Localization of the binding site of the C-terminal domain of cardiac myosin-binding protein-C on the myosin rod

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cMyBP-C [cardiac (MyBP-C) myosin-binding protein-C)] is a sarcomeric protein involved both in thick filament structure and in the regulation of contractility. It is composed of eight IgI-like and three fibronectin-3-like domains (termed C0–C10). Mutations in the gene encoding cMyBP-C are a principal cause of HCM (hypertrophic cardiomyopathy). cMyBP-C binds to the LMM (light meromyosin) portion of the myosin rod via its C-terminal domain, C10. We investigated this interaction in detail to determine whether HCM mutations in β myosin heavy chain located within the LMM portion alter the binding of cMyBP-C, and to define the precise region of LMM that binds C10 to aid in developing models of the arrangement of MyBP-C on the thick filament. In co-sedimentation experiments recombinant C10 bound full-length LMM with a K_d of 3.52 μM and at a stoichiometry of 1.14 C10 per LMM. C10 was also shown to bind with similar affinity to LMM containing either the HCM mutations A1379T or S1776G, suggesting that these HCM mutations do not perturb C10 binding. Using a range of N-terminally truncated LMM fragments, the cMyBP-C-binding site on LMM was shown to lie between residues 1554 and 1581. Since it had been reported previously that acidic residues on myosin mediate the C10 interaction, three clusters of acidic amino acids (Glu^{1554}/Glu^{1555}, Glu^{1571}/Glu^{1573} and Glu^{1578}/Asp^{1580}/Glu^{1581}/Glu^{1582}) were mutated in full-length LMM and the proteins tested for C10 binding. No effect of these mutations on C10 binding was however detected. We interpret our results with respect to the localization of the proposed trimeric collar on the thick filament.

Key words: cardiomyopathy, co-sedimentation, light meromyosin, myosin-binding protein-C, myosin rod, thick filament.

INTRODUCTION

MyBP-C (myosin-binding protein-C) is a modular protein with the cMyBP-C (cardiac MyBP-C) consisting of 11 domains (referred to as C0–C10), eight of which are IgI-like with the other three being fibronectin-3 domains. MyBP-C has been hypothesized to contribute to myosin filament structure through an interaction at its C-terminus with the filament backbone, and to have a role in the regulation of contraction via an interaction at its N-terminus with the subfragment-2 region of myosin (reviewed in [1]). The precise arrangement and function of MyBP-C in the sarcomere are yet to be fully elucidated, although a trimeric collar arrangement around the thick filament has been proposed [2]. MyHCs (myosin heavy chains) form the bulk of the myosin molecule and are made up of globular heads, which take part in the cross-bridge cycle, and an α-helical coiled-coil rod region. The formation of this coiled-coil structure is mediated by the seven-amino-acid (heptad) motif that repeats throughout the rod sequence. The *a* and *d* positions of the heptad repeat (*abcdefg*) are typically small hydrophobic residues that stabilize the interaction of the two helical chains [3]. There is also a marked 28-residue periodicity defined by regular charge periodicity [4]. The LMM sequence periodicity is interrupted by four single amino acids, termed skip residues, which are believed to contribute to the flexibility of the myosin rod [5]. The myosin rod can be separated by proteolytic cleavage into myosin subfragment-2 (S2) and LMM (light meromyosin), which forms the backbone of the thick filament.

It has been well documented that the C-terminal C10 domain of MyBP-C is responsible for binding to the LMM portion of the myosin rod [6–9] and that this interaction, along with the presence of other MyBP-C C-terminal domains, is necessary for proper localization of MyBP-C in the sarcomere [10,11]. It is also likely that the correct localization of MyBP-C on the thick filament is necessary for MyBP-C-mediated regulation of contraction. Previous work examining the interaction between MyBP-C and LMM has suggested a binding stoichiometry of 0.6–0.7:1 [6–9], and at less than 1:1, this suggests a single binding site for C10 on LMM. However, while the binding motifs for other thick filament-associated proteins titin and myomesin have been located [12,13], the specific binding site for MyBP-C has not been reported.

