif X was expressed in inclusion bodies, which were washed in detergent buffer (0.2 M NaCl, 1% w/v deoxycholic acid, 1% NP-40, 20 mM Tris, pH 7.0) prior to solubilization in 6 M urea and binding buffer. Pure fractions of Motif X were pooled, as judged by Tricine gel electrophoresis (Schagger and von Jagow 1987), diluted to 0.1 mg mL^{-1} and refolded slowly against sequential dialysis of decreasing concentrations of urea in the required CD or sedimentation assay buffers.

Bovine hearts (left ventricles) were obtained from Woy Woy abattoirs (NSW, Australia) and used for the preparation of bovine cardiac myosin according to the method of Tonomura et al. (1966).

Circular dichroism spectroscopy

Samples used for CD analysis in CD buffer (60 mM NaF, 20 mM Na_3PO_4, pH 7.0) were diluted to 0.1 mg mL^{-1}. A Jasco J-720 CD spectropolarimeter (Jasco, Tokyo, Japan) was calibrated with a

known ammonium-D-camphor-10-sulfonic acid (CSA) reference standard (Woody 1995). Protein samples were analyzed in a 0.1 cm pathlength quartz cuvette, using a temperature regulated cuvette holder, in a N_2 atmosphere. Four scans were collected for analysis over the range 260–190 nm using the following spectral parameters: resolution 0.2 nm, band width 1 nm, sensitivity 10 mdeg, response time 1 s and scan rate 20 nm min^{-1}.

Ellipticity values were converted to mean residue weight ellipticities ($[\theta]_{MRE}$) using the equation:

$$[\theta]_{MRF} = ([\theta] \times 100 \times MRW)/(c \times d) \quad (1)$$

where θ is the baseline corrected ellipticity, MRW is the mean residue weight, c is the concentration in mg mL^{-1}, and d is the optical pathlength in cm. $[\theta]_{MRE}$ has the units deg cm^{-2} $dmol^{-1}$.

Thermal denaturation curves

The optical activity of wild-type and mutant Motif X was measured as a function of temperature to detect thermal denaturation of the protein. The optimal wavelength to monitor thermal denaturation for Motif X was found to be 200 nm, corresponding to the region in which there is significant positive ellipticity due to β-sheet, compared to significant negative ellipticity due to random coil (Politou et al. 1994a, 1994b).

To obtain the thermal denaturation curve data, the temperature was increased at a rate of 20 °C per hour (to a maximum of 75 °C) and the ellipticity at the optimum wavelength (200 nm) recorded for 1 min at 1 or 2 °C increments. Spectral parameters were: 1 s step resolution, response time 0.5 s, sensitivity 20 mdeg and bandwidth 1.0 nm. In addition, spectra between 260 and 190 nm were recorded at 10 °C intervals to monitor the unfolding transition. Thermal denaturation curves of at least two different preparations were recorded for each protein. Calculated $[\theta]_{MRE}$ values were plotted against cell temperature and thermal denaturation curves fitted using a non-linear regression iterative process with the Marquardt-Levenberg algorithm in Sigmaplot V.2 (Jandel Scientific).

Sedimentation binding to myosin

The ability of various MyBP-C IgI domains to bind to myosin was determined using an established sedimentation binding assay (Okagaki et al. 1993). Bovine ventricular myosin was clarified (12,100×*g*, 20 min, 4 °C), then dialyzed against 100×volume of filament buffer (100 mM KCl, 20 mM Tris-HCl, pH 7.0) to obtain synthetic myosin filaments. Prior to the binding assay, motif domains in filament buffer were also clarified by centrifugation (12,100×*g*, 20 min).

Wild-type and mutant Motif X were mixed at varying molar ratios with myosin filaments (2 μM) for 10 min at 22 °C. The concentration of Motif X was increased from zero to a three-fold molar excess over myosin to demonstrate saturation. Filaments were sedimented at 8400×*g* for 15 min and pellets analyzed by 16.5% Tricine gel electrophoresis. Gels were analyzed using Phoretix 1D software. The intensity of the bound IgI domains were normalized to the intensity of the corresponding myosin regulatory light chain.

