

Review Article

Myosin Binding Protein-C Slow: An Intricate Subfamily of Proteins

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Myosin binding protein C (MyBP-C) consists of a family of thick filament associated proteins. Three isoforms of MyBP-C exist in striated muscles: cardiac, slow skeletal, and fast skeletal. To date, most studies have focused on the cardiac form, due to its direct involvement in the development of hypertrophic cardiomyopathy. Here we focus on the slow skeletal form, discuss past and current literature, and present evidence to support that: (i) MyBP-C slow comprises a subfamily of four proteins, resulting from complex alternative shuffling of the single MyBP-C slow gene, (ii) the four MyBP-C slow isoforms are expressed in variable amounts in different skeletal muscles, (iii) at least one MyBP-C slow isoform is preferentially found at the periphery of *M*-bands and (iv) the MyBP-C slow subfamily may play important roles in the assembly and stabilization of sarcomeric *M*- and *A*-bands and regulate the contractile properties of the actomyosin filaments.

1. Introduction

Myofibrils, the workhorses of skeletal muscle, consist of interdigitating thick and thin filaments, and their associated membrane systems [1]. Muscle contraction and relaxation is mediated by the sliding of thick myosin filaments past thin actin filaments, under the strict regulation of Ca^{2+} release and reuptake via the sarcoplasmic reticulum (SR) [2]. In addition to housing the basic thick and thin filaments, the sarcomere also contains several accessory proteins that are involved in the assembly, maintenance, and regulation of contractile activity [1]. Myosin Binding Protein-C (MyBP-C) comprises a family of accessory proteins that contributes to the assembly and stabilization of thick filaments, and regulates the formation of cross-bridges between myosin and actin by interacting directly with both filamentous systems (as reviewed in [3]).

MyBP-C was originally identified from mammalian skeletal muscle as an impurity of isolated myosin. Using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Star and Offer were the first to separate a number of unidentified myosin-associated proteins that were consistently found in preparations of purified myosin [4]. MyBP-C, originally

termed C-protein for its location on SDS-PAGE as band C, was further characterized as a myosin binding protein of ~140 kDa using a combination of biochemical methods, ranging from gel filtration, to ammonium sulfate fractionation and single molecule electron microscopy [4–6]. The location of MyBP-C at striped intervals within the C-zone of the A-band of skeletal muscle was first observed with X-ray diffraction and immunoelectron microscopy [7], further supporting its association with the thick myosin filaments. Subsequent studies revealed that MyBP-C is arranged along the length of the A-band in 7–9 transverse stripes that are ~43 nm apart, with ~2–4 molecules of MyBP-C associating with each myosin cross-bridge [8–11].

The family of MyBP-C contains three isoforms: cardiac, slow skeletal, and fast skeletal, which are encoded by separate genes; in humans, these map to chromosomes 11, 12, and 19, respectively [12, 13]. The different isoforms have been cloned and sequenced from various species, including human, chicken, rabbit and mouse, allowing a thorough comparison of their molecular composition and primary sequence [13–16]. An ~65% identity is shared among the individual isoforms across species, while an ~50% homology is shared among the human cardiac, slow and fast forms.

The core structure of MyBP-C is composed of seven immunoglobulin (Ig) domains and three fibronectin type III (Fn-III) repeats, numbered from the NH₂-terminus as C1–C10 [17]. The C1 domain is flanked by two unique motifs, one enriched in proline and alanine residues, termed proline/alanine rich motif and a conserved linker, referred to as MyBP-C motif. Notably, the cardiac isoform possesses three additional features, which are absent from the skeletal forms of the protein. These include an Ig domain at the extreme NH₂-terminus of the molecule, termed C0, a unique 9-residues long insertion within the MyBP-C motif containing phosphorylation sites necessary for the protein's regulatory role in contraction, and a 28-amino acids long loop in the middle of the C5 domain [12, 16].

In mammals, cardiac MyBP-C is expressed early in development, along with titin and myosin [18, 19]. The skeletal isoforms of MyBP-C, however, are detected later in development, after the expression of titin and myosin, with the expression of slow MyBP-C preceding that of fast [18, 20]. By contrast, in chicken skeletal muscles the slow and fast isoforms appear concurrently at the end of late embryogenesis [21]. As development proceeds, though, the amounts of the slow form diminish, while the expression of the fast form persists through adulthood [21]. The fast and slow skeletal isoforms have been shown to coexpress in the same muscle type and can even coexist in the same sarcomere; expression of the cardiac isoform, however, is restricted to the developing and mature heart [15, 18, 22–24]. Interestingly, a recent study demonstrated that the expression of MyBP-C slow is not restricted to skeletal muscle but is also evidenced in the right atrium and interatrial septum of adult mammalian cardiac muscle [25].

