

Research Article

Functional Differences between the N-Terminal Domains of Mouse and Human Myosin Binding Protein-C

Justin F. Shaffer,^{1,2} Peony Wong,¹ Kristina L. Bezold,¹ and Samantha P. Harris¹

¹Department of Neurobiology, Physiology and Behavior, University of California-Davis, Davis, CA 95616-8519, USA

²Department of Bioengineering, University of Washington, Seattle, WA 98195-5061, USA

Correspondence should be addressed to Samantha P. Harris, samharris@ucdavis.edu

Received 16 December 2009; Accepted 31 January 2010

Academic Editor: Henk L. M. Granzier

Copyright © 2010 Justin F. Shaffer et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The N-terminus of cMyBP-C can activate actomyosin interactions in the absence of Ca^{2+} , but it is unclear which domains are necessary. Prior studies suggested that the Pro-Ala rich region of human cMyBP-C activated force in permeabilized human cardiomyocytes, whereas the C1 and M-domains of mouse cMyBP-C activated force in permeabilized rat cardiac trabeculae. Because the amino acid sequence of the P/A region differs between human and mouse cMyBP-C isoforms (46% identity), we investigated whether species-specific differences in the P/A region could account for differences in activating effects. Using chimeric fusion proteins containing combinations of human and mouse C0, Pro-Ala, and C1 domains, we demonstrate here that the human P/A and C1 domains activate actomyosin interactions, whereas the same regions of mouse cMyBP-C are less effective. These results suggest that species-specific differences between homologous cMyBP-C isoforms confer differential effects that could fine-tune cMyBP-C function in hearts of different species.

1. Introduction

Myosin binding protein-C (MyBP-C) is a regulatory and structural protein associated with the A-bands (thick filaments) of vertebrate striated muscle sarcomeres. Regulatory effects are mediated in part by the N-terminus of cardiac cMyBP-C, which is made up of modular immunoglobulin- (Ig-) like domains, termed C0 at the N-terminus, followed by domains C1 through C10 (Figure 1(a)). Between the C0 and C1 domains there is a sequence of ~50 amino acids that contains a high percentage of proline and alanine residues (referred to as the Pro-Ala rich region (P/A)). Between the C1 and C2 domains there is a stretch of ~100 highly conserved amino acids referred to as MyBP-C motif or M-domain. The M-domain is phosphorylated by β -adrenergic agonists and phosphorylation increases the rate of cross-bridge cycling [1], thereby contributing to increased inotropic responses of the heart [2]. Phosphorylation reduces binding of the M-domain to actin [3] and myosin S2 in vitro [4], but the precise mechanism(s) by which phosphorylation accelerates actomyosin interactions is not well understood.

In efforts to uncover mechanisms by which the N-terminus of cMyBP-C affects cross-bridge cycling several groups have investigated the effects of recombinant proteins containing N-terminal cMyBP-C domains on actomyosin interactions. For instance, Razumova et al. [5] investigated effects of N-terminal domains in in vitro motility assays and found that recombinant proteins containing the C1 through C2 domains of mouse cMyBP-C (referred to as C1C2, inclusive of the M-domain) could activate thin filament motility even in the absence of Ca^{2+} . Incubation of permeabilized rat trabeculae with C1C2 also increased Ca^{2+} sensitivity of force and increased the rate of tension redevelopment (k_{tr}) [6]. The activating effects of C1C2 were attributed to the combined effects of the C1 and M-domains because these domains together were necessary and sufficient to increase Ca^{2+} sensitivity of force, whereas other domains including the C0, P/A, and C1 domains had little, if any, effect on force activation. However, in apparent contrast to these results, Herron et al. [7] found that a recombinant protein comprised of the human C0, P/A, and C1 domains (i.e., C0C1) activated tension and increased

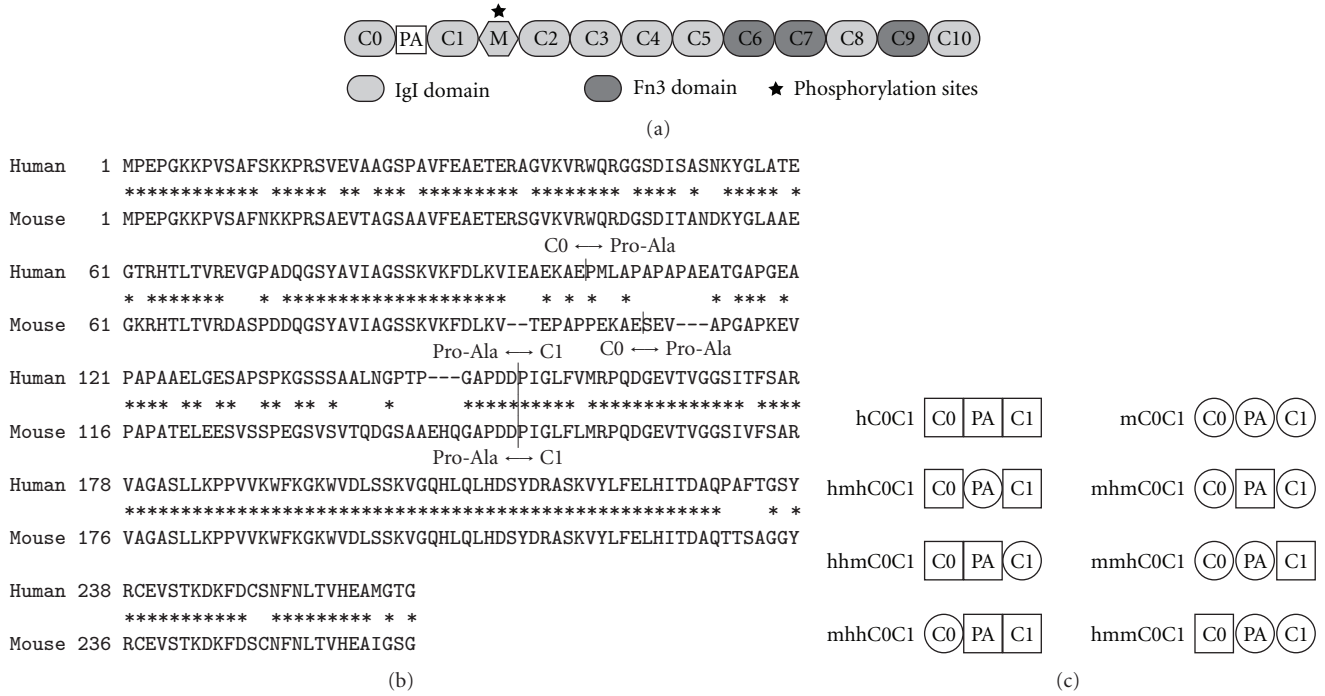


FIGURE 1: Schematic of full-length cMyBP-C, domain boundaries, and recombinant chimeric mouse and human C0C1 proteins. (a) Schematic of full-length cMyBP-C. The Pro-Ala rich region is denoted by a box between the C0 and C1 domains. (b) Amino acid sequence alignment of mouse and human C0, P/A, and C1 domains. Arrows indicate domain boundaries. Asterisks denote identical residues. (c) Schematic of recombinant chimeric proteins used in this study. Squares represent human domains; circles represent mouse domains.

k_{tr} in permeabilized myocytes from human myocardium, whereas C1C2 (which lacked the P/A region) had no effect. These authors concluded that the P/A rich region was required to activate tension and increase k_{tr} .