Modeling

Structural modeling of the Motif X wild-type and mutant domains was performed with the Homology module of the InsightII 98.0 (MSI, San Diego, Calif., USA) molecular modeling package. The crystal structure of an homologous protein, telokin (1tlk) (Holden et al. 1992), was used as a template. The wild-type and mutant Motif X sequences of human cardiac MyBP-C were aligned with the telokin sequence using the Dayhoff Evolutionary Mutation Matrix (PAM250) (Dayhoff et al. 1983) with a gap penalty of 10 residues, and a gap length penalty of 1.65. The α-carbon coordinates of telokin were then threaded onto the Motif X models over all structurally conserved regions (SCRs). For each segment between SCRs, 10 candidate loops were generated with random peptide dihedral angles ϕ,ψ and extended side chains. The conformations with the lowest root mean square difference (RMSD) of the flanking SCRs were chosen.

An optimal set of side-chain conformations was found using the algorithm of Novotny (Mas et al. 1992), as implemented in the auto rotamer tool of Homology. The side-chain conformation search was performed over all SCRs and loops simultaneously. Energies were calculated using the consistent valence force field (CVFF) with a nonbond cutoff of 8.0 Å. The algorithm was terminated when either the potential energy difference between cycles was less than 0.05 kcal mol^{-1} or a maximum of 50 cycles was reached.

Lastly, model structures obtained were energy minimized to relieve any remaining steric overlap. The relax tool of Homology was used with CVFF and a nonbond cutoff distance of 8.0 Å for 100 steps of the steepest decent algorithm, followed by further energy minimization using the conjugate gradient method until the maximum derivative was less than 5.0 kcal mol^{-1} $Å^{-1}$.

Results

Secondary structure of Motif X deduced by CD spectroscopy

CD spectroscopy was used to assess the secondary structure of the recombinant wild-type and mutant Motif X modules at 17 °C (Figs. 1a and 2a). From sequence homology studies, Motif X is predicted to be a β-sandwich IgI immunoglobulin domain (Harpaz and Chothia 1994). The major secondary structural element seen in both these spectra is consistent with a predominantly β-sheet secondary structure (Sreerama et al. 1999). The CD spectrum of the β-sheet is of low amplitude (minimum $[\theta]_{MRE}$ approximately -7×10^{-3} deg cm^2 $dmol^{-1}$), has minima at approximately 216–218 nm and a positive band at approximately 190 nm. These spectra are also similar to the spectra obtained from several IgI domains that form the titin molecule (Politou et al. 1994a, 1994b, 1996).

Secondary structure predictions on the Motif X sequence estimate the secondary structure content as: α-helix, less than 5%; β-strand, 65–75%; and random coil, 25–35% (including the 6-His tag) (Chou and Fasman 1978). With the α-helical content limited to $< 5\%$, decomposition of the CD spectra yields a unique solution of 65–75% β-strand, in good agreement with the secondary structure predictions and the atomic structure of IgI domains (Holden et al. 1992; Pfuhl and Pastore 1995). A slight increase in random coil, as reflected by a decrease in amplitude of the peak at 195 nm, corresponds well with a random-coil structure of the six duplicated residues in the mutant.

Thermal denaturation of Motif X detected by CD spectroscopy

Thermal denaturation curves were obtained to investigate the effect of the six amino acid duplication mutation on the structural stability of Motif X. Ellipticity of Motif