To date, much of our knowledge on the molecular properties and functional activities of MyBP-C originates from the numerous studies that focus on the cardiac isoform. The reader is referred to excellent reviews discussing the structure of cardiac MyBP-C, its key roles in maintaining the normal structure of thick filaments and regulating cross-bridge cycling, and its involvement in the development of hypertrophic cardiomyopathy [3, 26, 27]. Here, we will focus on the slow form(s) of MyBP-C found in skeletal muscles. We will review past and current literature, discuss its role in the organization and stabilization of thick filaments, and provide evidence that MyBP-C slow comprises a subfamily of four alternatively spliced proteins that are expressed in variable amounts in slow and fast twitch skeletal muscles.

2. Materials and Methods

2.1. Reverse Transcription-Polymerase Chain Reaction. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA) from P1 rat skeletal myotubes cultured for seven days and from adult rat extensor digitorum longus (EDL), flexor digitorum brevis (FDB), tibialis anterior (TA), gastrocnemius (gastroc), quadriceps (quad), and soleus muscles. Aliquots containing ~5 µg of RNA were reverse transcribed using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) following the

manufacturer's instructions. PCR amplification of MyBP-C slow transcripts was performed with primer sets that flanked each of the three insertions; for the NH₂-terminal insert: Forward-1 (F1): 5' CCAGAACCCACTAAGAAAG 3' and Reverse-1 (R1): 5' GATCCTCGAGGTGCACTT CAA-GATCAA 3', for the insert within Ig7: Forward-2 (F2): 5' GATCGAATTCAGCCC TCCTACTCTT 3' and Reverse-2 (R2): 5' GATCCTCGAGGGGCTCGCTGGCACCA 3' and for the COOH-terminal insert: Forward-3 (F3): 5' CACC-CATGTTTACTCAACCCT 3' and Reverse-3 (R-3) 5' GTGA-CAATATACATTGAA 3'. To amplify the COOH-terminal insert from EDL and TA muscles, it was necessary to reamplify 0.5 µL of the original PCR for an additional 30 cycles for a total of 80 cycles. All other PCRs were carried out for 50 cycles. PCR products were analyzed by electrophoresis in 1% agarose gels and their authenticity was verified by sequence analysis.

2.2. Generation of Protein Lysates from P1 Myotubes and Adult Rat Muscle. Homogenates of P1 myotubes cultured for seven days as well as of EDL, FDB, TA, gastrocnemius, quadriceps and soleus muscles of adult Sprague-Dawley rats (Zivic-Miller Laboratories, Zelienople, PA) were prepared at RT with a Brinkman Polytron homogenizer at setting 3 (VWR, West Chester, PA) in 10 mM NaPO₄, pH 7.2, 2 mM EDTA, 10 mM NaN₃, 120 mM NaCl, 0.5% deoxycholate, 0.5% NP-40, supplemented with Complete protease inhibitors (Roche, Indianapolis, IN). Each sample (~60 µg) was solubilized in 4xNuPAGE reducing sample buffer (Invitrogen, Carlsbad, CA) at 90°C for 5 minutes, fractionated by 4–12% SDS-PAGE BisTris gel using MES running buffer (Invitrogen, Carlsbad, CA), transferred to nitrocellulose and probed with an antibody that recognizes the slow forms of MyBP-C (300 ng/mL, Abnova) or with an antibody that specifically recognizes the COOH-terminal insert [28]. After incubation with the appropriate secondary antibodies, immunoreactive bands were visualized with a chemiluminescence detection kit (Tropix, Bedford, MA).

2.3. Immuno-Electron Microscopy. Immuno-electron microscopy was performed as previously described with minor modifications [29, 30]. Briefly, adult mouse FDB skeletal muscle was fixed both *in situ*, via whole animal perfusion-fixation and *ex vivo* with 2% paraformaldehyde in PBS. Samples were snap-frozen in a slush of liquid N₂, cryosectioned (~20 µm thick), and incubated overnight at 4°C with the antibody that specifically recognizes the COOH-terminal insert of MyBP-C slow. Samples were incubated with secondary goat antirabbit IgG adsorbed on 1 nm gold particles (Nanoprobes Incorporated; Yaphank, NY) and subsequently with fluorescein-conjugated anti-goat IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Samples were enhanced with silver (HiQ Silver Kit, Nanoprobes) for 5 minutes, fixed overnight at 4°C in 2% glutaraldehyde and 5 mg/mL tannic acid in 0.2 M cacodylate buffer and postfixed with 50 mM acetate buffer 1% osmium tetroxide. They were further stained *en bloc* for 2 hours with 1% uranyl acetate in 65% ethanol,

dehydrated, and embedded in araldite (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin (60–90 nm) sections were prepared with an MT5000 ultramicrotome (LKB instruments Inc., Gaithersburg, MD), mounted on grids, labeled with 1% uranyl acetate followed by Reynolds lead citrate, and examined with a Philips-201 electron microscope.

