Cardiac myosin binding protein-C phosphorylation regulates the super-relaxed state of myosin

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Phosphorylation of cardiac myosin binding protein-C (cMyBP-C) accelerates cardiac contractility. However, the mechanisms by which cMyBP-C phosphorylation increases contractile kinetics have not been fully elucidated. In this study, we tested the hypothesis that phosphorylation of cMyBP-C releases myosin heads from the inhibited super-relaxed state (SRX), thereby determining the fraction of myosin available for contraction. Mice with various alanine (A) or aspartic acid (D) substitutions of the three main phosphorylatable serines of cMyBP-C (serines 273, 282, and 302) were used to address the association between cMyBP-C phosphorylation and SRX. Single-nucleotide turnover in skinned ventricular preparations demonstrated that phosphomimetic cMyBP-C destabilized SRX, whereas phospho-ablated cMyBP-C had a stabilizing effect on SRX. Strikingly, phosphorylation at serine 282 site was found to play a critical role in regulating the SRX. Treatment of WT preparations with protein kinase A (PKA) reduced the SRX, whereas, in nonphosphorylatable cMyBP-C preparations, PKA had no detectable effect. Mice with stable SRX exhibited reduced force production. Phosphomimetic cMyBP-C with reduced SRX exhibited increased rates of tension redevelopment and reduced binding to myosin. We also used recombinant myosin subfragment-2 to disrupt the endogenous interaction between cMyBP-C and myosin and observed a significant reduction in the population of SRX myosin. This peptide also increased force generation and rate of tension redevelopment in skinned fibers. Taken together, this study demonstrates that the phosphorylation-dependent interaction between cMyBP-C and myosin is a determinant of the fraction of myosin available for contraction. Furthermore, the binding between cMyBP-C and myosin may be targeted to improve contractile function.

MYBPC3 | myosin S2 | myofilament | sarcomere | SRX

Cardiac myosin binding protein-C (cMyBP-C) is a thick filament-associated sarcomeric protein present in vertebrate cardiomyocytes (1). It consists of 11 Ig-like and fibronectin-like domains, termed C0–C10, and localizes as seven to nine stripes in the inner two thirds of each half A-band, termed the C-zone (2). Cardiac contractility is modulated by cMyBP-C through its ability to transiently interact with thick and thin filaments to regulate actomyosin interactions (3–5). The importance of cMyBP-C is highlighted by the fact that mutations in the gene encoding it, *MYBPC3*, are the most common cause of hypertrophic cardiomyopathy (HCM) (6). cMyBP-C primarily mediates contractility through its phosphorylation status. cMyBP-C is highly phosphorylated, with three phosphorylatable serines within the M-domain, namely serines 273, 282, and 302, all of which have been intensively characterized (7–9). These sites are differentially phosphorylated by a range of kinases, including protein kinase A (PKA), protein kinase C, and Ca²⁺/calmodulin-dependent protein kinase II (8). The importance of cMyBP-C phosphorylation is more evident by the pathological consequences of its dephosphorylated state in many cardiac diseases (10, 11). Transgenic overexpression of phosphomimetic cMyBP-C was shown to prevent disease development in cMyBP-C–null mice, whereas dephosphorylated cMyBP-C could not (12, 13). Despite its clear role in cardiac function in health and disease, the precise mechanism(s) by which cMyBP-C

phosphorylation increases cardiac inotropy (contraction of myocardium) and lusitropy (myocardial relaxation) has/have not been fully elucidated.

It is possible that cMyBP-C phosphorylation regulates cardiac function by modulating the myosin super-relaxed state (SRX), a subpopulation of myosin heads characterized by their highly inhibited ATP turnover (reviewed in ref. 14). Myosin heads in the SRX adopt an evolutionarily conserved conformation of myosin II, termed the interacting-heads motif (IHM), which is suggested to be a fundamental regulator of muscle contractility (15). Through multiple inter- and intramolecular interactions, myosin heads in the SRX adopt an ordered, quasi-helical arrangement around the thick filament backbone, where they cannot bind actin (16). These interactions reduce ATP turnover of SRX myosin to a rate approximately 10 times slower than that of myosin in the disordered-relaxed state (DRX), commonly known as the detached state, in which relaxed myosin heads protrude into the interfilament space (17). Thus, the ratio of SRX to DRX myosin heads determines the energy utilization of the myofilaments and the number of myosin heads that can contribute to contraction.