A potential explanation for the different conclusions reached in the two studies is that species-specific differences are responsible for the different functional effects observed for the mouse versus human proteins. Consistent with this idea, the full-length cMyBP-C protein is quite similar between mouse and human isoforms (89% identity, Table 1) and the C1, M, and C2 domains are highly conserved (>90% identity), but domains near the N-terminus show greater sequence divergence with 81% and 46% identity in the C0 domain and P/A regions, respectively (Figure 1(b)). The large difference in sequence homology in the P/A region is primarily due to differences in the content of proline and alanine residues: in the human P/A region, proline and alanine account for 51% of the sequence, while the mouse isoform contains only 28% proline and alanine. Interestingly, a similar trend was noted for a P/A rich sequence in fast skeletal myosin essential light chains (ELC1) where Bicer and Reiser [8] found that P/A content scaled with mammalian size.

The purpose of the present study was to determine whether species-specific differences in the P/A rich regions of human and mouse cMyBP-C can account for functional differences in the ability of recombinant proteins from the two species to activate actomyosin interactions. Results show that both the human P/A rich region and the C1 domains

can activate actomyosin interactions, whereas the mouse P/A and C1 domains are less effective. These results thus reconcile apparently disparate experimental results and raise the possibility that species-specific variations in cMyBP-C regulatory domains contribute to cross-species variations in cardiac function.

2. Methods

2.1. Protein Cloning, Expression, and Purification. The recombinant mouse protein mC0C1 (containing the C0, P/A, and C1 domains, Figure 1(c)) was subcloned from a full-length mouse cDNA (GenBank gi:3747133), expressed in M15 cells, and purified using Ni-NTA affinity chromatography as previously described [5]. Recombinant human hC0C1 was cloned from a human cDNA obtained by RT-PCR from whole heart human total RNA (Stratagene, La Jolla, CA) using a one-step RT-PCR kit (Invitrogen, Carlsbad, CA). A PCR product encoding hC0C1 was generated using gene specific primers flanking the desired domains (forward primer 5'-GGCCCATATGCCTGAGCCGGGAAGAAG-3', reverse primer 5'-GGCCAAGCTTTCATCCGGTGCCATGGCCTCGTG-3') and cloned into the pQE-2 expression vector (Qiagen, Valencia, CA) using NdeI and HindIII restriction sites on the upstream and downstream primers, respectively. Expression and purification of hC0C1 were as described previously [5]. Protein concentrations were determined by measuring light absorbance at 280 nm (corrected for turbidity at 310 nm) using extinction coefficients

TABLE 1: Sequence identity between domains of human and mouse cMyBP-C.

Protein or Domain	Number of Amino Acids		Percent Identity
	<i>Mouse</i>	<i>Human</i>	
cMyBP-C	1270	1274	89
C0C2	447	451	84
C0	104	101	81
Pro-Ala	46	51	46
C1	111	111	90
M	105	107	91
C2	81	81	96

calculated from Swiss Institute for Bioinformatics software [9].

2.2. Engineered Chimeric C0C1 Proteins. The In-Fusion PCR Cloning System (Clontech, Mountain View, CA) was used according to manufacturer's instructions and the protocol by Zhu et al. [10] to create seamless chimeric C0C1 proteins consisting of various combinations of mouse and human C0, P/A, and C1 domains. Boundaries of the C0, P/A, and C1 domains are shown in Figure 1(b) and were as described by Gautel et al. [11] and as listed for human and mouse cMyBP-C on the Universal Protein Resources Databank (UniProt) [12]. PCR products encoding the mouse and human domains were amplified from mouse and human cMyBP-C cDNA with additional 15 bp flanking sequences at the 5' and 3' ends that overlap with the adjacent segment of the construct (Table 2, Figure 1(c)). The pQE-2 expression vector (Qiagen) was digested with NdeI and HindIII and gel-purified. PCR products and the digested pQE-2 vector were mixed at a 1 : 2 : 2 : 2 molar ratio with the In-Fusion enzyme according to manufacturer's instructions and transformed into Top10 cells (Invitrogen). Correctly ligated plasmids were chosen after selection with carbenicillin and sequences were verified by DNA sequencing at the UC Davis DNA Sequencing Facility. Selected clones were transformed into M15 cells for expression and purification as described previously [5]. Chimeric C0C1 proteins used in this study are shown schematically in Figure 1(c).

2.3. Native Protein Purification. Heavy meromyosin (HMM) and S1 were prepared from rabbit psoas skeletal myosin via α -chymotryptic digest as described [13]. Bovine cardiac F-actin, tropomyosin, and troponin were purified from ether powder as described [14–16]. Thin filaments, comprised of F-actin and regulatory proteins, were reconstituted in AB buffer (in mmol/L: 25 KCl, 25 imidazole (pH 7.4), 4 MgCl₂, 1 EGTA, and 1 DTT) by combining at an actin:tropomyosin:troponin ratio of 4:1:1 for in vitro motility assays and at 7:2:2 for ATPase assays. The thin filament mix was labeled with rhodamine-phalloidin according to Homsher et al. [17] and used in in vitro motility assays.

2.4. Mechanical Force Measurements. Treatment of all animals was in strict accordance with guidelines and protocols

established by the University of California Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (200–250 g) were euthanized by intraperitoneal injection of sodium pentobarbital. Hearts were then rapidly excised and right ventricles were dissected in a Ringer's solution at pH 7.4 (in mmol/L: 100 NaCl, 24 NaHCO₃, 2.5 KCl, 1 MgSO₄, 1 Na₂HPO₄, and 1 CaCl₂). Trabeculae were permeabilized in situ by incubation of splayed ventricles overnight in a relaxing solution containing 50% glycerol and 1% Triton X-100 (Sigma, St. Louis, Missouri) at 4°C. Individual trabeculae were dissected free from ventricle walls, pinned to the bottom of a sylgar-coated Petri dish, and stored for up to one week in glycerinated relaxing solution at –20°C.