3. Results and Discussion

3.1. MyBP-C Slow: A Subfamily of Proteins. To date, four different MyBP-C slow transcripts have been identified in human skeletal muscle referred to as variants 1–4 (Figure 1; accession numbers NM_002465, NM_206819, NM_206820, and NM_206821, respectively). The four variants differ from one another at three regions, due to alternative splicing events that result in inclusion of exons 3 and 4 in the proline/alanine-rich motif, exon 23 in the Ig7 domain and exon 31 at the extreme COOH-terminus (Figure 1(a)); these encode novel sequences of 25 (Figure 1(b)), 18 (Figure 1(c)), and 26 (Figure 1(d)) amino acids, respectively. Analysis of the primary sequence of the four MyBP-C slow variants indicated that variants 1 and 2 contain the NH₂-terminal insertion located in the proline/alanine rich motif, variant 3 carries the insertion within domain Ig7, while variant 4 also contains the unique COOH-terminal region (Figure 1(a)). Notably, variant 3 is the prototypical human isoform of MyBP-C slow that was characterized by Furst and colleagues in 1992 [14].

To study the relative expression of the four MyBP-C slow transcripts in different rat skeletal muscles, we used RT-PCR analysis to amplify the unique regions described above. To this end, we prepared cDNAs from a panel of adult and developing rat skeletal muscles that contained distinct compositions of slow and fast twitch skeletal myofibers. These included extensor digitorum longus (EDL; ~90:10, fast:slow; [31, 32]), flexor digitorum brevis (FDB; ~80:20, fast:slow; [33]), tibialis anterior (TA; ~70:30, fast:slow; [34]), gastrocnemius (gastroc; ~40:60, fast:slow; [35]), quadriceps (quad; ~60:40, fast:slow; [36]), soleus (20:80, fast:slow; [35]), and hindlimb skeletal myotubes of postnatal day 1 (P1) rat pups (Figure 2). Primer sets were designed to flank each of the three novel insertions (Figures 2(a)–2(c), cartoons in the upper left corner). Amplification of two PCR products with distinct sizes within each reaction indicated the presence of a mixed population of transcripts that contained (larger size product) and lacked (smaller size product) the respective insertion. On the contrary, amplification of one PCR product indicated the presence of a homogeneous population of transcripts that either included or excluded the corresponding insertion, depending on its size. Accordingly, PCR products that carry the NH₂-terminal, Ig7 and COOH-terminal inserts would be ~600, ~310, and ~350 nucleotides long, respectively, whereas PCR products that lack them would be ~530, ~260, and ~290 nucleotides long, respectively.

All skeletal muscles examined, independent of their fiber type composition, contained sufficient amounts of MyBP-C

slow transcripts to be amplified by conventional RT-PCR. Figure 2(a) shows the results following amplification of the NH₂-terminal insertion located within the proline/alanine rich motif. All muscle samples express MyBP-C slow transcripts that include the NH₂-terminal insert, albeit to varying degrees, as seen by the presence of an ~600 nts amplicon (Figure 2(a), upper band). This finding suggested that all muscles tested express variants 1 and/or 2, with EDL, TA, and soleus containing the highest amounts. Notably, all seven muscles also contain different amounts of transcripts that lack the NH₂-terminal insert, as shown by the presence of an ~530 nts product, indicating that they also express variants 3 and/or 4, with FDB showing the highest levels (Figure 2(a), lower band). Contrary to EDL, TA, soleus, and FDB that appear to preferentially contain MyBP-C slow variants that either include (EDL, TA and soleus) or exclude (FDB) the NH₂-terminal insert, gastroc, quad, and P1 myotubes show similar amounts of both amplification products, suggesting that transcripts possessing and lacking the NH₂-terminal insert may exist in similar levels within these muscles (Figure 2(a) lanes 4–5 and 7).

Next, we extended our analysis to the second novel insertion located within Ig7. As before, our primers set was designed to amplify MyBP-C slow variants that contained or skipped the Ig7 insert. Only soleus and P1 myotubes possess transcripts that include the unique insertion within Ig7, as indicated by the presence of an ~310 nts band (Figure 2(b), lanes 6 and 7, upper band), corresponding to variant 3. As expected, all skeletal muscles tested contained transcripts that lack the Ig7 insertion, as shown by the amplification of an ~260 nts product, representing variants 1, 2 and/or 4. Thus, variant 3 is restricted to soleus muscle and developing myotubes.