Previously, we have demonstrated that cMyBP-C stabilized the SRX in that mice lacking cMyBP-C, or humans with cMyBP-C mutations, exhibited a significant shift of myosin heads from the SRX to the DRX (18, 19). Here, we tested the hypothesis that phosphorylation of cMyBP-C may promote a loss of SRX myosin heads, thereby regulating the number of force-producing myosin heads. To test this hypothesis, single-nucleotide turnover was

Significance

Cardiac myosin binding protein-C (cMyBP-C) is a sarcomeric protein closely linked to cardiac contractility. Importantly, although the phosphorylation status of cMyBP-C regulates the force and rate of cardiac contraction, the exact molecular mechanism(s) remain(s) unclear. Previously, loss of, or mutations in, cMyBP-C resulted in a loss of the inhibited super-relaxed state (SRX) of myosin, which explains the hypercontractile phenotype. Thus, we asked if cMyBP-C phosphorylation would increase contractile function by the release of myosin heads from SRX to a state more conducive to their interaction with actin. We found that a site-specific, phosphorylation-dependent interaction between cMyBP-C and myosin does modulate the fraction of SRX myosin that controls contractility.

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measured in four transgenic cMyBP-C phosphomimetic mouse models compared with wild-type (WT) controls. Based on these results, we measured force production, rate of tension redevelopment, and binding between myosin and cMyBP-C. Results tended to show that a stable SRX reduced maximal force production, which correlated with the interaction between myosin and cMyBP-C. Therefore, we reasoned that molecules that inhibit the interaction between cMyBP-C and myosin may, at the same time, increase contractility. It was found that competitive inhibition of myosin–cMyBP-C interaction does indeed reduce the number of myosin heads in the SRX and, strikingly, also resulted in increased force production and rate of force generation. Thus, regulatory mechanisms in the biology of cMyBP-C and its role in the SRX are revealed. Furthermore, we demonstrate that the interaction between cMyBP-C and myosin may be targeted to increase cardiac contractility.

Results

Transgenesis Reveals cMyBP-C Phospho-Regulation of the SRX. To test the hypothesis that phosphorylation of cMyBP-C can mediate the SRX, the fluorescent ATP analog 2'-3'-O-(N'-methylanthraniloyl)-ATP (mant-ATP) was used in a pulse-chase to measure the single-nucleotide turnover in detergent-skinned multicellular ventricular preparations from WT, cMyBP-C phosphomimetic (DDD; aspartate mutations in 273, 282, and 302 sites), and phospho-ablated (AAA; alanine mutations in 273, 282, and 302 sites) mice. An in-depth description of this assay is provided in *SI Appendix, Measurement of the SRX*. By Western blot, it was determined that WT controls were ~50% phosphorylated at each of these serines (*SI Appendix, Fig. S2*). Representative traces from each model are shown in Fig. 1A. Consistent with previously published data (19), WT mice had a P2 of $17 \pm 2\%$ and a lifetime of ATP turnover (T2) of 130 ± 11 s (Fig. 1C). The same experiment in AAA mice yielded a P2 of $15.2 \pm 0.7\%$ and a T2 of 112 ± 12 s, indicating that no changes in the SRX occurred in these mice. However, when

DDD mice were assayed, a significant shift of myosin from the SRX to the DRX was observed compared with WT mice (P1, $79 \pm 1\%$ vs. $88 \pm 1\%$; $P < 0.0001$; P2, $17 \pm 2\%$ vs. $8 \pm 1\%$; $P = 0.0001$). No difference in T2 was observed between these groups.