The autosomal dominant disorder HCM (hypertrophic cardiomyopathy) is caused by mutations in genes encoding contractile proteins. The two most commonly affected genes are those encoding cMyBP-C and βMyHC, each accounting for at least one third of reported mutations [14,15]. Many of the cMyBP-C mutations are predicted to result in truncated proteins lacking the C10 domain and these mutants are unlikely to incorporate correctly into the thick filament [1]. Most βMyHC mutations cause single amino acid substitutions within the myosin head and these result in alterations in motor function [16]. However, a few missense HCM mutations have been shown to lie in the filament-forming LMM region of βMyHC and these are most unlikely to have a direct impact on crossbridge activity. We hypothesize that at least some of the βMyHC LMM mutations may affect cMyBP-C attachment to the thick filament and hence act in a similar way to truncation mutations in cMyBP-C.

In order to test our hypothesis, we examined the ability of two HCM βMyHC LMM mutations identified in our laboratory [17] to bind the C10 domain of cMyBP-C. Furthermore, we have defined the location of C10 binding on LMM using a series of

Abbreviations used: DTT, dithiothreitol; MyBP-C, myosin-binding protein-C; cMyBP-C, cardiac MyBP-C; HCM, hypertrophic cardiomyopathy; LMM, light meromyosin; MyHC, myosin heavy chain.

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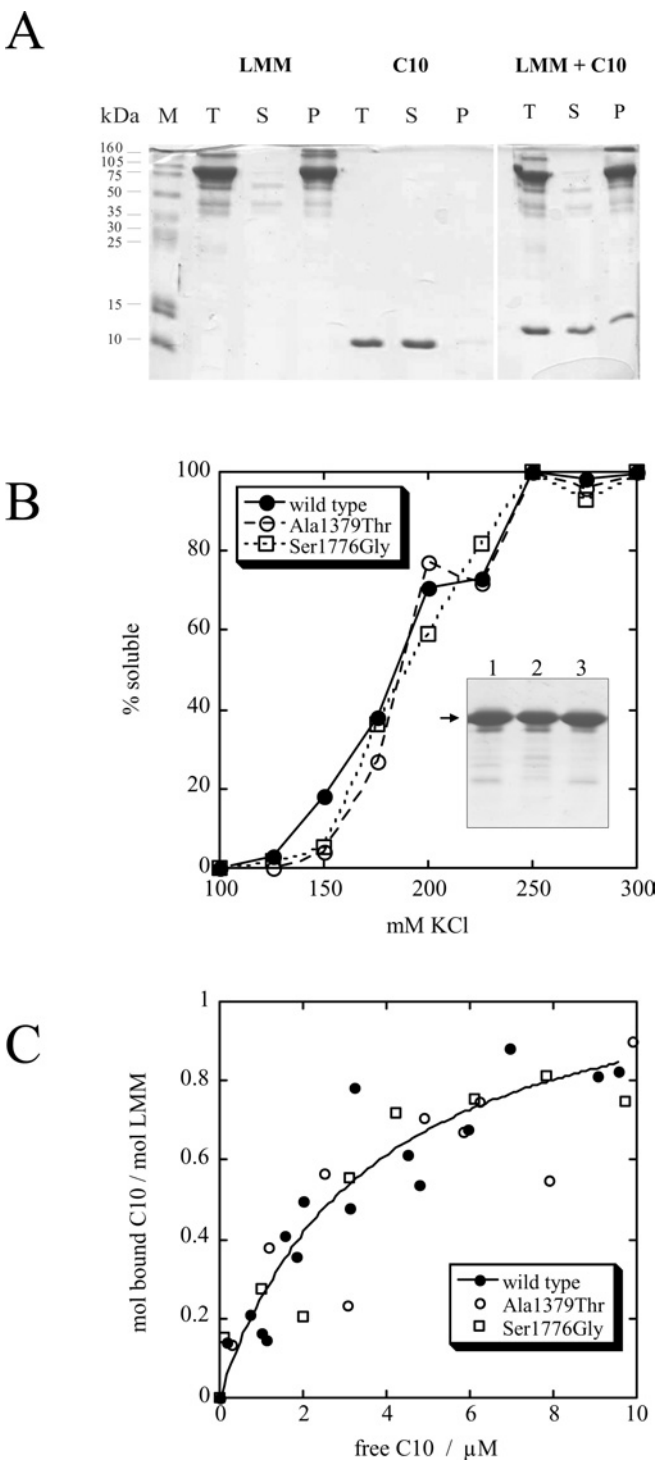

