Myosin Binding Protein C, a Phosphorylation-Dependent Force Regulator in Muscle That Controls the Attachment of Myosin Heads by Its Interaction With Myosin S2

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Abstract—Myosin binding protein C (MyBP-C) is one of the major sarcomeric proteins involved in the pathophysiology of familial hypertrophic cardiomyopathy (FHC). The cardiac isoform is *tris*-phosphorylated by cAMP-dependent protein kinase (cAPK) on β-adrenergic stimulation at a conserved N-terminal domain (MyBP-C motif), suggesting a role in regulating positive inotropy mediated by cAPK. Recent data show that the MyBP-C motif binds to a conserved segment of sarcomeric myosin S2 in a phosphorylation-regulated way. Given that most MyBP-C mutations that cause FHC are predicted to result in N-terminal fragments of the protein, we investigated the specific effects of the MyBP-C motif on contractility and its modulation by cAPK phosphorylation. The diffusion of proteins into skinned fibers allows the investigation of effects of defined molecular regions of MyBP-C, because the endogenous MyBP-C is associated with few myosin heads. Furthermore, the effect of phosphorylation of cardiac MyBP-C can be studied in a defined unphosphorylated background in skeletal muscle fibers only. Triton skinned fibers were tested for maximal isometric force, Ca^{2+}/force relation, rigor force, and stiffness in the absence and presence of the recombinant cardiac MyBP-C motif. The presence of unphosphorylated MyBP-C motif resulted in a significant (1) depression of Ca^{2+}-activated maximal force with no effect on dynamic stiffness, (2) increase of the Ca^{2+} sensitivity of active force (leftward shift of the Ca^{2+}/force relation), (3) increase of maximal rigor force, and (4) an acceleration of rigor force and rigor stiffness development. *Tris*-phosphorylation of the MyBP-C motif by cAPK abolished these effects. This is the first demonstration that the S2 binding domain of MyBP-C is a modulator of contractility. The anchorage of the MyBP-C motif to the myosin filament is not needed for the observed effects, arguing that the mechanism of MyBP-C regulation is at least partly independent of a "tether," in agreement with a modulation of the head-tail mobility. Soluble fragments occurring in FHC, lacking the spatial specificity, might therefore lead to altered contraction regulation without affecting sarcomere structure directly. **(*Circ Res*. 2000;86:51-58.)**

Key Words: myosin binding protein C ■ familial hypertrophic cardiomyopathy ■ protein phosphorylation ■ contraction regulation

Familial hypertrophic cardiomyopathy (FHC) is caused by mutations in muscle proteins, the known ones of which are sarcomeric proteins. Mutations in the cardiac myosin binding protein C (MyBP-C) gene on chromosome 11 are a frequent cause of FHC (reviewed in Reference 1; see also References 2–8). Despite this obvious importance in the pathology of hereditary cardiac disease, very little is known about the physiological function of this protein. MyBP-C is a modular muscle protein of the intracellular immunoglobulin superfamily[1,9] (Figure 1A), which is expressed in at least three isoforms, its cardiac isoform being strictly specific for heart muscle in mammals.[10,11] The C-terminal region interacts with the light meromyosin portion (LMM) of myosin as well as with titin, thus anchoring the protein to the thick filament shaft and specifying its sarcomeric localization.[1]

Cardiac MyBP-C is phosphorylated in a dynamic way by cAMP-dependent protein kinase (cAPK), suggesting a role in the β-adrenergic regulation of muscle contraction.[12–17] Phosphorylation occurs at three sites in an MyBP-C–specific domain in the N-terminal region.[17] This 100-residue region, the MyBP-C motif, is highly conserved between all isoforms of MyBP-C and between species.[17,18] N-terminal cardiac MyBP-C fragments are directed to the A band in neonatal rat cardiomyocytes.[18,19] Recently, we could show that the MyBP-C motif binds to the proximal 126 residues of the myosin S2 segment, close to the lever arm domain of the myosin head.[18] This segment of myosin S2 is identical between all sarcomeric myosin isoforms and vertebrate species, and the interaction with MyBP-C is independent of the MyBP-C, or myosin isoform.[18] We