Last, we generated the appropriate primers to amplify the region flanking the COOH-terminal insert of MyBP-C slow. With the exception of quadriceps muscle, all other muscles tested, contained an amplification product of ~350 nucleotides, that includes the novel COOH-terminal insert detected only in variant 1, with soleus and FDB showing the highest amounts (Figure 2(c), lanes 6 and 2, upper band). A second amplification product of ~290 nucleotides was also detected in all seven muscles, which lacks the unique COOH-terminal insertion, present in variant 1, but includes the common COOH-terminal region shared by variants 2, 3, and 4 (Figure 2(c), lower band).

The RT-PCR data is summarized in Table 1. Taken together, our results suggest that: (i) mRNA encoding MyBP-C slow is present in all skeletal muscles examined, regardless of fiber type composition or age, and (ii) complex alternative shuffling of the single MyBP-C slow gene in the various muscles results in the differential expression of the four MyBP-C slow isoforms.

To correlate the mRNA expression of the transcripts that contain or lack the three novel insertions, as seen by the RT-PCR, to the expression of the proteins that they encode, we used western blot analysis. Homogenates from EDL, FDB, TA, gastroc, quad, soleus, and P1 skeletal myotubes were separated on 4–12% gradient gel, which our laboratory has previously shown to provide optimal