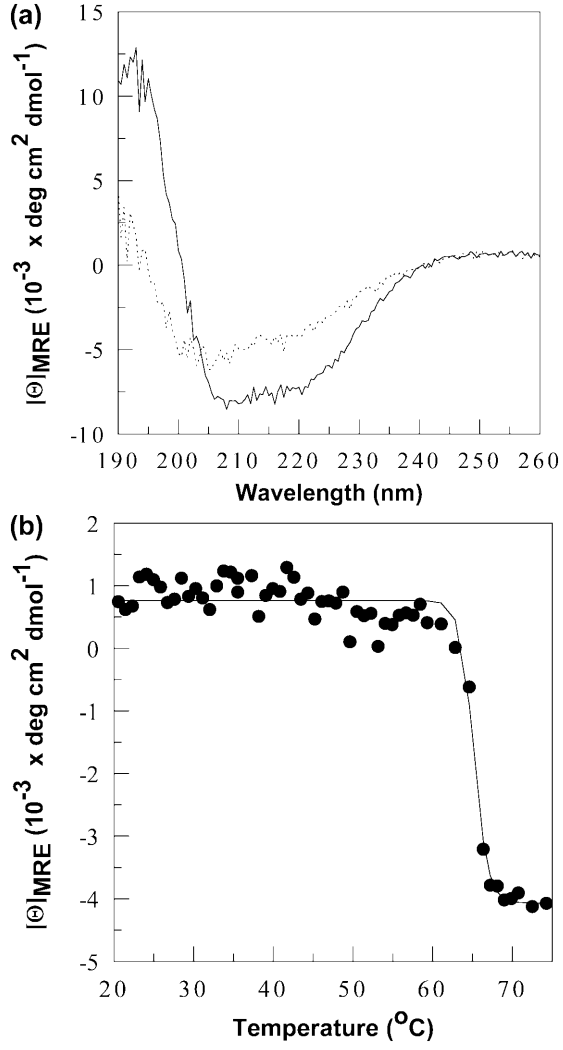

Fig. 1a, b. Thermal denaturation of wild-type Motif X by CD. **a** The wavelength scan at the two extreme temperatures (17 °C, *solid line* and 75 °C, *dotted line*) of the melting curve for wild-type Motif X. The major secondary structural element seen in the 17 °C spectrum is characteristic of predominantly β-sheet secondary structure. Note also that at wavelengths below approximately 195 nm there is a noticeable decrease in signal-to-noise, due to the exponentially increasing absorption of the sample at these wavelengths. Ellipticity was measured at 200 nm as a function of corrected temperature. The melting transition curve in **b** has a midpoint of 65 °C

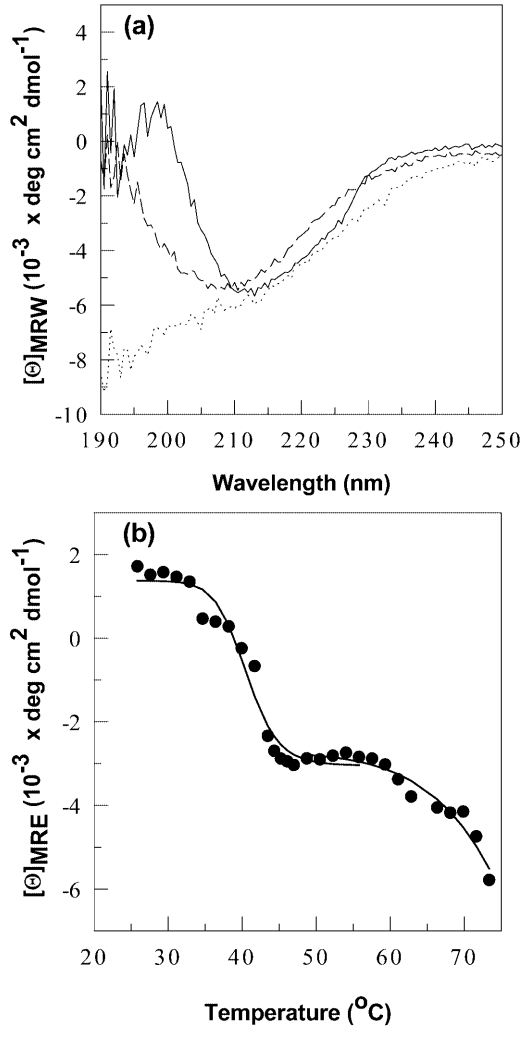

Fig. 2a, b. Thermal denaturation of mutant Motif X. **a** The wavelength scan at three temperatures (17 °C, *solid line*; 52 °C, *dashed line*; and 75 °C, *dotted line*) of the melting curve for mutant Motif X. Ellipticity was measured at 200 nm as a function of corrected temperature. The two melting transition curves in **b** have midpoints of 40 °C and > 65 °C