Having determined that phosphomimetic cMyBP-C destabilizes the SRX in transgenic mice, we explored the possibility of site-specific phospho-regulation of the SRX, and, to accomplish this, two additional models were used. In the first mouse model, DAD, the PKC-phosphorylatable sites ser-273 and ser-302 were mutated to aspartic acid, and the remaining site, ser-282, was mutated to alanine. The second model, ADA, reversed the condition of the first in that ser-282 was mutated to aspartic acid and ser-273 and ser-302 were mutated to alanine. Multicellular preparations were tested to measure the SRX in these models, as described earlier (traces in Fig. 1B). Interestingly, the SRX in DAD mice was similar to that in WT and AAA mice (P1, $76 \pm 3\%$; P2, $18 \pm 2\%$; Fig. 1C and D). Strikingly, however, the slow phase of single-nucleotide turnover in ADA mice was significantly reduced compared with WT experiments, indicating a shift of myosin heads from the SRX to the DRX (P1, $85 \pm 1\%$; P2, $8.8 \pm 0.5\%$; both $P < 0.01$ vs. WT). As ser-282 phosphorylation was sufficient to shift myosin heads from the SRX to the DRX, these data implicate phosphorylation of ser-282 as the primary cMyBP-C phosphorylation site that regulates the SRX. No changes were found in the lifetime of ATP turnover in the fast or slow phases (*SI Appendix, Fig. S3*).

cMyBP-C Is the Main PKA-Mediated Regulator of the SRX. It was apparent that the amino acid substitutions used to mimic phosphorylation of cMyBP-C might be completely representative of the structural changes accompanying endogenous serine phosphorylation. Indeed, a recent report suggests that this may be the case (20). Thus, to test whether destabilization of the SRX, as observed in phosphomimetic hearts, was representative of endogenously phosphorylated cMyBP-C, WT preparations were pretreated with PKA to phosphorylate myofilament targets before performing single-nucleotide turnover assays. PKA treatment of WT myofilaments elicited close to a 50% increase in phosphorylation for each serine site (*SI Appendix, Fig. S2*). Following PKA phosphorylation, mant-nucleotide turnover was faster than that of untreated samples (Fig. 2A). This corresponded to a 10% increase in the P1 ($79 \pm 1\%$ vs. $89 \pm 1\%$; $P < 0.0001$, Fig. 2D). Concurrently, the P2 of PKA-treated samples was significantly decreased compared with nontreated samples ($18 \pm 1\%$ vs. $8 \pm 1\%$; $P < 0.0001$; Fig. 2F) in a manner that closely resembled results from DDD mice. Whereas the lifetime of the fast phase was unaffected (Fig. 2C), a slight, near-significant increase was observed in the lifetime of SRX ATP turnover (120 ± 8 s vs. 160 ± 12 s; $P = 0.0523$; Fig. 2E). However, this could be attributed to the instability of fitting a second exponential to such a fast-decaying curve.

It is also possible that other PKA targets within the myofilament may have contributed to the decrease in SRX myosin observed upon PKA phosphorylation. To address this concern, we also compared the SRX in AAA mice with or without PKA treatment. As seen in Fig. 2B, the traces of AAA and AAA+PKA are nearly indistinguishable, and subsequent fittings were unchanged (Fig. 1C and D). In sum, these data confirm that cMyBP-C phosphorylation does indeed modulate the SRX in cardiac muscle, thus determining the fraction of myosin heads available for contraction. Furthermore, as PKA treatment of AAA fibers containing nonphosphorylatable cMyBP-C had no effect on the SRX, it can be inferred that cMyBP-C is the primary target for PKA-mediated regulation of the SRX.

Modulation of the SRX by cMyBP-C Phosphorylation in Turn Regulates Force and Rate Development. To determine whether changes in the fraction of SRX myosin affects contractile function, we measured force generation and tension redevelopment in skinned LV myocardial preparations from each group (Fig. 3A and B). The