Steady state force and rate of tension redevelopment (k_{tr}) measurements were performed as previously described [6]. Briefly, permeabilized trabeculae were mounted between a force transducer (model 403A, Aurora Scientific Inc.) and a torque motor (Model 312-Cl, Aurora Scientific Inc). Sarcomere length was adjusted to $\sim 2.3 \mu\text{m}$ in relaxing solution and monitored throughout the course of the experiment using an inverted microscope (Olympus IX-71) fitted with a 12-Mega pixel digital camera (Olympus DP70). Relaxing, preactivating, and Ca²⁺-activating solutions were prepared as previously described using a custom software package [18, 19]. Solutions were maintained at 0.18 M ionic strength and pH 7.0 at 15°C (in mmol/L: 15 phosphocreatine, 15 EGTA, at least 40 MOPS, 1 free Mg²⁺, 135 Na⁺ + K⁺, 1 DTT, 250 units ml⁻¹ creatine kinase (CK), and 5 ATP). Ca²⁺ concentration (reported as pCa = $-\log[\text{Ca}^{2+}]$) was established by varying amounts of CaCl₂. Recombinant proteins were added to relaxing and preactivating solutions by buffer exchange using desalting spin columns (Pierce, Rockford, IL).

2.5. In Vitro Motility Assays. In vitro motility assays were performed as described previously [5]. Briefly, HMM was applied to a nitrocellulose-coated coverslip followed by incubation with bovine serum albumin (BSA) to prevent non-specific adsorption of thin filaments or recombinant proteins to the motility surface. Shredded actin filaments followed by ATP were then added to block nonfunctional myosin heads (dead-heads). Next, rhodamine-phalloidin labeled bovine cardiac thin filaments (4:1:1 actin:tropomyosin:troponin) were added, followed by a motility buffer containing AB buffer, 2 mM ATP, and an oxygen-scavenging system to limit photo-bleaching. Recombinant cMyBP-C proteins were dialyzed into AB buffer and added to the slide surface in the motility buffer. Motility was viewed using an Olympus IX-71 microscope with an Hg-arc lamp, TRITC filter, and a 100 \times /1.4 NA oil-immersion objective. Video files were recorded using a Q-Imaging Retiga Exi digital camera and ImageProPlus software. Filament motility was analyzed using custom software developed in LabView and NI Vision Development (National Instruments, Austin, TX) kindly provided by Dr. Michael Regnier (University of Washington).

2.6. ATPase Assay. ATPase assays were performed by mixing 0.2 μM myosin S1 and 3.5 μM reconstituted

TABLE 2: Primers used to create recombinant chimeric C0C1 proteins.

cMyBP-C domain	Primer pairs (upstream/downstream primer)
Mouse C0	5'-ATCACCATCACCATATGCCGGAGCCAGGGAAGAAACCA -3'/ 5'-AGGGGCCAGCATGGGTTCTGCCTTCTCTGGAGGGGC -3'
Mouse P/A	5'-TCTGAAGTTGCTCCAGGAGCC -3'/ 5'-GTCATCAGGGGCTCCCTGATG -3'
Mouse C1	5'-GGAGCCCCCGATGACCCATTGGCCTCTTTCTGATGCGA -3'/ 5'-TCAGCTAATTAAGCTTTCATCCAGAACCAATCGCCTCATGGAC -3'
Human C0	5'-ATCACCATCACCATATGCCTGAGCCGGGAAGAAGC -3'/ 5'-TGGAGCAACTTCAGACTCTGCCTTCTCTGCCTCATGAC -3'
Human P/A	5'-CCCATGCTGGCCCTGC -3'/ 5'-GTCATCGGGGCTCCAGG -3'
Human C1	5'-GGAGCCCCTGATGACCCATTGGCCTCTTCGTGATGC -3'/ 5'-TCAGCTAATTAAGCTTTCATCCGGTGCCCATGGCCT -3'

bovine cardiac thin filaments (mixed at a 7:2:2 actin:tropomyosin:troponin ratio) in ATPase buffer (in mmol/L: 10 imidazole (pH 7.4), 2 MgCl₂, 1 EGTA, and 1 DTT) with or without 1 μ M recombinant cMyBP-C proteins. The addition of 1 mM ATP started the reaction and reactions were quenched at three different time points with stop solution (3.3% SDS, 0.12 M Na-EDTA, pH 7.4). Phosphate production was determined via colorimetric assay as described [20].

2.7. *Statistical Tests.* Data were compared using ANOVA followed by Bonferroni post-hoc comparisons. Significance was considered at $P < .05$.

3. Results

3.1. *Human C0C1 Increases Ca²⁺ Sensitivity of Tension in Permeabilized Trabeculae.* Effects of recombinant mouse and human C0C1 proteins (inclusive of the C0, P/A, and C1 domains) were first assessed in permeabilized rat cardiac trabeculae. Figure 2(a) shows effects of human (h)C0C1 and mouse (m)C0C1 on force generation at pCa 9.0 and at maximal Ca²⁺ activation (pCa 4.5). Neither hC0C1 nor mC0C1 affected resting force in the absence of Ca²⁺ (pCa 9.0) or maximal force at pCa 4.5 even at concentrations up to 80 μ M, the highest concentration used by Herron et al. [7]. This result is consistent with observations by Razumova et al. [6] who found that preincubation of rat trabeculae with 30 μ M mC0C1 did not affect force, but it differs somewhat from Herron et al. [7] who found that 30 μ M hC0C1 activated force generation even in the absence of Ca²⁺ at pCa 9.0. Because measurements by Herron et al. [7] were conducted primarily using permeabilized myocytes from human myocardium, species-specific differences (rat versus human) could potentially contribute to the different effects observed here. However, as shown in Figures 2(b) and 2(c), hC0C1 was effective at increasing force and k_{tr} at submaximal Ca²⁺ (pCa 5.3) near the half-maximal [Ca²⁺]₅₀ (pCa₅₀) required for maximal force generation [6]. Effects were significant after preincubation with 50 μ M hC0C1.

50 μ M mC0C1 also increased Ca²⁺ sensitivity of tension, albeit to a lesser extent than hC0C1, but had no effect on k_{tr} .