Figure 1 Initial characterization of the C10–LMM interaction

(**A**) The interaction of purified wild-type LMM and C10. Sedimentation experiments were carried out using 6 μM wild-type LMM and 6 μM C10. Aliquots of the mixtures before centrifugation [T (total)] and of the pellets (P) and supernatants (S) after centrifugation were analysed by SDS/PAGE and the gels stained with Coomassie Blue. The control data demonstrate that under the standard conditions used, LMM completely sediments and the C10 fragment in the absence of LMM remains soluble. Also shown are molecular mass markers (M). (**B**) Measurement of the salt dependence of solubility of wild-type and HCM mutant LMM. LMM (6 μM) [either wild-type (1) or the A1379T (2) or S1776G (3) mutants; purified proteins shown in inset] were sedimented under standard conditions in the presence of between 100 and 300 mM KCl. The amount remaining in the supernatant after centrifugation was determined by SDS/PAGE and scanning densitometry and plotted as a function of KCl concentration. Each LMM had a similar solubility profile with midpoint at approx. 175 mM KCl. (**C**) Quantification of the interaction of C10 and LMM. Co-sedimentation experiments were carried out using 4 μM LMM (either wild-type or the A1379T or S1776G mutants) and 0–12 μM C10. Aliquots of the mixtures before centrifugation and of the pellets and supernatants after centrifugation were analysed by SDS/PAGE and the gels stained with Coomassie Blue. The concentrations of bound and free C10 were calculated by scanning densitometry of the stained gels. The data for each LMM fragment were fitted to the equation, Bound $= B_{max} \times$ free/($K_d +$ Free), using Kaleidagraph (Synergy Software); the fitted curve for the wild-type data is shown.

deletion constructs in co-sedimentation experiments, and interpret these data with respect to the position of the proposed MyBP-C trimeric collar on the thick filament.

EXPERIMENTAL

Cloning, expression and purification of MyBP-C C10 and LMM deletion constructs

The C10 domain sequence (residues 1180–1274) was amplified from full-length human cMyBP-C cDNA and inserted into the bacterial expression vector pMW172 [18]. Transformed BL21 (DE3)pLysS cells were grown and induced with IPTG at 37 °C according to standard protocols [19]. Cells were lysed in the presence of protease inhibitors in 50 mM Tris/HCl (pH 7.5), 25 % (w/v) sucrose, 300 mM NaCl, 1 mM EDTA and 1 mM 2-mercaptoethanol by sonication. Soluble protein was purified from the lysis supernatant by cation-exchange chromatography on a MonoS column (Amersham Biosciences).

The full-length LMM DNA sequence (amino acids 1185–1935) was amplified from a human cardiac cDNA library (Clontech) and LMM deletion constructs were engineered from this initial clone using PCR. Missense mutations within the LMM sequence were inserted using a two-step PCR-based protocol. All LMM sequences were inserted into pET-28a (Novagen) and the proteins were overexpressed in BL21(DE3)pLysS cells. The harvested cell pellets were resuspended, in the presence of protease inhibitors, in 1 M KCl, 50 mM Tris/HCl (pH 7.5), 25 % sucrose, 1 mM EDTA and 1 mM 2-mercaptoethanol and lysed by sonication. The lysis supernatant was heated to 90 °C, rapidly cooled and then centrifuged at 45 000 ***g*** for 20 min at 4 °C. At high ionic strength, LMM does not denature upon heating and remains in soluble form [20]. The supernatant was filtered through glass wool and then diluted 10-fold with cold deionized water to allow LMM to polymerize. Upon centrifugation, LMM filaments formed a pellet which was resuspended in 2–5 ml of 1 M KCl, 20 mM imidazole (pH 7.0) and 1 mM DTT (dithiothreitol).

Co-sedimentation experiments

Proteins were dialysed into myosin binding buffer (100 mM KCl, 20 mM imidazole, pH 7.0 and 1 mM DTT). Precise quantities of protein were mixed in various ratios, or individually as control experiments, to a total volume of 100 μl, and ultracentrifuged at 80 000 rev./min for 10 min at 4 °C using a TLA-100 rotor (Beckman). Protein samples (30 μl) were taken from the total mixture (pre-centrifugation) and soluble fraction, and pelleted protein was resuspended in 93 μl of 1 × SDS/PAGE loading buffer. Samples were analysed by SDS/PAGE and the gels stained with Coomassie Blue. When required, protein bands were quantified by scanning densitometry and the data were fitted to the equation, Bound $= B_{max} \times$ free/($K_d +$ Free), using Kaleidagraph (Synergy Software). Three separate sets of data were generated and fitted for each LMM and the K_d and B_{max} expressed as means ± S.E.M.