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could also recently show that the phosphorylation of cardiac MyBP-C abolishes the interaction with S2,[20] for the first time identifying the molecular switch that is controlled by MyBP-C phosphorylation. It has been proposed that the interaction with myosin S2 could modulate the head-tail mobility of the two-headed sarcomeric myosin,[18] on the basis of the observation that antibody F_{ab} fragments against the same region of S2 affect the movement of the myosin heads,[21] which are sterically constrained.[22]

Given that most MyBP-C mutations that cause FHC are predicted to result in N-terminal fragments of the protein that contain the S2 binding site,[1] it should be important for the molecular understanding of the possible effects of these mutants to investigate the physiological function of the N-terminal regulatory domain of MyBP-C, especially whether its binding site on myosin S2 may have an effect on contraction properties.

A recently described transgenic animal model,[23] in which a truncated MyBP-C molecule was expressed in *trans* to the two normal alleles, resulted in myofibril disorders similar to those observed in FHC. Physiological experiments showed a leftward shift of the pCa/force relation and a depression of maximal Ca^{2+}-activated force. However, it is hard to attribute the changes in contractility observed in such a model to either the myofibril disarray or a direct effect of the truncated MyBP-C on contractility. Furthermore, adaptive changes in protein composition or their phosphorylation states may occur in vivo, which can also contribute significantly to changes in contractility.

We therefore aimed to perform a study on the direct effects on contractility of the S2 binding MyBP-C motif in a morphologically unaltered system, and using controlled phosphorylation states of the proteins involved.

Rationale of the Experimental Setup. In cardiac muscle, cAPK activity results in the phosphorylation of several proteins, including troponin I and MyBP-C.[12,15] Furthermore, cardiac muscle contains a high background activity of various kinases, including a calcium/calmodulin–activated protein kinase associated with cardiac MyBP-C.[16,17] It is therefore hardly possible to attribute the changes in contractility resulting from phosphorylation to any given single substrate protein or signaling pathway. Because the interaction of myosin S2 with MyBP-C is conserved between all myosin and MyBP-C isoforms known,[18] we used skinned skeletal muscle fiber experiments to investigate active and rigor force, Ca^{2+} sensitivity of the contractile proteins, and stiffness (a measure reflecting the number of attached myosin heads.[24]) We exposed fibers to unphosphorylated cardiac MyBP-C C1C2, *tris*-phosphorylated C1C2 (C1C2-P), and the N-terminal fragment of MyBP-C (C0C1; for domain nomenclature, see Figure 1A). This latter fragment does not bind to myosin,[18] but it is similar to C1C2 in size and charge. The soaking of skinned fibers with exogenous protein allows the investigation of the effects of defined molecular regions of MyBP-C and defined phosphorylation states. Given that the endogenous MyBP-C affects only every eighth myosin head,[25,26] there are seven times as many free binding sites on myosin S2 as occupied by endogenous MyBP-C.

Furthermore, the effect of the phosphoisoforms of cardiac MyBP-C can be studied in a defined unphosphorylated background in skeletal muscle fibers, because the skeletal isoforms are no cAPK substrates,[20] and no protein kinase treatment of the actomyosin system is necessary. Finally, the study of soluble MyBP-C fragments allows the investigation of the question whether thick-filament anchorage is necessary for the regulatory function or whether myosin S2 binding alone can modulate contractility.

Materials and Methods

Protein Expression and Purification

Soluble MyBP-C fragments were prepared essentially as described.[17,18,27] In some cases, proteins without His_6 tag were prepared.[20] Proteins were adjusted to 30 μmol/L. For confocal microscopy, C1C2 was labeled with tetramethylrhodamine-isothiocyanate essentially as described.[18,28] The labeled protein was adjusted to high relaxing (HR) buffer and to a protein concentration of 0.6 mg/mL. Phosphorylated C1C2 fragment was prepared as described previously[17,20] and in further detail online (see http://www.circresaha.org).