X was monitored over the range 17–75 °C at 200 nm to reflect the transition from β-sheet to random coil. Figure 1a shows the wavelength scans at the two extreme temperatures of the thermal denaturation curve for wild-type Motif X. There was a large decrease in the positive ellipticity in the range 205–190 nm, towards a negative ellipticity, and a smaller increase in the negative ellipticity in the range 230–205 nm, implying an increase of the fraction of random coil. However, the spectrum obtained for wild-type at 75 °C did not show a large negative peak at 200 nm that would be characteristic of random coil, indicating that, at these temperatures, the wild-type retained some β-sheet structure.

The thermal denaturation curve (Fig. 1b) shows the wild-type Motif X unfolded with a clear and sharp transition in secondary structure over a narrow temperature range of 58–68 °C. The midpoint of the sigmoidal transition (T_m) was 63 °C. This contrasts with the corresponding curve for the mutant protein (Fig. 2b), which exhibited a double unfolding transition over the range 34–74 °C. The first unfolding transition was fitted to a sigmoidal curve ($T_m = 40$ °C) and a second melting transition occurred above 57 °C but did not reach completion by 74 °C. The change in ellipticity at the first transition was similar for both proteins (a decrease in ellipticity of approximately 4.5×10^{-3} deg cm^2 dmol^{-1}), suggesting the unfolding of equivalent secondary structure components. This was also evident by comparing the full CD spectra. The 52 °C spectrum of the mutant protein in Fig. 2a exhibits the same features as the wild-type spectrum at 75 °C, implying similar secondary

structure at the endpoint of the first transition. Thus, the 23 °C shift in the first transition between the two protein forms implies a decrease in structural stability of mutant Motif X.

Sedimentation binding studies of Motif X to myosin filaments

The binding of wild-type and mutant Motif X to synthetic bovine cardiac myosin filaments was examined using a centrifugation binding assay (Okagaki et al. 1993). Figure 3 shows the protein composition of the pellet fractions when 2 μM myosin filaments were sedimented in the presence of 0–6 μM IgI Motif, either wild-type or mutant Motif X, or control Motif V. The myosin binding data for wild-type and mutant Motif X demonstrate that both sediment with myosin in a similar manner, while essentially no control Motif V binds to myosin (Fig. 4). The stoichiometry of binding at a 1:1 molar ratio is approximately 75% for both wild-type and mutant Motif X, and is also similar to that obtained previously for skeletal MyBP-C using the same centrifugation binding assay (Okagaki et al. 1993). Motif V is not predicted to bind to myosin (Okagaki et al. 1993).

The addition of up to a three-fold molar excess of IgI Motifs over myosin resulted in a slight increase in the binding of both Motif X samples, although at a three-fold molar excess, sedimentation remained in the range of 1.0–1.4 moles of Motif X per mole of myosin. There was no increase in the sedimentation of Motif V under the same conditions and the fraction of bound Motif X did not increase following longer incubation periods with the myosin filaments (to 4 h; data not shown). Notably, less than 1% of the wild-type or mutant Motif X sedimented in the absence of myosin filaments but greater than 95% of the myosin filaments sedimented under the same conditions.

Thus, these data show that the cardiac isoform of Motif X binds specifically to bovine cardiac myosin filaments, in the same manner to that seen previously for skeletal Motif X (Okagaki et al. 1993), and that the binding of Motif X to myosin is unaffected by the six amino acid duplication seen in the mutant Motif X.