3.2. *The Human Pro-Ala Rich Region Activates Filament Motility in the Absence of Ca²⁺.* Because results from permeabilized rat trabeculae suggested that species-specific differences contribute to differences in effects of hC0C1 and mC0C1, we sought to compare effects of hC0C1 versus mC0C1 in a defined system that minimizes variability in cross-species isoform expression. We therefore compared effects of hC0C1 and mC0C1 using in vitro motility assays with reconstituted cardiac thin filaments and skeletal HMM. Control values for average filament velocities in the absence (pCa 9) and presence (pCa 5) of Ca²⁺ were $0.3 \pm 0.1 \mu\text{m/s}$ ($n = 12$) and $3.8 \pm 0.5 \mu\text{m/s}$ ($n = 9$), respectively, demonstrating that reconstituted thin filaments were well regulated by Ca²⁺ under control conditions in the absence of added recombinant proteins. Figure 3(a) compares filament sliding speeds at pCa 9.0 in the absence or presence of hC0C1 or mC0C1. Under control conditions at low Ca²⁺, filament sliding speed was low and the vast majority of filaments were stopped (fraction moving was 4%). Addition of 1 μ M mC0C1 to motility buffers did not activate motility, whereas addition of hC0C1 significantly increased motility. These results are similar to those obtained in rat trabeculae (Figure 2) where hC0C1 increased Ca²⁺ sensitivity of tension and k_{tr} to a greater extent than mC0C1. They are also consistent with Herron et al. [7] who found that hC0C1 could activate tension development in myocytes in the absence of Ca²⁺.

To determine whether the P/A region contributes to the activating properties of hC0C1, chimeric C0C1 proteins were created that substituted human and mouse P/A regions. Figure 3(b) shows effects on filament motility of exchanging the human and mouse P/A domains in chimeric proteins. Insertion of the human P/A region into mouse C0C1 to create the chimeric protein mhmC0C1 (mouse C0, human P/A, and mouse C1) increased filament motility at pCa 9. This result demonstrates that the human P/A region but not the mouse P/A region is sufficient to confer activating effects on C0C1 proteins. However, activation was not complete

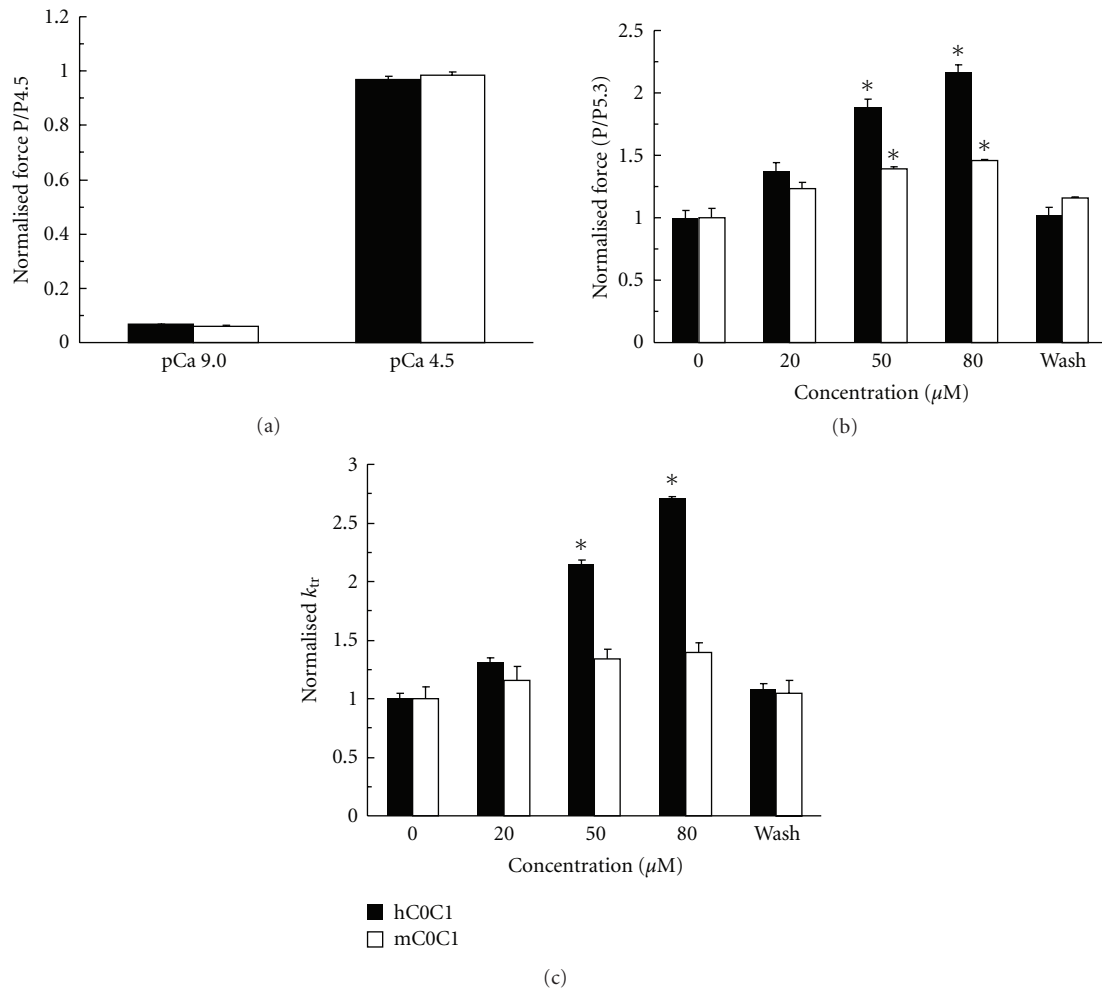


FIGURE 2: Effects of mC0C1 and hC0C1 on steady state force and rate of tension redevelopment (k_{tr}) in permeabilized rat trabeculae. (a) Preincubation of trabeculae with 80 μ M mC0C1 or hC0C1 did not affect resting force at pCa 9.0 or maximal Ca^{2+} activated force at pCa 4.5. However, hC0C1 increased force (b) and k_{tr} (c) at intermediate [Ca^{2+}] (pCa 5.3) with effects significant at 50 μ M. mC0C1 increased force at 50 μ M but did not significantly increase k_{tr} . Data are mean \pm SEM ($n = 4$ for hC0C1 and $n = 3$ for mC0C1). Asterisks denote significant differences in compared values obtained prior to protein addition ($P < .05$).

since sliding speeds were still somewhat less than in the presence of hC0C1 comprised of all human sequences. This suggests that domains outside of the P/A region must also contribute to the activating effects of hC0C1. Consistent with this idea, when the mouse P/A region was exchanged into hC0C1, that is, in hmhC0C1 (human C0, mouse P/A, and human C1), filament sliding speeds were increased relative to control but were less than in the presence of hC0C1. Collectively, these results demonstrate that the human P/A rich region is sufficient to activate motility in the absence of Ca^{2+} , but that the C0 or C1 domains must also contribute to the ability of hC0C1 to activate filament motility.