Muscle Fiber Preparation and Force Measurements

Muscle fibers were prepared as described previously.[29] Details are provided online (see http://www.circresaha.org). The concentrations of the experimental solutions were described previously[30,31] and are given in detail online (see http://www.circresaha.org). Solutions were adjusted to pH 7.0, and ionic strength was calculated to 175 mmol/L. All measurements were performed at room temperature (22°C). All solutions containing recombinant MyBP-C fragments were adjusted to 30 μmol/L of the respective protein and contained protease inhibitors.

Force measurements were carried out essentially as described.[30,31] Sarcomere length was adjusted from laser diffraction pattern to 2.5 μm.[32] Solutions with protein contained 30 μmol/L of MyBP-C fragments; some control experiments were carried out using equal concentrations of C0C1 fragment, which does not bind to myosin S2,[18] or control proteins without the His_6 tag. Details are published online (see http://www.circresaha.org).

Confocal Imaging

For confocal imaging, a single fiber was glued at its ends to a cover slide using silicon glue,[33] mounted into a 50 μL flow cell,[34] skinned for 5 minutes in skinning solution, and then incubated for 15 minutes at 22°C in HR solution containing either rhodamine-labeled cardiac C1C2 or C0C1 at a concentration of 0.6 to 0.8 mg/mL, similar to described protocols.[18,28] The fiber was washed briefly in HR and then imaged using a confocal laser scanning microscope (Fluoview, Olympus Optical C., Tokyo, Japan). Thickness of optical sections was $\approx$1 μm. Owing to the weak binding of C0C1, diffusion of the labeled protein reduced the signal rapidly and resulted in poor signals.

Western Blotting

Single fibers were subjected to the experimental procedures of force measurements and were briefly washed in HR at the end of an experimental series and subsequently freeze-dried. The dried fibers were solubilized in 10 μL Laemmli sample buffer, and the entire sample was loaded on 14% SDS polyacrylamide gels. The gels were blotted following standard procedures, and endogenous MyBP-C and the impregnated soluble fragments were detected using the antibodies against C0C1 and C1C2 described previously.[11]

Statistical Analysis

Normal distribution of each group was confirmed by application of the Kolmogorov-Smirnov test. One-way ANOVA was applied for comparison between different groups. When the differences between

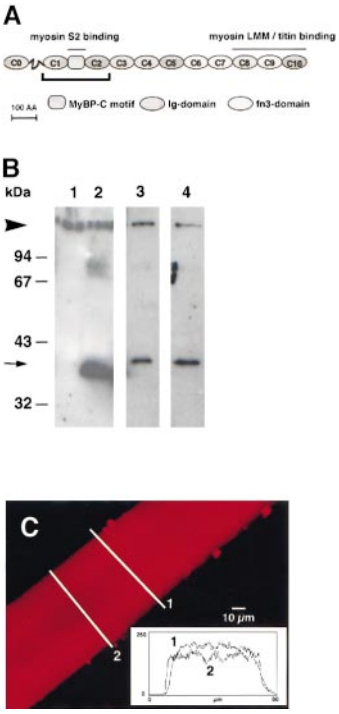

Figure 1. A, Domain structure of cardiac MyBP-C. Underlining indicates the C1C2 fragment used in the present study. B, Western blot of skinned fibers after incubation with recombinant MyBP-C fragments (as described in Materials and Methods) reveals the penetration of the fibers with protein solution to appreciable levels. In the case of C1C2, the blot was performed after several washes and reflects largely the sarcomere-bound form of the protein. Arrowhead indicates endogenous MyBP-C; arrow, exogenous protein fragment. 1, Control; 2, C0C1; 3, C1C2; and 4, C1C2-P. Molecular masses are given for a standard low molecular weight marker mix (BioRad). C, Confocal image of a single skinned skeletal fiber after incubation with rhodamine-labeled cardiac C1C2 (as described in Materials and Methods). Two densitometric slices through the fiber show a relatively homogenous distribution of labeled protein throughout the fiber diameter.