For determination of the salt dependence of the solubility of myosin fragments, myosin binding buffer with KCl concentrations of between 100 and 300 mM were used.

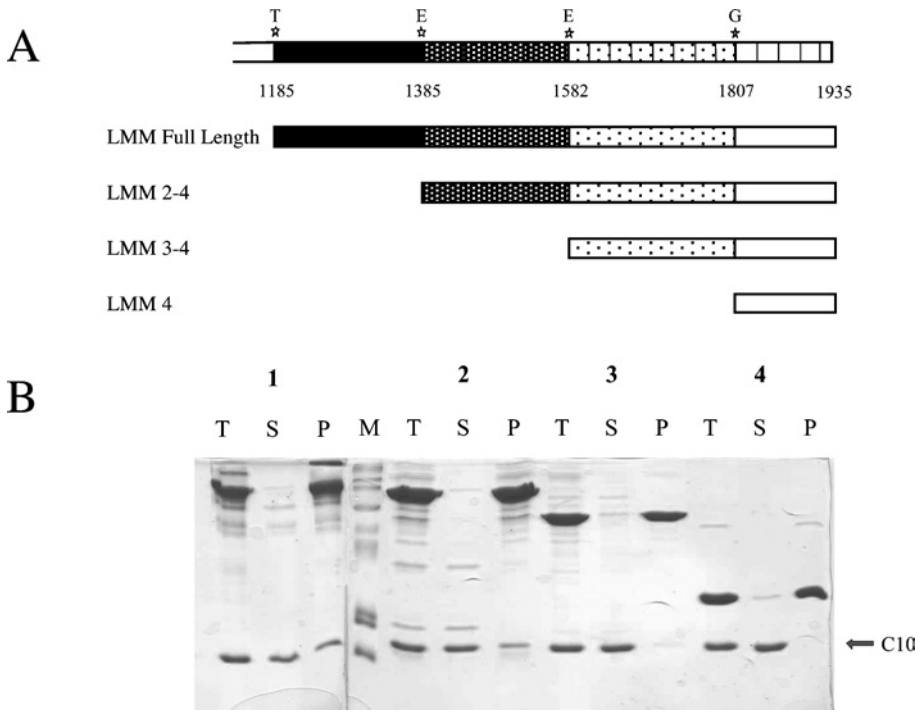

Figure 2 Interaction of C10 with LMM deletion fragments

(**A**) Cartoon showing full-length LMM, LMM 2–4, LMM 3–4 and LMM 4. The recombinant full-length LMM fragment extends from residues 1185 to 1935 of human βMyHC. The heptad repeat is broken at four places by the presence of single additional acids (so-called 'skip residues') and these are indicated by asterisks. The sequence can be further subdivided based on charge distribution into 28-amino-acid repeats [4] as indicated. LMM 2–4 = residues 1385–1935; LMM 3–4 = 1582–1935; LMM 4 = 1807–1935. (**B**) Co-sedimentation of C10 with full-length LMM, LMM 2–4, LMM 3–4 and LMM 4. All proteins were at 6 μM and co-sedimentation took place in myosin binding buffer at 250000 ***g*** for 10 min at 4 °C. (1) LMM + C10; (2) LMM 2–4 + C10; (3) LMM 3–4 + C10; (4) LMM 4 + C10. T = total, pre-centrifugation; S = supernatant, post-centrifugation; P = pellet, post-centrifugation.