Fig. 1. Comparison of SRX in skinned fibers from transgenic cMyBP-C phosphomimetics. (A and B) Mant-ATP single-nucleotide turnover experiments comparing WT with AAA and DDD (A) and WT with DAD and ADA (B). Between-group comparisons of amplitudes of fast phase (C) and slow phase (D) representing the fraction of SRX myosin, as determined from curve fitting with double exponential fits. Lifetimes of exponential fits are reported in *SI Appendix, Fig. S3*. $n = 8$ (24) for WT, $n = 5$ (16) for AAA, $n = 5$ (17) for DDD, $n = 3$ (13) for ADA, and $n = 4$ (13) for DAD (values inside parentheses represent number of fibers). Error bars indicate $\pm$ SEM (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

also destabilizes the SRX, we could specifically probe the effect of cMyBP-C phosphorylation (22). We first found that phosphorylation of cMyBP-C releases myosin heads from the SRX, as determined by single-nucleotide turnover from DDD and PKA-treated WT mice. Our analysis of chronically (phosphomimetic) and acutely (WT PKA-treated fibers) phosphorylated cMyBP-C was critical to confirm that cMyBP-C phosphorylation regulates the SRX. Indeed, although the use of phosphomimetic cMyBP-C allowed for the specific isolation of cMyBP-C-mediated effects, the use of PKA-treated fibers allowed us to circumnavigate any potential structural changes as a result of the aspartic acid mutation of cMyBP-C. Second, the phosphorylation state of serine 282 appears to be critical in regulating this effect on the SRX. Finally, this regulation is likely mechanistically driven by the phosphorylation-dependent interaction between cMyBP-C and myosin, providing a potential target to modulate contractile function.

cMyBP-C phosphorylation positively regulates cardiac inotropy and lusitropy; however, the mechanism(s) that underlie(s) such regulation is/are not fully understood. Specifically, phosphorylation of cMyBP-C increases the rate of force redevelopment and accelerates the rate of relaxation and force development during stretch activation in submaximally activated myocardial preparations (9, 38, 39). Furthermore, PKA treatment of skinned cardiac muscle significantly increases power output and speed of unloaded shortening (40–42), whereas AAA mice expressing phospho-ablated cMyBP-C develop diastolic dysfunction (9, 40). Our finding that cMyBP-C phosphorylation disrupts SRX myosin agrees with structural data (39, 43), and by increasing the fraction of possible cross-bridges, readily explains how cMyBP-C phosphorylation modulates many of the aforementioned phenomena such as increased k_{tr} and power output. It is less clear how cMyBP-C phosphorylation accelerates myocardial relaxation, but it likely results from structural changes within the catalytic domain of myosin that accelerate ADP release.

Strikingly, we also report a strong dependence on the phosphorylation status of serine 282 in the cMyBP-C regulation of the SRX. Specifically, when just this site was mutated to mimic phosphorylation (i.e., ADA), we found that the change in SRX:DRX ratio was similar to that observed in DDD mice, whereas phosphomimetic substitution of PKC-phosphorylatable sites (i.e., DAD) exhibited stable SRX. Although structural studies have not been performed on these models, these data predict reduced IHM and disordered thick filaments in ADA mice, but stabilized thick filaments in DAD models. Supporting this, we found a reduced interaction between ADA C0C2 and myosin. Furthermore, force production in DAD skinned myocardial preparations was significantly reduced, with no changes in ADA mice, as previously reported (21). Although cMyBP-C phosphorylation is known to be high under physiological conditions, dobutamine treatment significantly increased phosphorylation of all three serine sites *in vivo* and *ex vivo* (8, 44). We found similar increases in the phosphorylation level of each site upon PKA treatment (*SI Appendix, Fig. S2*). This demonstrates that cMyBP-C regulation of the SRX is possible at physiological level. In general, we observed that greater stability of the SRX correlated with reduced maximal force production. An exception to this was the WT model, which exhibited a stable SRX while producing force similar to that of DDD and ADA models. The reason for this is uncertain. However, it is possible that the basal phosphorylation levels of WT cMyBP-C, although sufficient to maintain the SRX in relaxation, may allow for the recruitment of these myosin heads in response to calcium. Interestingly, it was recently demonstrated that phosphorylation of serine 302 reduces cardiomyocyte fractional shortening in response to PKC, which agrees with our results (45). Furthermore, PKC depressed myofilament function and could not be rescued by PKA treatment (46). Although these effects can be attributed to contributions of cTnI and cMyBP-C phosphorylation, they agree with our findings that residue-specific phosphorylation of cMyBP-C

regulates contractile function. Conversely, phospho-ablation of only serine 302, leaving serines 273 and 282 unmutated (i.e., SSA), blunted the β -adrenergic response (7). The origin of these contrasts remains unknown, and because we did not quantify the SRX in the SSA model, it is possible that cMyBP-C regulation of the SRX is even more intricate.