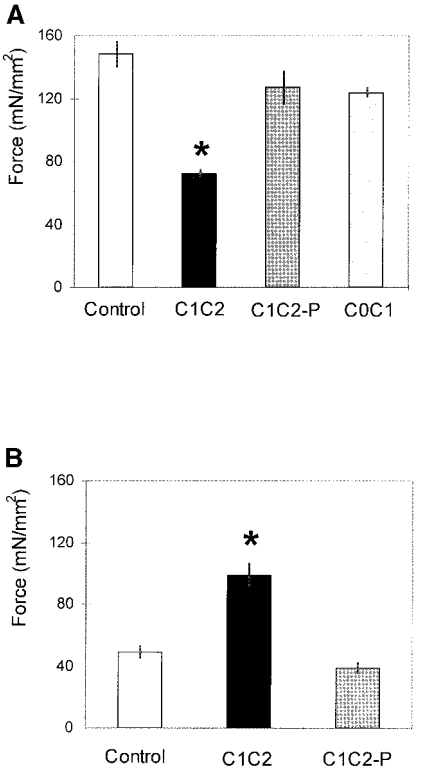

Figure 2. A, Maximal Ca^{2+}-activated isometric force (in the presence of 32 μmol/L Ca^{2+}). Comparison of unphosphorylated C1C2 fragment (C1C2, n=7), phosphorylated C1C2 fragment (C1C2-P, n=4), the control fragment C0C1 (C0C1, n=5), and the control (n=7). Results are mean±SEM. *$P<0.05$ compared with control. B, Maximal rigor force (in the absence of Ca^{2+}) for unphosphorylated C1C2 fragment (C1C2, n=7), phosphorylated C1C2 fragment (C1C2-P, n=4), and the control (n=7). Results are mean±SEM. *$P<0.05$ compared with control.

the groups were greater than would be expected by chance, the Bonferroni *t* test was applied. A significant difference was defined by a value of $P<0.05$. Data are presented as mean±SEM.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Penetration of Skinned Fibers by Soluble MyBP-C Fragments

To test the ability of the MyBP-C fragments used in the present study to diffuse into mouse skeletal muscle single skinned fibers, we assayed detergent-skinned fibers impregnated with the MyBP-C fragments C0C1 or C1C2 by Western blot (for domain nomenclature, see Figure 1A). The blots demonstrated the presence of endogenous MyBP-C in the control fibers and an additional band that could be visualized after impregnation with C0C1, C1C2, or the *tris*-phosphorylated C1C2 (Figure 1B). After exhaustive washing, these additional bands disappeared (not shown). This demonstrated that all MyBP-C fragments penetrate the skinned fibers and accumulate at appreciable levels. This observation was independent of the presence of the His_6 tag. Densitometry of the blots[35] showed that for C1C2, the approximate ratio of added MyBP-C fragment to endogenous protein was between 6:1 and 10:1. This suggests that the majority, if not all, of the free binding sites on myosin S2 (see Introduction) were saturated by exogenous protein. To visualize the distribution of the protein in the fiber, we used the rhodamine-labeled cardiac C1C2 fragment.[18] The images show the protein associated to the sarcomeres in constant ≈1.6-μm-broad stripes, giving rise to a characteristic cross-striated pattern (Figure 1C; see also online supplementary information, http://www.circresaha.org). This is in agreement with the A band localization reported previously,[18,19] which reflects the myosin S2 binding properties of the MyBP-C motif[18] (Figure 1C). Measurements of the signal intensity of C1C2 in the equatorial section plane of the fiber revealed a relatively homogeneous distribution of the protein with a signal decrease of only ≈10% toward the center of the fiber (Figure 1C, inset). This analysis demonstrates that the recombinant protein fragment diffuses almost entirely into the skinned fiber preparations within a relatively short incubation period of 15 minutes and binds to the sarcomere as evidenced by the A band cross-striation pattern described previously[18] (Figure 1C).