3.3. Human C1, but not C0, Activates Motility in the Absence of Ca^{2+} . To determine whether human C1 or C0 domains also contribute to the activating effects of hC0C1, we created additional chimeric proteins that substituted human and mouse C0 and C1 domains. To assess if the C1 domain is

required for activating effects, 1 μ M mmhC0C1 or hhmC0C1 proteins were added to in vitro motility assays. As shown in Figure 3(c), adding the human C1 to mouse C0 and P/A domains, (mmhC0C1) activated thin filament motility at pCa 9, demonstrating that the human C1 domain can also confer activating effects. However, filament velocity was reduced compared to hC0C1 (Figure 3(a)), indicating that other domains (e.g., P/A; Figure 3(b)) contribute to the activating effects of hC0C1. Conversely, when the mouse C1 domain was added to human C0 and P/A domains (hhmC0C1) activation occurred but to a lesser extent than hC0C1 (Figure 3(a)). These results show that mouse C1 cannot substitute for human C1 and cannot restore full activating effects when expressed with the human C0 and P/A domains.

To assess whether the human C0 domain also contributes to activating effects of hC0C1, hmmC0C1 and mhhC0C1 proteins were created and their effects in motility assays

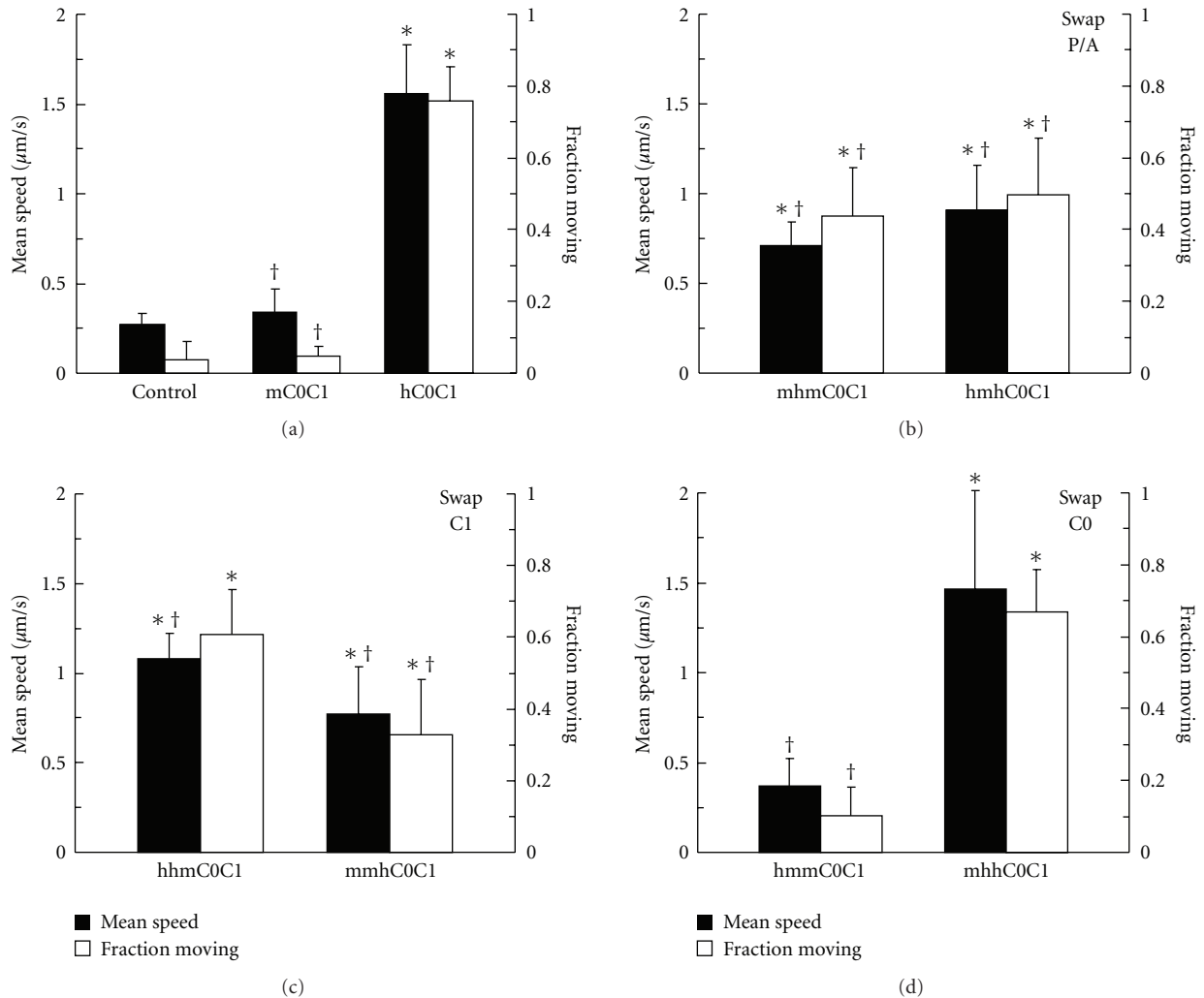


FIGURE 3: Effects of mouse and human C0, P/A, and C1 domains on motility of regulated thin filaments (F-actin + Tm + Tn) at pCa 9. (a) Compared to control experiments in the absence of added protein ($n = 12$), $1 \mu\text{M}$ hC0C1 significantly activated filament sliding speed motility and the fraction of filaments moving ($n = 8$), whereas $1 \mu\text{M}$ mC0C1 did not affect motility ($n = 9$). (b) Substitution of the human for mouse P/A domain in the mouse C0C1 backbone (mhmC0C1) activated motility ($n = 7$), whereas substitution of the mouse P/A domain into the human backbone (hmhC0C1) depressed motility relative to hC0C1 ($n = 5$). (c) Exchange of mouse and human C1 domains. The human C1 domain (mmhC0C1) activated motility (compared to mC0C1, $n = 5$), whereas the mouse C1 domain (hhmC0C1) depressed motility (compared to hC0C1, $n = 6$). (d) Exchange of mouse and human C0 domains. The human C0 (hmmC0C1, $n = 5$) and mouse C0 (mhhC0C1, $n = 10$) domains did not affect thin filament motility (compared to mC0C1 and hC0C1 controls, resp.). Data are mean \pm SD. Asterisks (*) and crosses (†) denote significant differences compared to mC0C1 and hC0C1, respectively, ($P < .05$).

were assessed at pCa 9. As shown in Figure 3(d), when the human C0 domain was added to mouse P/A and C1 domains (hmmC0C1), activation of filament motility did not occur and effects of hmmC0C1 were not different from the parent mC0C1. Similarly, when the mouse C0 domain was expressed with the human P/A and C1 domains (mhhC0C1), filament motility was the same as in the presence of hC0C1 (Figure 3(a)). These results show that C0 does not contribute to the activating effects of hC0C1 in motility assays, and that the human P/A and C1 domains together are sufficient to account for the full activating properties of hC0C1 on actomyosin interactions in the absence of Ca^{2+} .