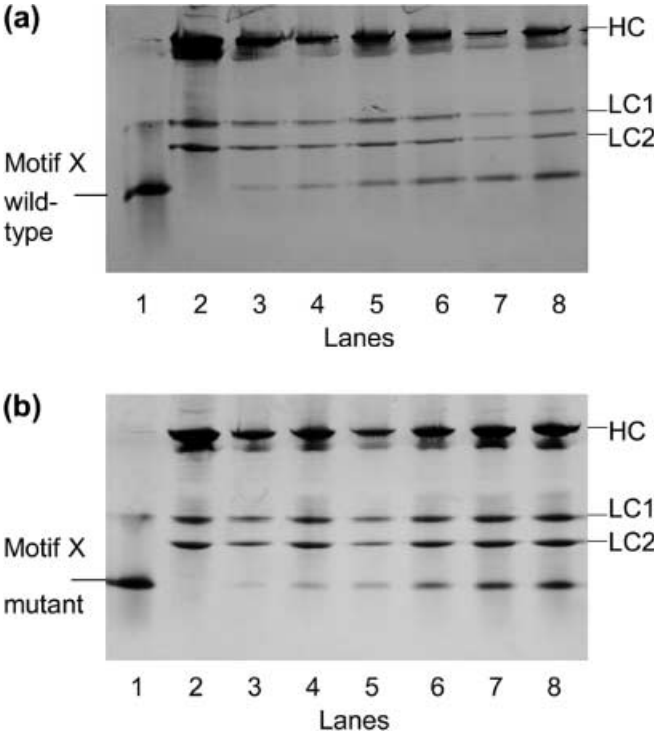

Fig. 3a, b. Binding of wild-type and mutant Motif X to synthetic bovine cardiac myosin filaments. Fractions containing different molar ratios of Motif X to myosin filaments were sedimented at 8400×*g* for 15 min. **a** Wild-type Motif X and **b** mutant Motif X sedimentation assays. The pellet fraction was analysed using 16.5% Tricine gel electrophoresis. The positions of the myosin heavy chain (HC), myosin light chains (LC1 and LC2) and Motif X are shown. In these experiments, myosin concentration was 2 μM. *Lane 1*: Motif X; *lane 2*: myosin filament; *lanes 3–8* contain 2 μM myosin filament with increasing concentrations of Motif X (0.5, 1.0, 1.5, 2.0, 4.0 and 6.0 μM, respectively). Note that, in *lanes 3–8*, both wild-type and mutant Motif X were observed to co-sediment with the myosin filaments

Molecular modeling

We modeled the wild-type and mutant forms of cardiac Motif X in order to predict structural changes that may occur within the mutant form of Motif X. Nine amino acid residues from skeletal Motif X that affect myosin binding have been identified using a mutagenesis/sedimentation assay (Miyamoto et al. 1999), suggesting surfaces of skeletal Motif X that may be involved in

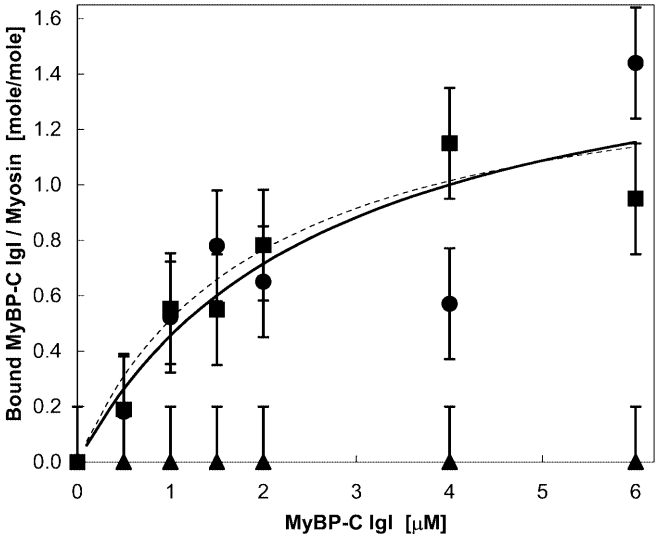

Fig. 4. Densitometric analysis of the pellet fraction from binding of the IgI domains Motif X and Motif V to synthetic bovine cardiac myosin filaments. The molar stoichiometry of MyBP-C IgI/mole of myosin was plotted against MyBP-C (mole) IgI added for triplicate data sets (±SE). Motif X wild-type curve (*circles*, *solid line*); Motif X mutant curve (*squares*, *dashed line*); Motif V curve (*triangles*)

binding to the LMM region of myosin. These residues were transposed onto our model of cardiac Motif X, to predict the possible spatial relationship of the FHC mutant duplication to the predicted myosin binding surfaces.