Several fibers were assayed for the presence of C1C2 by Western blotting after an experimental series. In all fibers, we

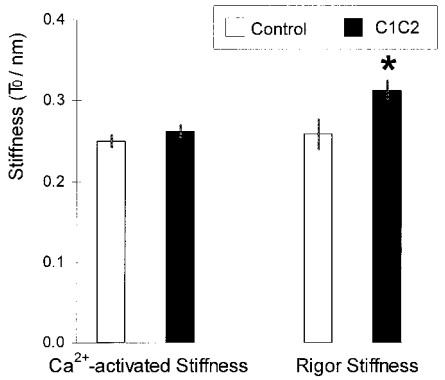

Figure 3. Maximal Ca^{2+}-activated dynamic stiffness (per half-sarcomere; in the presence of 32 μmol/L Ca^{2+}) and rigor stiffness normalized to T_0; comparison between unphosphorylated C1C2 fragment (C1C2, n=7) and the control (n=7). T_0 indicates maximum Ca^{2+}-activated tension. Results are mean±SEM. *$P \leq 0.05$ compared with control.

detected the recombinant, soluble MyBP-C fragments at apparently constant levels, in agreement with the confocal images obtained with fluorescently labeled protein (examples shown in Figure 1B).

Ca^{2+}-Activated Isometric Force and Rigor Force

Skinned fibers were incubated with MyBP-C fragments. Maximal Ca^{2+}-activated force was significantly reduced after incubation with C1C2 compared with the control fibers (by 51%, $P<0.05$; Figure 2). In contrast, there was no significant reduction after incubation with C1C2-P (Figure 2A). Changes in maximal Ca^{2+}-activated force were reversible by washing out C1C2 (not shown). Similarly, the C0C1 fragment resulted in no significant change of the maximal Ca^{2+}-activated force (Figure 2A). We conclude that MyBP-C fragments containing the MyBP-C regulatory domain, but not the neighboring domains, influence the attachment of myosin heads in response to the Ca^{2+}-induced activation state of the thin filament.

Rigor force develops on ATP depletion of muscle when myosin heads attach to the actin filaments in a noncycling state. Because only about one ATP turnover occurs in a "depletion rigor," under our experimental conditions, this force is usually ≈30% of the active force that a muscle develops when rigor is induced starting from the relaxed state. Rigor force was significantly increased after incubation with C1C2 (by 102%, $P<0.05$) compared with the control, which was in contrast to C1C2-P, where no significant change was seen (Figure 2). Changes in rigor force were reversible also by washing out C1C2.

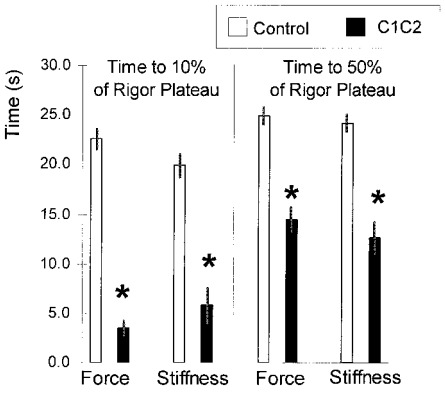

Figure 4. Rigor kinetics. Comparison between unphosphorylated C1C2 fragment (C1C2, n=7) and the control (n=7). Results are mean±SEM. *$P<0.05$ compared with control.

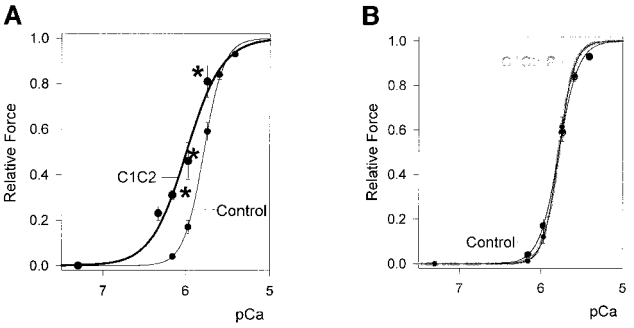

Figure 5. pCa/force relations of unphosphorylated MyBP-C C1C2 fragment (A; C1C2, n=5) and phosphorylated MyBP-C C1C2 fragment (B; C1C2-P, n=4) compared with the pCa/force relation of the control (n=7). *$P \leq 0.05$ compared with control.