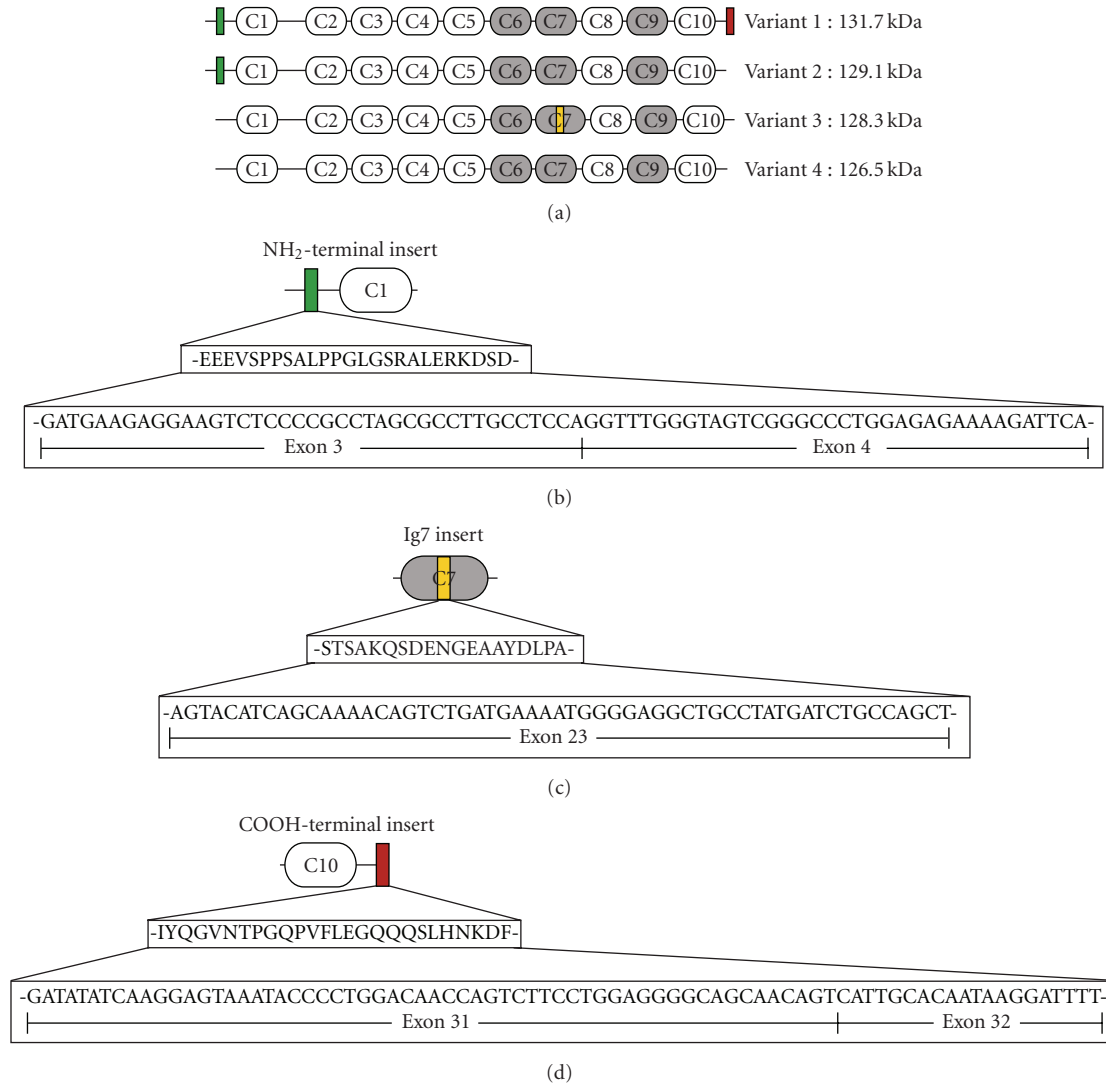


FIGURE 1: (a): Schematic representation of MyBP-C slow variants 1–4, showing their common structural motifs and novel insertions; white and grey ovals represent Ig and FN-III domains, respectively, while green, yellow, and red rectangles correspond to the NH₂-terminal, Ig7, and COOH-terminal inserts, respectively. The NH₂-terminal insert is present in variants 1 and 2, while the Ig7 and COOH-terminal inserts are present in variants 3 and 1, respectively. Variant 4 does not contain any of the three inserts. (b–d): Complex alternative splicing events result in the inclusion of exons 3 and 4, exon 23 and exon 31 that encode the NH₂-terminal (b), Ig7 (c), and COOH-terminal (d) novel insertions. The amino acid and nucleotide compositions of the three insertions are shown.

separation of the MyBP-C slow isoforms (see Section 2, and [28]). Homogenates were probed with a commercial antibody that recognizes a region within domain C5 common to all MyBP-C slow variants (Figure 3(a)). We were able to resolve at least 3 immunoreactive bands based on their distinct electrophoretic mobilities. The top band may represent variant 1 (~132 kDa, Figure 3(a), top panel, marked with a blue dot), the middle band may correspond to variants 2 and/or 3 (~129 and ~128 kDa, respectively, Figure 3(a), top panel, denoted with a red dot), and the bottom band may represent variant 4 (~126 kDa, Figure 3(a), top panel, marked with a green dot). The cartoon shown in the bottom panel of Figure 3(a) illustrates a representative image of several western blots, analyzed at

varying exposure times. The dotted lines indicate immunoreactive bands that only become evident after periods of long exposure.

Consistent with the RT-PCR data, our immunoblots also demonstrated the presence of at least one form of MyBP-C slow in each skeletal muscle tested. EDL and gastrocnemius possess three immunoreactive bands with the most prominent being the middle one (Figure 3(a), lanes 1 and 4). FDB also contains three distinct bands, however, the top and middle bands appear to be of similar intensities and occasionally appear as a broad, single band (Figure 3(a), lane 2). Similar to FDB, TA also shows two closely migrating bands, but lacks the bottom one (Figure 3(a) lane 3). Contrary to the rest of the muscles analyzed, quadriceps