3.4. Activating Effects Are Independent of Myosin S2. When expressed in rat neonatal cardiomyocytes, hC0C1 localized to sarcomere A-bands [7], suggesting that interactions of hC0C1 with myosin or other thick filament proteins are required for the observed activating effects. Consistent with this idea, the C0 domain was reported to bind to myosin S1 [21] and C1 can bind to myosin S2 [22]. Therefore to test whether interactions with myosin S2 are required for the activating effects of the human P/A and C1 domains, effects of chimeric C0C1 proteins were assessed in ATPase assays using myosin S1 (without S2) and regulated thin filaments. As shown in Figure 4, under control conditions in

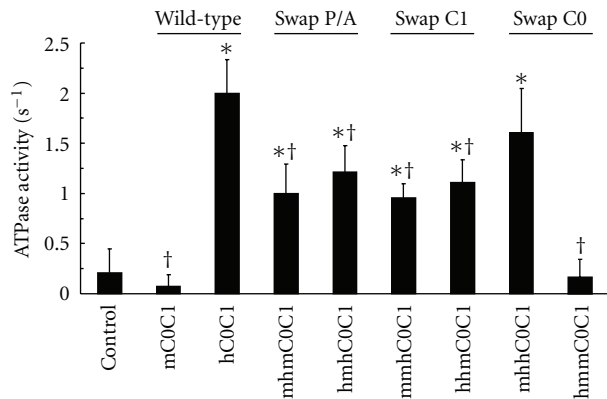


FIGURE 4: Effects of mouse and human C0, P/A, and C1 domains on thin filament activated myosin S1 ATPase assays at pCa 10 in the absence of myosin S2. 1 μ M mC0C1 had no effect on ATPase activity ($n = 9$), whereas 1 μ M hC0C1 increased ATPase activity ($n = 9$). The human P/A and C1 domains each activated ATPase activity (mhmC0C1 ($n = 7$) and mmhC0C1 ($n = 5$), resp.) to intermediate values. The mouse P/A and C1 domains (hmhC0C1, ($n = 5$) and hhmC0C1, ($n = 6$), resp.) depressed ATPase activity when compared to hC0C1. The human C0 domain (hmmC0C1, $n = 6$) had no activating effects on actin-activated S1 ATPase activity, while the mouse C0 domain (mhhC0C1, $n = 4$) did not affect activity when compared to hC0C1. Data are mean \pm SD. Asterisks (*) and crosses (†) denote significant differences when compared to mC0C1 and hC0C1, respectively, ($P < .05$).

the absence of recombinant proteins, ATPase rates were low ($0.2 \pm 0.2 \text{ sec}^{-1}$, $n = 17$) at low Ca^{2+} (pCa 10) and increased ($3.3 \pm 1.0 \text{ sec}^{-1}$, $n = 16$) in the presence of maximal Ca^{2+} (pCa 3). These results demonstrate that the thin filaments were well regulated by Ca^{2+} . Similar to the results obtained in the *in vitro* motility assays, addition of 1 μ M hC0C1 activated ATPase activity at pCa 10, whereas mC0C1 did not significantly affect ATPase rates. Addition of the human P/A domain or the C1 domains to the mouse C0C1 backbone (mhmC0C1 or mmhC0C1) also activated ATPase activity in the absence of Ca^{2+} , but not to the full extent of hC0C1. Substituting the mouse P/A and C1 domains in the human C0C1 backbone (hmhC0C1 and hhmC0C1) reduced ATPase activity when compared to hC0C1. Similar to motility assays (Figure 3(d)), exchanging the C0 domains (hmmC0C1 and mhhC0C1) had no effect on the ATPase rates when compared to mC0C1 and hC0C1 controls (Figure 4). Taken together, these results confirm conclusions from the *in vitro* motility assays that *either* the human P/A or C1 domains can confer activating properties to the hC0C1 protein, but that *both* domains are required for full effects. However, because all activating effects occurred in the absence of myosin S2, results from the ATPase assays demonstrate that activation does not require interactions of hC0C1 with myosin S2.

4. Discussion

The major result from this study is that species-specific differences between mouse and human cMyBP-C contribute to functional differences in the activities of recombinant

cMyBP-C proteins. In particular, we found significant sequence divergence (46% identity) in a proline-alanine (P/A) rich region near the N-terminus of the molecule. The human P/A rich region but not the mouse P/A region promoted actomyosin interactions as shown by the ability of proteins containing the human P/A region to increase thin filament motility and actomyosin ATPase activity even in the absence of Ca^{2+} (Figure 3). Interestingly, the human C1 domain but not mouse C1 also conferred activating effects even though the sequences of the C1 domains are much more similar (90% identity). Taken together, these results suggest that even modest sequence variations in conserved domains can lead to significant functional differences between homologous cMyBP-C proteins.

Results from this study partially reconcile disparate results from two previously published studies. Using human cMyBP-C recombinant proteins, Herron et al. [7] attributed activating effects to the P/A rich region, whereas Razumova et al. [6], using mouse recombinant proteins, found that the C1 and M domains together were required to activate force. In the present study we performed a side-by-side comparison of the effects of mouse and human C0C1 proteins and found in good agreement with Herron et al. [7] that the human P/A region can confer activating effects on actomyosin interactions, whereas also in good agreement with Razumova et al. [6] that the mouse P/A region does not. Thus, species-specific differences can account in part for the different conclusions reached in the two studies.

The precise mechanism(s) by which the P/A and C1 domains promote actomyosin interactions is not known, but interactions with either thick filaments [23] or actin [3] are possible. In support of the former, hC0C1 localizes to sarcomere A-bands when expressed in rat neonatal cardiomyocytes [7] and C1 can bind to myosin S2 [22]. However, the present results exclude interactions with thick filaments because myosin S2 was not required for hC0C1 to increase acto-S1 ATPase activity (Figure 4). Furthermore, while C0 was reported to bind to myosin S1 [21], C0 made little or no contribution to the activating effects of hC0C1 (Figures 3 and 4). Thus, interactions with myosin do not appear important for the activating effects of hC0C1, although interactions with other thick filament proteins (e.g., myosin light chains) cannot be excluded. Alternatively, the P/A region could interact with thin filaments to promote activation. In support of this idea Squire et al. [24] proposed that the P/A region of human cMyBP-C binds actin based on sequence similarity to a P/A rich segment found in essential myosin light chains (MLC). Although in a previous study we found little evidence that the mouse P/A region binds to actin because mC0C1 bound weakly if at all to F-actin in cosedimentation assays [3], Kulikovskaya et al. [25] reported that human C0C1 can bind actin. Side-by-side comparisons of binding affinity are needed, but these data are thus in the right direction for the Pro-Ala sequence to contribute to functional effects in human (e.g., because of greater actin binding affinity) but exert less effects in mouse because of reduced interactions with actin [6, 7].