Dynamic Stiffness Measurements

Stiffness is regarded as a measure that mainly reflects the number of attached myosin crossbridges (myosin heads)[24,36] and could therefore give important information on whether the changes in active, or rigor, force observed above are caused by inducing the detachment of myosin heads in active force (stiffness would decrease) or by a change in the rate of reattachment of myosin heads (stiffness would not change despite a drop in active force). Similarly, the increase in rigor force can be analyzed for changes in the number of attached heads, and the kinetics of head attachment. We therefore measured the effects of C1C2 on active and rigor stiffness, given that this protein, but not its phosphorylated form C1C2-P, influences both active and rigor force. We observed that stiffness under Ca^{2+}-activating conditions did not change by the presence of C1C2, whereas rigor stiffness increased slightly, but significantly, under the influence of C1C2 (Figure 3).

Kinetics of Rigor Force and Rigor Stiffness

After incubation with C1C2, force increased within a shorter time to 10% and 50% of maximal rigor force in comparison to the control, $P<0.05$ (Figure 4). In parallel to the strongly accelerated development of rigor force, rigor stiffness development was also accelerated significantly (Figure 4).

pCa/Force Relation

The pCa/force relation (Figure 5) revealed a shift to the left and thus to lower Ca^{2+} concentrations of the pCa/force curve after incubation with C1C2 (ΔpCa_{50}0.21; Figure 5A). In contrast, the presence of C1C2-P induces no changes of the Ca^{2+} sensitivity of active force (ΔpCa_{50}0.02; Figure 5B). The Hill coefficient (h) was significantly smaller after incubation with C1C2 compared with the control ($\Delta h = -1.37$, $P<0.05$), whereas it revealed no significant change after incubation

with C1C2-P (Δh0.76). Similarly, C0C1, which has no significant effect on active force, does not alter the pCa/force relation ($\Delta pCa_{50} = -0.06$) or the Hill coefficient (Δh0.47). These changes were completely reversible by washing out the proteins (not shown). The presence of C1C2 shifts the $[Ca^{2+}]_{50}$ from 1.86 μmol/L to 1.15 μmol/L and thus increases Ca^{2+} sensitivity by 38%.

Discussion

Regulation of muscle contraction is primarily achieved by the Ca^{2+}-sensitive troponin/tropomyosin complex, which, in response to a rise in intracellular Ca^{2+}, allows the binding of myosin heads and the generation of active force. In the heart, this process can be modulated after β-adrenergic stimulation by controlling the macroscopic Ca^{2+} flux within the cardiomyocyte by cAPK phosphorylation of L-type sarcolemmal Ca^{2+} channels,[37] which results in an increase of the mean open probability of the individual Ca^{2+} channels, or that of phospholamban, a protein controlling Ca^{2+} flux to the sarcoplasmic reticulum.[15,38] On the sarcomeric level, cAPK phosphorylation of troponin I,[15] which is involved in regulating Ca^{2+} sensitivity on the thin filament,[38,39] results in a decreased Ca^{2+} sensitivity. MyBP-C is phosphorylated synergistically with these contraction regulators.[15] However, although the effects of phosphorylation on Ca^{2+} channels, phospholamban, and troponin I are relatively well known, the function of MyBP-C is just emerging. Because MyBP-C is a modular protein, its functions can be dissected into defined fragments with specific functions.

In the present study, we show that the N-terminal S2 binding MyBP-C motif has pronounced effects on contractility. In active muscle, the presence of the protein fragment results in a decrease in active force, whereas stiffness (reflecting the number of attached myosin heads) is not affected. At the same time, Ca^{2+} sensitivity is increased significantly, requiring 38% less Ca^{2+} for the same activation levels, although at lower force output. These effects are completely reversible by phosphorylation of the MyBP-C motif by cAPK. MyBP-C is therefore a thick-filament–associated protein that can modulate Ca^{2+} sensitivity.

In rigor, myosin heads attach without cycling in a conformation believed to reflect the end state of the power stroke. Rigor force, being ≈30% of active force at the ionic strength used in our experiments,[30] is significantly increased by the MyBP-C motif concomitant with a smaller but significant increase in plateau rigor stiffness (Figures 2 and 4). Importantly, the kinetics of rigor force development is greatly accelerated, with a parallel increase in stiffness. Both effects are reverted by phosphorylation of the MyBP-C motif. Given that the attachment of rigor heads under our experimental conditions should be largely independent of thin-filament activation because it occurs in absence of Ca^{2+}, effects on the thick filament rather than thin filaments attribute mainly for these changes. In vitro actin binding of MyBP-C has been reported.[40,41] The A band localization of the fragments used in this and other studies and the effects of phosphorylation on force and stiffness, which reflect the biochemical effects on the interaction with myosin S2,[20] make a thick-filament effect most likely.