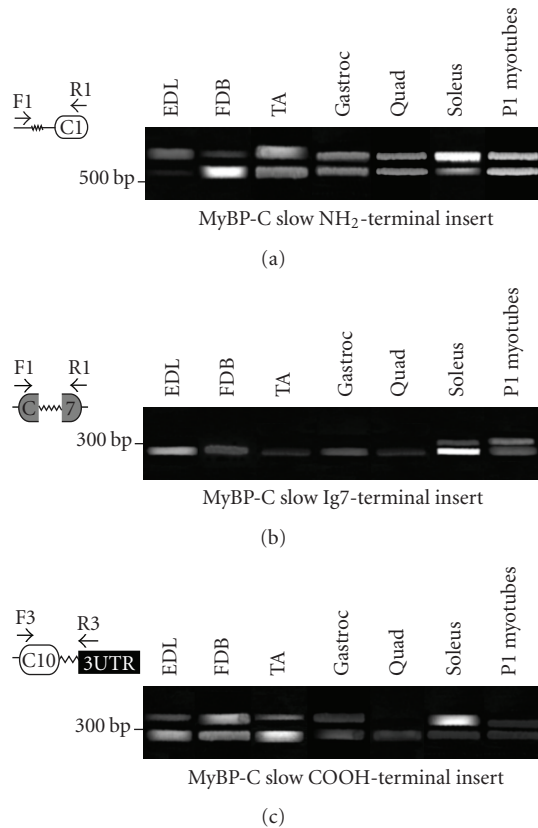


FIGURE 2: RT-PCR analysis using cDNA generated from developing and adult rat extensor digitorum longus (EDL), flexor digitorum brevis (FDB), tibialis anterior (TA), gastrocnemius (gastroc), quadriceps (quad) and soleus skeletal muscles and primer sets designed to flank the NH₂-terminal (a), Ig7 (b), and COOH-terminal (c) insertions. A schematic representation of the amplified region is shown in the upper left corner of each panel. White and grey ovals depict Ig and FN-III domains, respectively, while the 3' UTR is shown as a black line. The zig-zag lines denote the three novel insertions. In each amplification reaction, the top band corresponds to transcripts that include the insertion of interest, whereas the bottom band corresponds to transcripts that lack it. (a): All skeletal muscles tested contain a mixed population of MyBP-C slow variants that contain and lack the NH₂-terminal insertion, as indicated by the presence of two amplification products. (b): On the contrary, only soleus and developing myotubes express transcripts that include the Ig7 insertion. (c): With the exception of quadriceps muscle that only contains MyBP-C slow variants that lack the COOH-terminal insertion, all other muscles examined contain a mixed pool of transcripts that carry and lack the novel COOH-terminal insertion.

appears to contain only the middle band, although, the bottom one becomes evident after long exposure times (Figure 3(a) lane 5). Soleus expresses high amounts of the upper and lower bands, but moderate to low amounts of the middle one (Figure 3(a) lane 6). Finally, developing P1 skeletal myotubes show only one immunoreactive band with the same mobility as the top one, however, following longer exposure, the middle immunoreactive band is also apparent (Figure 3(a) lane 7).

TABLE 1: Tabulated summary of the RT-PCR data shown in Figure 2. The plus (+) and minus (–) signs denote the presence or absence of mRNAs encoding the three regions of interest, respectively; the relative abundance of the respective transcripts is illustrated by the number of plus signs.

Muscle Type	NH ₂ -insert	Ig7-insert	COOH-insert
Extensor Digitorum Longus	++	–	+
Flexor Digitorum Brevis	+	–	++
Tibialis Anterior	+++	–	+
Gastrocnemius	++	–	+
Quadriceps	++	–	–
Soleus	++++	+	+++
P1 Myotubes	+++	++	+

We also probed the same muscle homogenates with an antibody that is specific for the COOH-terminal insert and therefore only recognizes MyBP-C slow variant 1 (Figure 3(b)). With the exception of quadriceps, all of the other muscles were immunopositive for variant 1. This finding is in agreement with our immunoblot data shown in panel A, and the RT-PCR analysis shown in Figure 2(c), which revealed that quadriceps was the sole muscle to lack the top immunoreactive band (i.e., variant 1) and the COOH-terminal insert, respectively. Notably, MyBP-C slow variant 1 appears to be expressed more abundantly in soleus muscle and least prominently in developing myotubes, while the remaining muscles tested contained intermediate amounts. The slight differences in the sizes of the bands detected in the different muscles are likely due to posttranslational modifications, as it has previously been shown that MyBP-C slow is capable of phosphorylation [37, 38].

Taken together, our RT-PCR and immunoblotting data (summarized in Table 2) clearly indicate that all skeletal muscles tested, apart from quadriceps, express variant 1, albeit to different extents, with soleus and FDB containing the highest amounts. Variants 2 and 3 have complementary expression profiles, with variant 2 being preferentially expressed in muscles that have a higher composition of fast twitch fibers, such as EDL, FDB, TA, gastrocnemius and quadriceps, and variant 3 being selectively present in slow twitch muscles, like soleus, and developing myotubes. Finally, variant 4 is detected in all muscles examined, with the exception of TA and developing myotubes. It therefore appears that different forms of MyBP-C slow are present within the same muscle, independently of its fiber type composition, developmental stage, or age.

3.2. MyBP-C at the A-band. Early studies have confirmed the direct interaction between MyBP-C and myosin and identified the sites of binding on both proteins. The light meromyosin (LMM) region of the myosin rod, that forms the backbone of the thick filament, binds the COOH-terminal C10 region of all three MyBP-C isoforms [39–41]. Positively charged residues on the surface of the C10 domain mediate binding to LMM [42], however, the interaction is significantly strengthened by the presence of the C8