RESULTS

The interaction of C10 with LMM and the influence of βMyHC HCM mutations

The binding of recombinant C10 domain to myosin was carried out using a recombinant LMM fragment of human βMyHC corresponding to residues 1185–1935, that is from the first skip residue to the C-terminus. Affinity measurements were performed by co-sedimentation in myosin binding buffer containing 100 mM KCl using 4 μM LMM and 0–12 μM C10. LMM forms filaments at this ionic strength and under the sedimentation conditions used all of the LMM is found in the pellet (Figures 1A and 1B), whereas the C10 domain remains soluble (Figure 1A). These experiments showed that C10 bound with a K_d of 3.52 ± 0.28 μM ($n = 3$) and at a maximum stoichiometry of 1.14 ± 0.09 C10/LMM ($n = 3$), consistent with reports of a single binding site for C10 on myosin [6,7,9] (Figure 1C). Similar experiments were carried out with full-length LMM containing either A1379T or S1776G, both mutations reported to cause HCM [17]. These proteins were purified as for wild-type LMM (Figure 1B, inset) and the mutations were found not have a significant effect on filament solubility (Figure 1B). C10 bound each with similar affinity to wild-type LMM [A1379T $K_d = 3.08 \pm 0.32$ μM ($n = 3$), S1776G $K_d = 3.82 \pm 0.16$ μM ($n = 3$)] and with stoichiometry again close to unity (1.02 ± 0.07 and 1.16 ± 0.11 C10/LMM for A1379T and S1776G respectively), thus suggesting that these HCM mutations do not affect cMyBP-C attachment to the thick filament (Figure 1C).

C10 binding to LMM deletion fragments

Location of the region on myosin responsible for C10 binding was attempted by generating a series of recombinant N-terminally truncated LMMs. All of the expressed fragments contained the C-terminal 'assembly competence domain' (amino acids 1871–1899), which is necessary for proper formation of myosin filaments at physiological ionic strengths [21], and so could be used in co-sedimentation experiments. Initially, the skip residues in the LMM sequence were used as markers to define the N-terminus of the deletion fragments and the expressed fragments were termed LMM 2–4 (starting at the second skip residue Glu^{1385}), LMM 3–4 (starting at the third skip residue Glu^{1582}) and LMM 4 (starting at the final skip residue Gly^{1807}) (Figure 2A). Co-sedimentation experiments were carried out using 6 μM C10 and 6 μM of the LMM fragments. C10 was shown to co-sediment with full-length LMM and LMM 2–4, but not with LMM 3–4 or LMM 4 (Figure 2B). Titration experiments, carried out using constant 4 μM LMM 2–4, showed that the K_d of C10 for this fragment was 3.35 ± 0.17 μM ($n = 3$), not significantly different from full-length LMM.

The above data suggest that the C10-binding site lies between the second and third skip residues (amino acids 1385 and 1582) of human βMyHC. To obtain finer definition of the region involved, further N-terminal truncations of LMM were designed by successive deletion of 28-amino-acid repeats between the second and third skip residues (Figure 3A). Initially, a deletion construct was designed with the N-terminus at LMM residue 1497 (LMM 2.5–4) and it was found that C10 did indeed bind to this fragment