Previous ultrastructural investigations have shown that phosphorylation of MyBP-C extends the myosin heads from the backbone of the filament and increases their degree of order and/or alters their orientation.[42,43] Whether anchorage of MyBP-C to the thick-filament backbone was essential for this function (as a regulated tether) or whether the interaction of the phosphorylated region alone would result in regulatory effects could not be answered on this level. Our results suggest that the MyBP-C motif can control contractility in a phosphorylation-dependent way that does not require the anchorage of the domain to the thick filament via its LMM binding C-terminus. This is consistent with a model in which the MyBP-C motif controls the mobility of the myosin head-tail junction and thereby affects the attachment rates and/or states of myosin heads. The reduction in active force with constant stiffness and with an increased Ca^{2+} sensitivity suggests that the addition of the soluble MyBP-C S2 binding domain promotes the attachment of myosin heads. Structural investigations have shown that during muscle activation from the rigor state, weak crossbridges evolve from rigor bridges. Attached crossbridges, presumed to be weakly attached, increase at early times when tension is low.[44] Our experiments with soluble MyBP-C fragments suggest that the extension of crossbridges from the filament backbone may not be the sole regulatory mechanism of MyBP-C and that an anchorage-independent influence of crossbridge attachment exists as well.

Effective Concentration of Myosin Heads Is Regulated by the MyBP-C Motif

Two factors are importantly influencing the binding of myosin heads to actin: (1) the activation state of the thin filament and (2) the effective concentration of myosin heads. Because myosin and actin form insoluble filaments, the latter is greatly influenced by the distance of the myosin heads relative to actin. The degree of thin-filament activation (reflected in the Ca^{2+} concentration needed for half-maximal force) needed for a certain number of myosin heads to attach is therefore also dependent on the effective concentration of myosin heads. The leftward shift of the Ca^{2+}-force relationship in the presence of the MyBP-C motif, with constant stiffness at maximal activation, is best explained by a facilitated attachment of a subgroup of myosin heads by increasing their effective concentration. At the same time, the slope of the Ca^{2+}-force relationship is reduced (Figure 3), suggesting a reduced cooperativity of the Ca^{2+} activation of active force. Interestingly, the reduction of active force even at almost saturating protein concentration in our experiments never exceeded 50%. The apparently facilitated attachment of myosin heads in the presence of the MyBP-C motif is also seen in rigor, where rigor force and stiffness increase simultaneously, and both the kinetics of force and stiffness development are accelerated strongly (Figures 2 and 4). Given that rigor force under our experimental conditions (low Ca^{2+}) is largely independent on thin-filament activation, it should rather reflect changes in myosin conformation.

Previous skinned fiber experiments, in which MyBP-C was extracted,[45] showed an increased active tension at submaximal concentrations of Ca^{2+}, with little effect on maximum

tension. The effects on the pCa/force relationship are partly reproduced in our experiments, where both competition with endogenous protein and occupation of free binding sites occur: a surplus of the MyBP-C regulatory domain decreases the maximal Ca^{2+}-activated force. However, extraction studies are difficult to control, and protein redistribution and/or changes on the ultrastructural level can lead to additional effects. Studies on cardiomyocytes also showed that increases in cAPK activity and phosphorylation of troponin I and C protein lead to a significant decrease in tension-generating ability at a given submaximal Ca^{2+} concentration, in the absence of an effect of cAPK on unloaded shortening velocity.[46] In other words, the presence of unphosphorylated MyBP-C is accompanied by a leftward shift of the pCa/force relationship and the phosphorylation of MyBP-C with a rightward shift. These data are partly contradictory to the extraction data mentioned above but agree well with our observations in skinned fibers. The ability of unphosphorylated MyBP-C to lead to an increased Ca^{2+} sensitivity can also be deduced from the data presented in a mouse transgenic model,[23] where the effects of soluble MyBP-C on cardiac contractility are similar to our results.