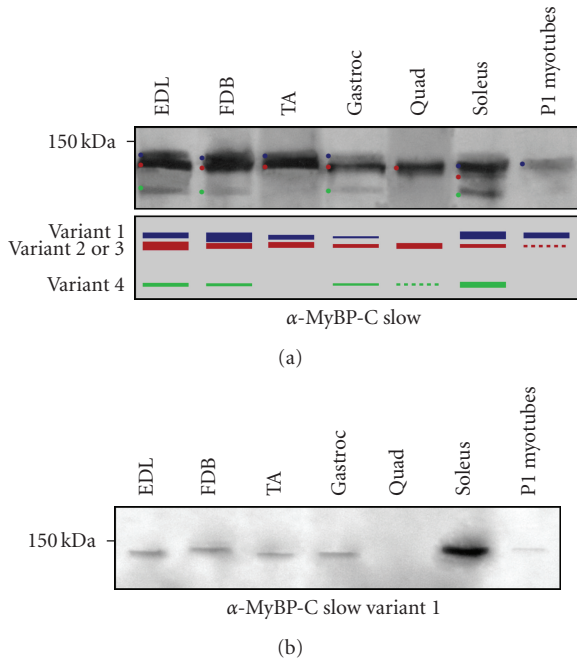


FIGURE 3: Western blot analysis of protein homogenates prepared from rat skeletal muscles and blotted with antibodies to the fifth Ig domain that recognize all MyBP-C slow variants (a) or to the novel COOH-terminal insertion that specifically recognizes variant 1 (b). (a) Top panel: With the exception of quadriceps, all other muscles express variant 1, represented by the upper most band, marked by a blue dot. All adult skeletal muscles tested, but not the developing myofibers, express variants 2 and/or 3, corresponding to the band with the intermediate mobility, and denoted with a red dot. As variants 2 and 3 have similar molecular weights (129 and 128 kDa, respectively), it is not feasible to separate them in the current gel system. Moreover, variant 4 is detected in homogenates prepared from EDL, FDB, gastrocnemius, and soleus, and is represented by the lower immunoreactive band, marked with a green dot. Bottom panel: A cartoon showing the presence of the different MyBP-C slow variants in developing and adult skeletal muscles. Dotted lines correspond to immunoreactive bands, which are evident only after long exposure times. (b) In agreement with the immunoblot shown in panel (a), antibodies specific for the novel COOH-terminal insert demonstrated that EDL, FDB, TA, gastrocnemius, soleus, and P1 skeletal myotubes express variant 1, with soleus containing the highest amounts.

and C9 regions. Another interaction between the MyBP-C motif, located at the NH₂-terminus of MyBP-C and subfragment 2 (S2) of myosin has been also identified, albeit of weaker affinity [43]. In addition to binding myosin, MyBP-C associates with actin, in a Ca²⁺ dependent manner, through its NH₂-terminal proline/alanine rich motif [44–46]. The ability of MyBP-C to directly interact with both myosin and actin facilitates its role as a regulator of cross-bridge formation during contraction. Interestingly, at low ionic strength MyBP-C inhibits actomyosin ATPase activity, while at higher ionic strength it acts as a mild activator [47, 48].

In addition to myosin, MyBP-C also associates with titin at the A-band [14, 49–51]. The COOH-terminal C8-C10

TABLE 2: MyBP-C slow variants 1–4 are present in varying amounts in different skeletal muscles. A plus (+) sign indicates the presence of the respective variant in a select muscle. The relative abundance of variants 1–4 in the skeletal muscles tested is illustrated by the number of plus signs.

Muscle Type	Variant 1	Variant 2	Variant 3	Variant 4
Extensor Digitorum Longus	++	+++	—	++
Flexor Digitorum Brevis	+++	++	—	++
Tibialis Anterior	++	++	—	—
Gastrocnemius	+	+	—	+
Quadriceps	—	++	—	—
Soleus	++++	—	+	+++
P1 Myotubes	++	—	+	—

domains of MyBP-C directly bind to the 11-domain super-repeat [Ig-(FN-III)₂-Ig-(FN-III)₃-Ig-(FN-III)₃] present in the C-zone portion of titin, and specifically the first Ig domain, although flanking motifs further strengthen the interaction [52, 53]. Notably, the arrangement of MyBP-C in 11 transverse stripes at regular intervals of ~43 nm corresponds to that of the 11-domain super-repeat of titin, to which MyBP-C binds. Consequently, it has been suggested that binding to titin's super-repeats specifies the subsarcomeric distribution of MyBP-C in the C-zone of the A-band [52].

Recent studies have proposed that MyBP-C forms a trimeric “collar” around each thick filament in which domains C5–C7 of one molecule overlap with domains C8–C10 of the neighboring molecule [54, 55]. In vitro binding studies using the respective peptides postulated that this arrangement might apply to the cardiac and fast skeletal isoforms, but not the slow isoform. Interestingly, for these studies Flashman and coworkers generated a recombinant protein that contained domains C8–C10, present in all four slow variants, followed by the novel COOH-terminal insertion, present only in variant 1. It is, therefore, possible that the presence of the COOH-terminal 26-amino acids may inhibit binding of the C8–C10 domains of variant 1 to the C5–C7 domains of the neighboring molecule of MyBP-slow. Indeed, this is consistent with our recent studies indicating that variant 1 is preferentially localized at the periphery of the M-band [28]. Conversely, an interaction between the respective motifs present in variants 2–4 is likely, as these contain a short COOH-terminus following domain C10 that, similar to the cardiac and fast isoforms, consists of four amino acids.