The functional significance of the Pro-Ala rich sequence of cMyBP-C *in vivo* is not known. However, the Pro-Ala

rich sequences have been identified in other thick filament regulatory proteins including cardiac and skeletal isoforms of myosin essential light chains (MLC) [26]. In these proteins the Pro-Ala sequences modulate cross-bridge cycling rates and shortening velocity (V_{\max}) by binding to actin [27]. The proline-alanine rich regions of different MLC isoforms slow cross-bridge kinetics either by binding directly to actin [28] or by functioning as a rigid spacer arm that extends an actin binding site located near the N-terminus of the MLC out toward the thin filament [29]. In either case, interactions with actin are thought to create a drag that limits filament sliding and slows cross-bridge cycling and shortening velocity [30]. Consistent with this idea, atrial myocytes that express an atrial MLC isoform with reduced affinity for actin have nearly twice the V_{\max} and maximal power output than ventricular myocytes expressing an MLC that binds to actin with greater affinity [26].

By analogy with MLC, it is possible that the Pro-Ala rich region of cMyBP-C performs a similar role and contributes to the ability of cMyBP-C to limit myocyte shortening velocity, cross-bridge cycling, and power output [31]. If so, then the species-specific differences described here between the Pro-Ala regions of mouse and human cMyBP-C could serve to fine-tune shortening velocity to optimize power output (the product of force and velocity) such that contractile efficiency is maximized in hearts that contract under different hemodynamic loads and at different speeds. Consistent with this idea, we found that the percentage of proline and alanine residues in the Pro-Ala region varies inversely with heart rate in mammals such that larger mammals have a greater proportion of Pro-Ala residues, Shaffer and Harris [32]. Thus, decreased Pro-Ala content of cMyBP-C in small mammals could accelerate cross-bridge cycling kinetics, whereas increased Pro-Ala content in larger mammals could slow cycling rates. Species-specific changes in cMyBP-C could thereby fine-tune larger shifts in cross-bridge cycling kinetics that occur due to differences in isoform expression of other contractile proteins such as in myosin heavy chain that shifts from fast α -MHC (high ATPase activity and cross-bridge cycling) expressed in small mammals to slow β -MHC expressed in larger mammals [33–36]. Such systematic changes in cross-bridge cycling kinetics could also tune cardiac relaxation rates, for instance, to ensure adequate diastolic filling times even at high heart rates in small mammals.

In summary, results presented here demonstrate significant species-specific differences in the ability of the P/A rich region and C1 domains of mouse versus human cMyBP-C to activate actomyosin interactions. These differences suggest that the function of cMyBP-C varies in different species and raises the intriguing possibility that cMyBP-C fine-tunes cardiac contraction in different animals to better match contractile speed to hemodynamic load. Experiments to test these ideas are in progress.

Acknowledgments

The authors thank Elaine Hoye and David Dai for assistance in protein expression and purification, and Gordon Freeman

for helpful advice regarding use of the In-Fusion cloning system. They also thank Martha Mathiason and Mike Regnier for motion analysis software for filament motility assays. Support for this study was provided by NIH HL080367 to SPH and a NSF Graduate Research Fellowship to JFS.

References

- [1] J. E. Stelzer, J. R. Patel, J. W. Walker, and R. L. Moss, "Differential roles of cardiac myosin-binding protein C and cardiac troponin I in the myofibrillar force responses to protein kinase A phosphorylation," *Circulation Research*, vol. 101, no. 5, pp. 503–511, 2007.
- [2] C. W. Tong, J. E. Stelzer, M. L. Greaser, P. A. Powers, and R. L. Moss, "Acceleration of crossbridge kinetics by protein kinase A phosphorylation of cardiac myosin binding protein C modulates cardiac function," *Circulation Research*, vol. 103, no. 9, pp. 974–982, 2008.
- [3] J. F. Shaffer, R. W. Kensler, and S. P. Harris, "The myosin-binding protein C motif binds to F-actin in a phosphorylation-sensitive manner," *Journal of Biological Chemistry*, vol. 284, no. 18, pp. 12318–12327, 2009.
- [4] M. Gruen, H. Prinz, and M. Gautel, "cAPK-phosphorylation controls the interaction of the regulatory domain of cardiac myosin binding protein C with myosin-S2 in an on-off fashion," *FEBS Letters*, vol. 453, no. 3, pp. 254–259, 1999.
- [5] M. V. Razumova, J. F. Shaffer, A.-Y. Tu, G. V. Flint, M. Regnier, and S. P. Harris, "Effects of the N-terminal domains of myosin binding protein-C in an in vitro motility assay: evidence for long-lived cross-bridges," *Journal of Biological Chemistry*, vol. 281, no. 47, pp. 35846–35854, 2006.
- [6] M. V. Razumova, K. L. Bezold, A.-Y. Tu, M. Regnier, and S. P. Harris, "Contribution of the myosin binding protein C motif to functional effects in permeabilized rat trabeculae," *Journal of General Physiology*, vol. 132, no. 5, pp. 575–585, 2008.
- [7] T. J. Herron, E. Rostkova, G. Kunst, R. Chaturvedi, M. Gautel, and J. C. Kentish, "Activation of myocardial contraction by the N-terminal domains of myosin binding protein-C," *Circulation Research*, vol. 98, no. 10, pp. 1290–1298, 2006.
- [8] S. Bicer and P. J. Reiser, "Variations in apparent mass of mammalian fast-type myosin light chains correlate with species body size, from shrew to elephant," *American Journal of Physiology*, vol. 292, no. 1, pp. R527–R534, 2007.
- [9] E. Gasteiger, A. Gattiker, C. Hoogland, I. Ivanyi, R. D. Appel, and A. Bairoch, "ExpASY: the proteomics server for in-depth protein knowledge and analysis," *Nucleic Acids Research*, vol. 31, no. 13, pp. 3784–3788, 2003.
- [10] B. Zhu, G. Cai, E. O. Hall, and G. J. Freeman, "In-fusionTM assembly: seamless engineering of multidomain fusion proteins, modular vectors, and mutations," *BioTechniques*, vol. 43, no. 3, pp. 354–359, 2007.
- [11] M. Gautel, O. Zuffardi, P. Freiburg, and S. Labeit, "Phosphorylation switches specific for the cardiac isoform of myosin binding protein-C: a modulator of cardiac contraction?" *EMBO Journal*, vol. 14, no. 9, pp. 1952–1960, 1995.
- [12] E. Jain, A. Bairoch, S. Duvaud, et al., "Infrastructure for the life sciences: design and implementation of the UniProt website," *BMC Bioinformatics*, vol. 10, article 136, 2009.
- [13] S. S. Margossian and S. Lowey, "Preparation of myosin and its subfragments from rabbit skeletal muscle," *Methods in Enzymology*, vol. 85, part B, pp. 55–71, 1982.
- [14] J. D. Potter, "Preparation and properties of the components from troponin," *Methods in Enzymology*, vol. 85, part B, pp. 241–263, 1982.