Current structural models for the SRX suggest exceptional importance for myosin S2 in the formation of the IHM whereby the mesa of blocked and free heads cradles the proximal region of myosin S2 (24, 34). Furthermore, exciting new work has demonstrated the biochemical presence of an inhibited SRX-like state in soluble myosin that is dependent on the presence of a proximal myosin S2 (29, 30). Given the intrinsic capacity of myosin to form an IHM, it is interesting that cMyBP-C exhibits such dramatic effects on the SRX in muscle fibers (18, 19, 31). It seems reasonable to argue that cMyBP-C acts to stabilize one IHM, allowing the formation of neighboring IHMs in a cooperative manner throughout the C-zone. Proximal myosin S2 also binds to N-terminal domains of cMyBP-C, and in the model proposed by Nag and colleagues, it would likely act to stabilize the IHM (34). Our results demonstrate that exogenous myosin S2 can destabilize the SRX, which agrees with the aforementioned model (34). Excess myosin S2 has previously been demonstrated to block the interaction between full-length myosin and cMyBP-C, suggesting that this is indeed the mode of action in our assays (35). Increased contractility of intact cardiomyocytes upon myosin S2 permeabilization has also been described, supporting our findings that this peptide increased force production and rate of tension redevelopment (47).

We and others have determined that ~40–50% of myosin occupies the biochemical SRX state in skinned myocardial fibers. However, X-ray diffraction of intact trabeculae suggests a far greater number of myosin heads occupying the structural OFF-state during diastole (48). Furthermore, under resting conditions, treatment of intact trabeculae with isoprenaline, a β_1 -agonist, did not destabilize the OFF-state of myosin motors, whereas direct PKA phosphorylation of skinned trabeculae could (39, 49). However, consistent with the model proposed in this paper, isoprenaline treatment resulted in a reduced intensity of the M1 cluster related to cMyBP-C (49). It is possible that the loss of osmotic compression associated with the skinning process promotes propensity toward loss of thick filament stability and subsequent myosin head disorder. An excellent editorial piece has recently addressed these disparate findings, suggesting that, within the intensely crowded milieu of the intact lattice, small movements of the myosin heads may not be detectable (50). Alternatively, multiple biochemical states of myosin may exist within the structural OFF-state, such as between blocked and free myosin heads. Clearly, extensive investigations are still required to characterize the relationship between the biochemical and structural properties of these states in physiologically relevant settings.

In conclusion, we have defined a regulatory mechanism of the SRX by phosphorylation of cMyBP-C, significantly advancing our understanding of the mechanisms controlling SRX biology. Additionally, we demonstrated that the phospho-dependent interaction between cMyBP-C and myosin could be targeted to improve contractile function. Future work will further characterize the specific interaction between cMyBP-C and myosin and define in depth the effects of exogenous myosin S2 on cardiac function.

Materials and Methods

Transgenic Mouse Models. An expanded description of the study methods and materials is provided in *SI Appendix*. Transgenic mice overexpressing phosphomimetic cMyBP-C have been previously characterized (8, 12, 13). Mice were deeply anesthetized by isoflurane inhalation, followed by cervical dislocation and bilateral thoracotomy. Hearts were excised, snap-frozen in liquid nitrogen, and stored at -80°C until use. All animal experiments were approved by the institutional animal care and use committees at Loyola University Chicago and University of Cincinnati and followed the policies described in the *Guide for the Use and Care of Laboratory Animals* published by the National Institutes of Health (51).

In Vitro Assays. To measure the SRX, single-nucleotide turnover assays were performed by using skinned LV from the free wall as described in *SI Appendix* (19). Cardiac muscle mechanics were performed by using the Aurora Scientific 1400A permeabilized fiber system (18, 19, 22). Recombinant peptides were produced in *Escherichia coli* by using the pET28⁺ expression system and purified by His-tag affinity purification, and cosedimentation assay was performed as described previously (4). Statistical analysis was performed by using GraphPad Prism 7 with significance accepted at $P < 0.05$.

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