A wild-type Motif X structure was modeled by homology, based upon the coordinates of the 2.8 Å resolution structure of telokin, followed by energy minimization. The differences between the α-carbon coordinates of the structurally conserved segments of Motif X model and telokin, expressed as an RMSD, was 0.6 Å. These differences are considered small; for example, the RMSD between the smooth and skeletal isozymes of muscle myosin was 0.8 Å (Dominguez et al. 1998; Rayment et al. 1993). Additionally, energy minimization of the crystal structure of telokin results in a 0.6 Å RMSD between the telokin crystal structure and the minimized telokin structure. The absence of any significant rearrangement of the homology modeled Motif X structure upon energy minimization increases our confidence in the proposed structure.

A similar strategy was employed in modeling the Motif X mutants. Mutant Motif X contains a duplication of six residues (1248–1253; Gly-Gly-Ile-Tyr-Val-Cys). Since homology modeling depends on the choice of a template structure, we constructed three different mutant structures using either telokin or Motif X wild-type model as the template structure (Fig. 5). The structure built on telokin, using identical methods to that used for the wild-type, is shown in Fig. 5b, where comparison of structurally conserved regions between the mutant and wild-type model reveals RMSD = 0.9 Å. The two other structures begin with the wild-type Motif X model and thread the mutant sequence starting from either the N-terminus (Fig. 5a) or the C-terminus (Fig. 5c). As is clearly displayed in the overlays of the mutant and wild-type models, the position of the inserted loop is not unique; therefore, any interpretation of the insert structure would be misleading. More revealing is the increase of the RMSD in all mutant models by 0.9, 0.7 and 1.0 Å for models a, b and c, respectively. These differences represent the shift in position of the β-strand backbone, which is likely to result in a change in the strength of the ternary interactions between the strands. Such a change is consistent with the changes seen in the CD spectra of the mutant and the shift in the thermal denaturation curve of the first transition (Figs. 1b and 2b).

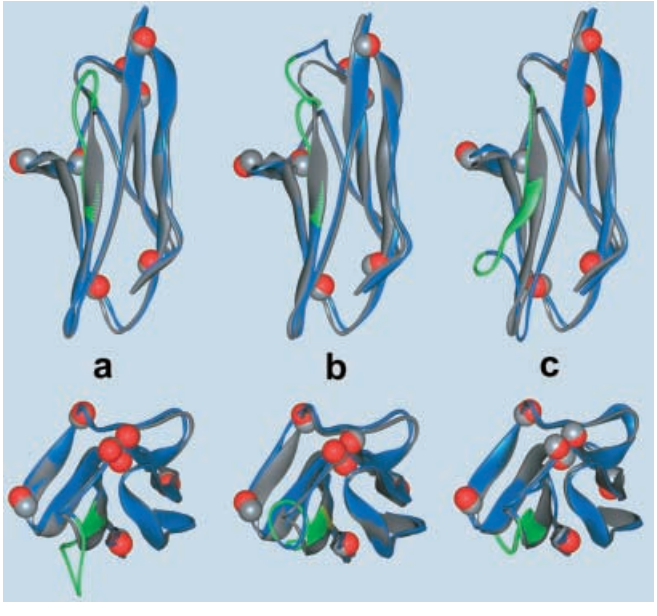

Fig. 5a–c. Molecular models of Motif X wild-type and mutant structures. Overlays of the wild-type and mutant structures built by homology building: **a** mutant built from the Motif X wild-type starting at the N-terminus; **b** Motif X mutant and wild-type modeled from telokin structure; **c** as in **a** but threading from the C-terminus. *Green* shows the original six amino acid residues and the mutant insert. *Red* shows the residues involved in myosin binding (Miyamoto et al. 1999)