- [15] J. D. Pardee and J. A. Spudich, "Purification of muscle actin," *Methods in Enzymology*, vol. 85, part B, pp. 164–181, 1982.
- [16] L. B. Smillie, "Preparation and identification of α - and β -tropomyosins," *Methods in Enzymology*, vol. 85, part B, pp. 234–241, 1982.
- [17] E. Homsher, B. Kim, A. Bobkova, and L. S. Tobacman, "Calcium regulation of thin filament movement in an in vitro motility assay," *Biophysical Journal*, vol. 70, no. 4, pp. 1881–1892, 1996.
- [18] D. A. Martyn, P. B. Chase, J. D. Hannon, L. L. Huntsman, M. J. Kushmerick, and A. M. Gordon, "Unloaded shortening of skinned muscle fibers from rabbit activated with and without Ca^{2+} ," *Biophysical Journal*, vol. 67, no. 5, pp. 1984–1993, 1994.
- [19] M. Regnier, A. J. Rivera, C.-K. Wang, M. A. Bates, P. B. Chase, and A. M. Gordon, "Thin filament near-neighbour regulatory unit interactions affect rabbit skeletal muscle steady-state force- Ca^{2+} relations," *Journal of Physiology*, vol. 540, pp. 485–497, 2002.
- [20] H. D. White, "Special instrumentation and techniques for kinetic studies of contractile systems," *Methods in Enzymology*, vol. 85, part B, pp. 698–708, 1982.
- [21] J. Flavigny, M. Souchet, P. Sébillon, et al., "COOH-terminal truncated cardiac myosin-binding protein C mutants resulting from familial hypertrophic cardiomyopathy mutations exhibit altered expression and/or incorporation in fetal rat cardiomyocytes," *Journal of Molecular Biology*, vol. 294, no. 2, pp. 443–456, 1999.
- [22] A. Ababou, E. Rostkova, S. Mistry, C. L. Masurier, M. Gautel, and M. Pfuhl, "Myosin binding protein C positioned to play a key role in regulation of muscle contraction: structure and interactions of domain C1," *Journal of Molecular Biology*, vol. 384, no. 3, pp. 615–630, 2008.
- [23] M. Gruen and M. Gautel, "Mutations in β -myosin S2 that cause familial hypertrophic cardiomyopathy (FHC) abolish the interaction with the regulatory domain of myosin-binding protein-C," *Journal of Molecular Biology*, vol. 286, no. 3, pp. 933–949, 1999.
- [24] J. M. Squire, P. K. Luther, and C. Knupp, "Structural evidence for the interaction of C-protein (MyBP-C) with actin and sequence identification of a possible actin-binding domain," *Journal of Molecular Biology*, vol. 331, no. 3, pp. 713–724, 2003.
- [25] I. Kulikovskaya, G. McClellan, J. Flavigny, L. Carrier, and S. Winegrad, "Effect of MyBP-C binding to actin on contractility in heart muscle," *Journal of General Physiology*, vol. 122, no. 6, pp. 761–774, 2003.
- [26] M. C. Schaub, et al., "Modulation of contractility in human cardiac hypertrophy by myosin essential light chain isoforms," *Cardiovascular Research*, vol. 37, no. 2, pp. 381–404, 1998.
- [27] O. A. Andreev, L. D. Saraswat, S. Lowey, C. Slaughter, and J. Borejdo, "Interaction of the N-terminus of chicken skeletal essential light chain 1 with F-actin," *Biochemistry*, vol. 38, no. 8, pp. 2480–2485, 1999.
- [28] I. P. Trayer, H. R. Trayer, and B. A. Levine, "Evidence that the N-terminal region of A1-light chain of myosin interacts directly with the C-terminal region of actin. A proton magnetic resonance study," *European Journal of Biochemistry*, vol. 164, no. 1, pp. 259–266, 1987.
- [29] D. J. Timson and I. P. Trayer, "The role of the proline-rich region in A1-type myosin essential light chains: implications for information transmission in the actomyosin complex," *FEBS Letters*, vol. 400, no. 1, pp. 31–36, 1997.
- [30] H. L. Sweeney, C. Reggiani, S. Lowey, et al., "Function of the N terminus of the myosin essential light chain of vertebrate striated muscle," *Biophysical Journal*, vol. 68, no. 4, supplement, pp. 112S–119S, 1995.
- [31] F. S. Korte, K. S. McDonald, S. P. Harris, and R. L. Moss, "Loaded shortening, power output, and rate of force redevelopment are increased with knockout of cardiac myosin binding protein-C," *Circulation Research*, vol. 93, no. 8, pp. 752–758, 2003.
- [32] J. F. Shaffer and S. P. Harris, "Species-specific differences in the Pro-Ala rich region of cardiac myosin binding protein-C," *Journal of Muscle Research and Cell Motility*, vol. 30, no. 7-8, pp. 303–306, 2009.
- [33] N. Hamilton and C. D. Ianuzzo, "Contractile and calcium regulating capacities of myocardia of different sized mammals scale with resting heart rate," *Molecular and Cellular Biochemistry*, vol. 106, no. 2, pp. 133–141, 1991.
- [34] B. Pope, J. F. Hoh, and A. Weeds, "The ATPase activities of rat cardiac myosin isoenzymes," *FEBS Letters*, vol. 118, no. 2, pp. 205–208, 1980.
- [35] I. Morano, H. Arndt, C. Gartner, and J. C. Ruegg, "Skinned fibers of human atrium and ventricle: myosin isoenzymes and contractility," *Circulation Research*, vol. 62, no. 3, pp. 632–639, 1988.
- [36] K. Schwartz, Y. Lecarpentier, and J. L. Martin, "Myosin isoenzymic distribution correlates with speed of myocardial contraction," *Journal of Molecular and Cellular Cardiology*, vol. 13, no. 12, pp. 1071–1075, 1981.