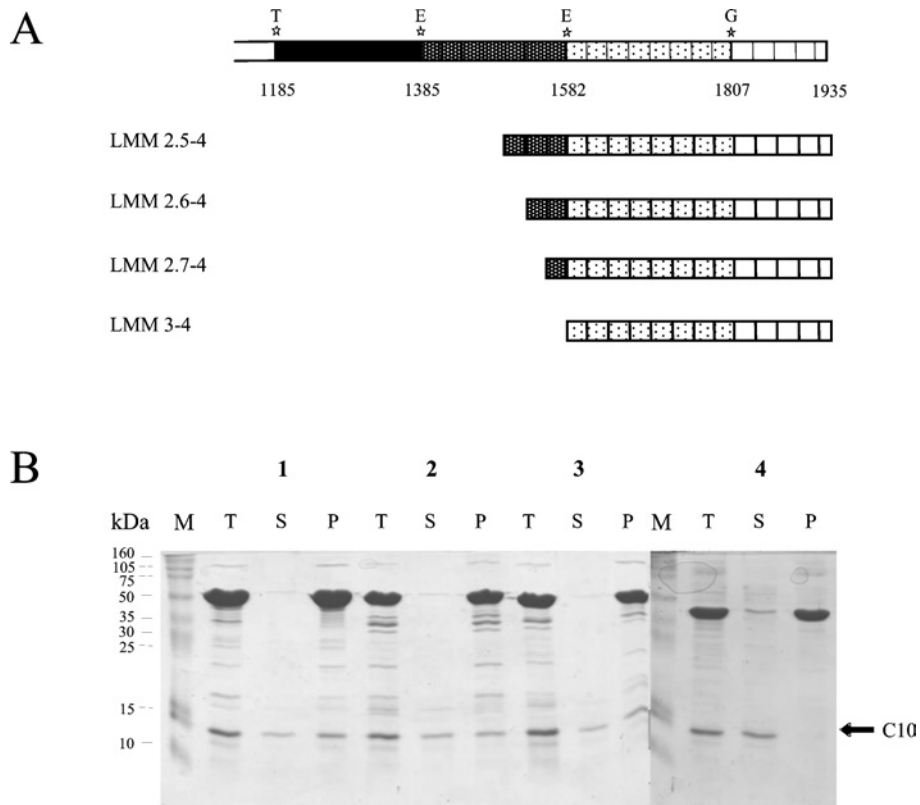

Figure 3 Fine mapping of the C10-binding site of LMM

(**A**) Cartoon showing LMM 2.5–4, LMM 2.6–4, LMM 2.7–4 and LMM 3–4. Further N-terminal deletions of LMM were designed by successive deletion of 28-amino-acid repeats between the second and third skip residues. LMM 2.5–4 = residues 1498–1935; LMM 2.6–4 = 1526–1935; LMM 2.7–4 = 1554–1935; LMM 3–4 = 1582–1935. (**B**) Co-sedimentation of C10 with LMM 2.5–4, LMM 2.6–4, LMM 2.7–4 and LMM 3–4. All proteins were at 6 μM and co-sedimentation took place in myosin binding buffer at 250000 ***g*** for 10 min at 4°C. (1) LMM 2.5–4 + C10; (2) LMM 2.6–4 + C10; (3) LMM 2.7–4 + C10; (4) LMM 3–4 + C10. T = total, pre-centrifugation; S = supernatant, post-centrifugation; P = pellet, post-centrifugation.

(Figure 3B). The C10-binding site therefore lies to the C-terminus of this residue, so deletion fragments LMM 2.6–4 and 2.7–4 were designed and prepared. The results in Figure 3(B) show that C10 does bind to both LMM 2.6–4 and LMM 2.7–4, and re-confirms that binding does not occur to LMM 3–4. This suggests that the binding site for C10 is between amino acids 1554 and 1581.

Identification of the residues involved in the interaction

Alignment of the identified C10-binding sequence of βMyHC LMM with the equivalent region from other mammalian species, non-mammalian species and different muscle isoforms shows this sequence to be highly conserved (Figure 4). There is a very high degree of identity with sequences from other mammalian species (e.g. 100 % identity with rat and mouse α-myosins) and also of conservative replacement of amino acids in non-mammalian species and non-striated muscle isoforms, although this high sequence homology was not unique to the 28-amino-acid region of interest (the same level of homology is also seen in the flanking repeats). A previous study has indicated that acidic residues on myosin mediate its interaction with the C10 domain [22]. In order to investigate whether acidic groups within the defined region were contributing to the binding, three different clusters of acidic amino acids occurring in surface positions of the heptad repeat (Glu^{1554}/Glu^{1555}, Glu^{1571}/Glu^{1573} and Glu^{1578}/Asp^{1580}/Glu^{1581}/Glu^{1582}) were individually mutated to their amide equivalents in full-length LMM and the proteins tested for C10 binding. Each mutant LMM sedimented as wild-type under the standard conditions used (results not shown). In co-sedimentation assays with C10, the measured affinities of each mutant LMM did not significantly vary from wild-type: K_d values for the Glu^{1554}/Glu^{1555}, Glu^{1571}/Glu^{1573} and Glu^{1578}/Asp^{1580}/Glu^{1581}/Glu^{1582} were 4.01 ± 0.31, 3.40 ± 0.11 and 3.80 ± 0.24 μM respectively ($n = 3$ in each case) compared with 3.52 ± 0.28 μM for wild-type. This suggests that the negative charge provided individually by these clusters does not contribute significantly to C10 binding.