Figure 5 also shows the locations of the key residues believed to be involved in myosin binding (Miyamoto et al. 1999). Irrespective of the actual structure of the inserted loop, comparison of the putative myosin binding sites in Fig. 5 (bottom row) reveals that: (1) the insert is on a protein surface not involved in myosin binding; and (2) the positions of residues implicated in myosin binding by site-directed mutagenesis (Miyamoto et al. 1999) do not change. The RMSD between the α-carbons of Val24, Pro30, Leu39, Glu43, Arg61, Lys62 and Arg91 [equivalent to Arg37, Lys43, Glu52, Asp56, Arg73, Arg74 and Arg103 in skeletal Motif X, respectively (Miyamoto et al. 1999)] was the same as the RMSD between the structurally conserved regions, in the range 0.7–1.0 Å. Thus, the movement of the myosin binding sites was the same as the movement of the whole structure and, most importantly, was very small. Most residues moved by 0.1–0.8 Å, except for Leu39 which was displaced by 1.2–1.6 Å, depending on the model.

In summary, the very small displacements of the myosin binding residues in the modeled structures of mutant Motif X provide a plausible structural explanation for the lack of change in myosin binding seen for mutant Motif X.

Discussion

The wild-type COOH-terminal immunoglobulin domain (Motif X) of human cardiac MyBP-C and an FHC-causing mutant form containing a six amino acid duplication (Watkins et al. 1995) were expressed and purified from a bacterial expression system. The data presented here show that both the recombinant wild-type and mutant Motif X domains fold into a β-sandwich secondary structure at physiological temperatures, consistent with the predicted IgI-type structure based on sequence homology comparisons. The structural stability of the mutant Motif X was found to be minimally decreased, as compared to that of the wild-type. The shift in the thermal denaturation temperature was

approximately 23 °C. The mutation did not detectably alter myosin binding, consistent with molecular modeling that predicts the mutant residues lie on a surface of Motif X that is not involved in myosin binding.

The atomic resolution structure of the Motif X of MyBP-C has not been determined. However, this domain shares a high degree of homology with members of the immunoglobulin superfamily, in particular the class of proteins termed IgI domains (Freiburg and Gautel 1996). Secondary structure predictions (Chou and Fasman 1978) and CD spectra both support this assignment. A similar IgI domain (m5 domain of titin) has been expressed in recombinant form, refolded using similar methods and the structure confirmed by NMR spectroscopy (Pfuhl and Pastore 1995). Thus, the high β-strand content seen in the CD spectra and the high thermal stability of the expressed Motif X makes us confident that the protein is folded in its native structure. While the thermal stability of the mutant Motif X was lower than the wild-type, the mutant thermal stability was still high, with the first transition, corresponding to only partial unfolding, occurring at 35 °C. Thus, we believe that slight structural instability of the mutant Motif X does not contribute to the pathogenesis of FHC.

Multi-phase denaturation has been observed for proteins belonging to the same IgI superfamily. An IgI domain of titin exhibits three transitions at 44, 46 and 53 °C (Politou et al. 1994a, 1994b). Titin is a fibrous elastic protein that is believed to be capable of stretch within the sarcomere, by first breaking weak inter-domain interactions and then by individual immunoglobulin domains unfolding (Means 1998; Tskhovrebova et al. 1997). The unfolding of titin at these relatively low temperatures indicates lower stability and may correlate with its function within the sarcomere (Politou et al. 1994a, 1994b, 1996). The higher stability of Motif X (first transition at 65 °C) might reflect its more passive role in the sarcomere, as there is no evidence to suggest that Motif X unfolds in response to stretch within the thick filament.