Cycling of Myosin Heads Is Controlled by the MyBP-C Regulatory Domain in a Phosphorylation-Dependent Manner

Active force is generated by the ATP-consuming power stroke of the myosin heads.[47] The myosin II in striated muscle is double headed. Increasing evidence points to an asymmetry in the myosin molecule,[48] with the two heads adopting distinct conformations. Because of predicted sterical constraints at the head-tail junction,[22] both heads cannot bind to actin in the same state and hence fulfill different tasks at given times during the crossbridge cycle.[49] The binding of the MyBP-C regulatory domain to myosin S2 close to the head-tail junction[18] suggests that it might act by modulating the head-tail mobility and hence the transition of force generating to less productive conformational states of the two myosin heads. Steric hindrance is particularly limiting in rigor, with one head presumably bound in a strained conformation. It appears that MyBP-C binding can relieve this strain partly, which could lead to an increase of the rigor force. This interpretation is in agreement with increasing evidence that points to a cooperativity of both myosin heads in the production of active force and movement. These studies suggest that single-headed myosin produces less force and smaller steps than double-headed myosin.[50] Interfering with the interplay of force production by both heads by altering their relative attachment is therefore predicted to reduce the active force output while the number of bound heads could remain constant ("locked"). Our data suggest that binding of the MyBP-C motif to myosin S2 facilitates the binding of subgroups of myosin heads, but the reduction of active force by 50% at constant stiffness at the same time shows that the active force production by the myosin heads is impaired, with the bound population preferentially in a conformation with lower forces per crossbridge. One possibility for this effect is an increase in detachment rate, which would result in a decrease of the duty cycle ratio. Effectively, higher Ca^{2+} concentrations are therefore needed for identical power output.

Because MyBP-C is anchored to the thick filaments in regular intervals but at low stoichiometry to myosin, two major consequences arise. First, although the soluble S2 binding fragment of cardiac MyBP-C can modulate contractility in a phosphorylation-dependent way, the other attachment sites at the C-terminus and presumably at the isoform-specific N-terminal tails are likely to contribute to regulation, in cardiac as well as in skeletal muscle. Second, the regulatory effect of MyBP-C may be rather modest, but the spatial specificity of this, because of the axial distribution of MyBP-C to the central A band, may be most relevant. This is particularly obvious for the increase in Ca^{2+}-activated force with increasing sarcomere length,[51] which is phosphorylation-regulated independently of phosphorylation of troponin I[52] or the isoform of troponin C[53] in cardiac muscle. However, it duly reflects the decreasing number of crossbridges associated with MyBP-C. The effects observed with soluble protein fragments in the present study are therefore unlikely to represent the complete regulation mechanism of MyBP-C. However, they highlight the importance of protein interactions with myosin S2 for the modulation of contractility, which had previously been inferred solely from work with S2-directed antibodies.[21,54,55] A possible function as a length sensor would be largely independent of fiber type and hence MyBP-C isoform but phosphorylation-regulated in the heart. This concept is in excellent agreement with recent data[53,56,57] and can now be experimentally tested.

Because cAPK-mediated phosphorylation abolishes the binding of the MyBP-C motif to myosin S2[20] and its effects on contractility, this regulation can now for the first time be described for an endogenous thick-filament protein on the molecular level. The cardiac isoform of MyBP-C is therefore truly a regulator of contraction, and soluble fragments of the protein generated in FHC could, were they to accumulate significantly, indeed lead to aberrant contraction regulation. The function of the additional N-terminal modules of MyBP-C, which are isoform specific, must now be resolved. It was proposed that these domains may interplay with the regulatory light chains by their proximity to this region[20] and may thus confer further regulatory input that might not be resolved with shorter protein fragments such as C0C1. Finally, which binding state of the two-headed myosin is affected by MyBP-C and whether intermediate states such as the weakly bound crossbridges[58] are involved can now be resolved using more refined techniques at the ultrastructural and single-molecule level.

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