DISCUSSION

One aim of this study was to test the hypothesis that HCM mutations within the LMM region of βMyHC may interfere with the attachment of cMyBP-C to the thick filament. By measuring the affinity of soluble recombinant C10 domain for filamentous LMM *in vitro*, it was found that wild-type LMM bound with a similar affinity to the A1379T and S1776G HCM mutants. This suggests that these particular HCM mutations do not act by reducing cMyBP-C–myosin interactions and are more likely to cause aberrant thick filament structure or stability. Furthermore, since our initial report in 2002 [17] of these two HCM mutations in the βMyHC LMM domain, at least another 17 missense HCM mutations in this region have been reported [14,23–25] as well as at least four mutations that cause dilated cardiomyopathy [26,27]. Interestingly, not one of these mutations affects a residue

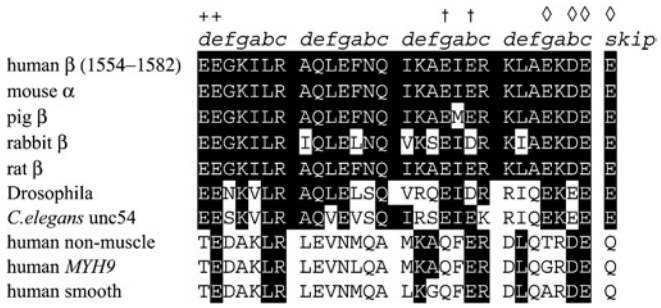

Figure 4 Alignment of the C10-binding sequence in a range of species and muscle MyHC isoforms

Residues identical with the human βMyHC sequence (residues 1554–1582) are boxed. The residues mutated in the present study are indicated above the sequence: '+' indicates Glu^{1554} and Glu^{1555}; †, amino acids Glu^{1571} and Glu^{1573}; ◇, Glu^{1578}, Asp^{1580}, Glu^{1581} and Glu^{1582}.

within the defined 28-amino-acid binding site, suggesting that cardiomyopathy mutations in LMM in general do not interfere with MyBP-C binding.

The expression and purification of deletion fragments of the LMM portion of the myosin rod and the filamentous nature of LMM at low ionic strengths have allowed determination of the region of LMM that binds C10 by co-sedimentation assays. The binding site is contained within a 28-amino-acid sequence, 1554–1581, immediately N-terminal to the third skip residue in the LMM sequence. This lies within the broadly defined myomesin and M-protein binding region (residues 1505–1673 of βMyHC) [12] but is distinct from the proposed titin-binding site (amino acids 1815–1831) [13]. C10 and myomesin or M-protein are unlikely to bind to the same LMM molecule, as the latter are structural proteins in the M band of the sarcomere, whereas MyBP-C occurs in the C zone. The residues of the proposed site are highly conserved within mammalian striated myosins (Figure 4). Earlier evidence had been put forward to suggest that the C10–myosin interaction involves acidic residues on LMM [22]; however, the mutation of residues Glu^{1554}, Glu^{1555}, Glu^{1571}, Glu^{1573}, Glu^{1578}, Asp^{1580}, Glu^{1581} and Glu^{1582} had no effect on C10 affinity, suggesting that these particular acidic groups are not involved.

The experiments carried out assume that all the deletion fragments form similar structures under the experimental conditions in such a way that C10 could bind to the protein given the presence of a binding site. All deletion constructs appeared to retain their insolubility in low-salt buffers, as none were detected in significant quantities in the soluble supernatant after centrifugation (see Figure 2). However, ordered filament formation may also be a factor necessary for C10 binding, and the insolubility of the fragments does not necessarily mean that ordered filaments are formed. It is possible that constructs LMM 3–4 and LMM 4, despite containing the assembly competence domain, could not form these filaments, and thus not bind C10. Myosin rod deletion constructs beginning at amino acid 1661 have been seen to form paracrystals; however, constructs beginning at amino acid 1758 could not [21]. The N-terminus of both these constructs falls between skip residues 3 and 4, suggesting that LMM 3–4 could form paracrystals in an equal manner to full-length LMM, yet C10 does not bind to this fragment, supporting our proposed binding site.

These data allow the prediction of the relative position of C10, and thus the MyBP-C collar, on the thick filament, and this will be useful in the development of models of thick filament structure. The precise position of titin, which interacts with both myosin and cMyBP-C, remains to be elucidated. This protein lies parallel to the myosin filament [28], with its 11×11 domain super-repeat of IgI-like and fibronectin-3 domains present in the A band [29,30]. Titin binds to MyBP-C domains C9 and/or C10 [31], both of these domains being arranged circumferentially to the MyBP-C–LMM binding site according the trimeric collar model [2]. Although it is known that it is the first domain in each titin 11×11 domain super-repeat that binds to MyBP-C [31], there has been no specific domain within the repeat identified as being responsible for myosin binding. Further work using, for example, antibody staining should be carried out to clarify this arrangement.

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