Myosin binding

The C-terminal region of skeletal MyBP-C (Motifs VII to X) binds to the LMM region of skeletal myosin (Moos et al. 1975; Starr and Offer 1978) and to titin (Freiburg and Gautel 1996; Furst et al. 1992; Koretz et al. 1993). Specifically, Motifs VII to X are required for correct incorporation into the C-zone of the sarcomere (Gilbert et al. 1996) and Motifs VIII to X are required for titin binding (Freiburg and Gautel 1996). However, skeletal Motif X alone can bind to the LMM region of skeletal myosin (Okagaki et al. 1993). Thus, the MyBP-C C-terminal region plays a role in filament formation in muscle (Okagaki et al. 1993). Our experiments confirm the binding of the cardiac isoform of recombinant Motif X to cardiac myosin filaments, implying a similar role for Motif X in filament formation in cardiac muscle to that seen in skeletal muscle. The stoichiometry of binding (0.75:1) was similar to that found previously for skeletal Motif X (0.7:1 Motif X:myosin) (Okagaki et al. 1993).

Our results also confirm that the mutant Motif X was capable of binding to filamentous myosin at a similar stoichiometry to the wild-type (0.75:1). Notably, the method we have used has proven to be sensitive for studying myosin/MotifX interactions (Miyamoto et al. 1999; Okagaki et al. 1993). This group demonstrated that the charge reversal of a single amino acid in Motif X at a putative myosin binding site was sufficient to abolish all myosin binding. These data indicate the sensitivity of this method for detecting perturbations in myosin binding resulting from mutations within Motif X. Thus, we conclude that the duplication-mutation in Motif X found in FHC does not alter myosin binding.

Our structural modeling data, based on the results of mutagenesis mapping experiments using skeletal isoforms (Miyamoto et al. 1999), provide an explanation for this result. Miyamoto showed that seven charged ''mapping'' residues, predicted to be involved in myosin binding on the basis of sequence conservation, abolished myosin binding when individually mutated. On the other hand, mutation of two other ''mapping'' residues did not alter binding. Our modeling of Motif X revealed that the ''mapping'' residues that interfered with myosin binding were located on two ''faces'' formed by strands B and E and by strand C of the β-sandwich (Fig. 5). On the other hand, the two ''mapping'' residue mutations that were found not to alter myosin binding mapped to a different surface of Motif X (centered on the F strand). Notably, one of these skeletal ''mapping'' residue mutations corresponded with a residue in the FHC mutant insert (cardiac Gly68; skeletal Gly80) and the other lies in the C strand directly adjacent to the insert on the F strand (cardiac Lys36; skeletal Asn49). Thus, the FHC six amino acid duplication mutation studied in this paper is in the middle of a region of Motif X that our modeling predicts is not involved in myosin binding (Fig. 5).

Additionally, our modeling predicts that the relative locations of known key myosin binding residues are not significantly altered by the FHC mutant insert (RMSD between the α-carbons of key residues 0.7–1.0 Å) compared to the wild-type Motif X model. Taken together, these data are consistent with the binding study results obtained here for mutant Motif X, namely that the FHC mutant duplication does not alter myosin binding, probably because it is neither located within, nor does it alter, the myosin binding surfaces.

The question that then arises is: how does the mutant insert in Motif X alter the function of MyBP-C sufficiently to cause the sarcomeric dysfunction that results in FHC in affected family members? The simplest hypothesis that emerges from these data is that the mutation affects some function of Motif X other than myosin binding. Two possible functional interactions of Motif X that could be altered by the duplication-mutation are

titin binding (Furst et al. 1992) or an alteration in an interaction that may occur with the adjacent Motif IX. These two possibilities are the subject of future research. Finally, we cannot exclude the possibility that the decrease in the structural stability of mutant Motif X that we measured may be sufficient to perturb MyBP-C function. For example, if significant strain was applied to MyBP-C during binding to the S-2 region of myosin, then mutant Motif X may be induced to unfold.

At the genetic level, mechanisms to explain autosomal dominant modes of inheritance include haploinsufficiency or a dominant-negative effect. Truncation mutations in MyBP-C, where the myosin and titin binding domains are lost, probably affect sarcomeric function by a haploinsufficiency mechanism, where truncated MyBP-C fails to incorporate into the sarcomere (Redwood et al. 1999). Our data show that the duplication mutation in Motif X does not alter myosin filament binding, making a haploinsufficiency mechanism unlikely and emphasizing the probability that this Motif X mutation alters a key function of MyBP-C, probably through a dominant-negative mechanism.

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