Through its interactions with myosin and titin, MyBP-C contributes to the stabilization and maintenance of the sarcomeric A-band. In vitro studies indicate that myosin filaments are capable of forming in the absence of MyBP-C, however, its addition results in increased filament length, and improved structure and uniformity across the filament [40, 56, 57]. Additionally, in vivo deletion of the myosin

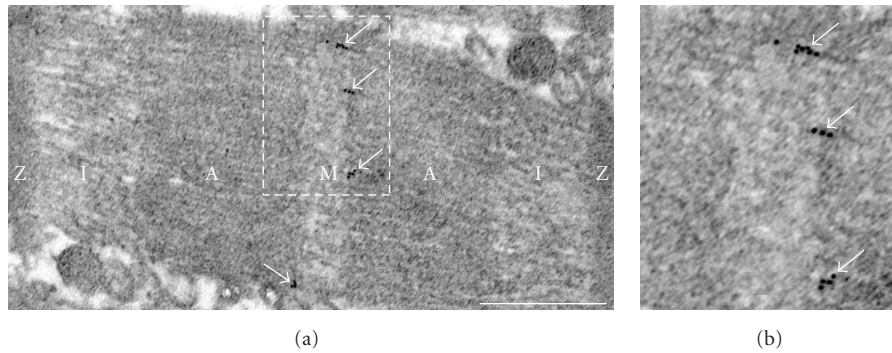


FIGURE 4: Ultrathin cryo-sections of adult mouse FDB skeletal muscle were labeled with antibodies specific for the COOH-terminal insertion present in MyBP-C slow variant 1. (a) Variant 1 was detected at the periphery of the *M*-band (arrows). The boxed area in (a) is blown up in (b) for ease of visualization of the immunolabeling. Scale bar corresponds to 0.5 μm .

and titin binding sites on MyBP-C results in disorganized *A*-bands [58, 59]. Consistent with this, the C8–C10 domains that harbor the binding sites for myosin and titin are deleted in patients suffering from familial hypertrophic cardiomyopathy [3, 60, 61]. Interestingly, though, normal *A*-bands are formed in animals deficient of cardiac MyBP-C [62, 63], suggesting the presence of a compensatory mechanism that maintains the myosin and titin filaments within the *A*-band of the sarcomere. Taken together, these studies suggest that MyBP-C, through its direct interaction with myosin and titin, and possibly its self-association, may stabilize the sarcomeric *A*-band.

In addition to maintaining the structure of thick filaments, MyBP-C may also play key roles in regulating contractile function by modulating the rate of cross-bridges formation. Consistent with this, in cardiomyocytes lacking MyBP-C, actomyosin filaments exhibited increased power output during contraction and faster rates of force development at half maximal Ca^{2+} activation [64]. Reintroduction of the NH_2 -terminal region of cardiac MyBP-C, containing the myosin S2 binding site (i.e., domains C1 and C2), enhanced Ca^{2+} sensitivity and restored the contractile properties of the null cardiomyocytes to normal levels [65], suggesting that cardiac MyBP-C contributes to the regulation of myofilament tension and their cycling rates.

Contrary to the cardiac isoform that has been directly implicated in the regulation of the contractile properties of cardiomyocytes (reviewed in [3]), studies focusing on the function of the skeletal forms of MyBP-C are limited. However, early studies have suggested that the role of MyBP-C in skeletal muscle likely parallels that of the cardiac isoform [66]. Consistent with this, the amounts of MyBP-C slow were recently found significantly increased in EDL muscles from a mouse model in which the kinase domain of titin was deleted [67]. Deficient EDL muscles exhibited reduced myofilament Ca^{2+} sensitivity and altered contractile properties, which were restored to normal levels upon extraction of MyBP-C slow. Contrary to the slow form of MyBP-C, the fast form was significantly downregulated in the same muscles, while the expression levels of other proteins of the *A*- and *M*-bands remained unaffected. It is, therefore, likely that the

kinase domain of titin affects the expression of genes involved in the regulation of myofilament Ca^{2+} sensitivity and force production. Experimental evidence has therefore started to emerge suggesting a key role for the skeletal forms of MyBP-C in modulating contractility, too.

3.3. Mybp-C Slow Variant 1 Selectively Concentrates at the *M*-Band. The presence of MyBP-C slow at the C-zones of the *A*-band has been studied extensively during the last three decades [3, 26, 68]. Recent studies from our laboratory, however, have provided evidence that at least one form of MyBP-C slow, specifically variant 1, has a unique topography in the muscle cell [28]. Detailed immunofluorescence studies combined with confocal microscopy demonstrated that MyBP-C slow variant 1 is selectively localized at the periphery of the *M*-band in adult rat soleus muscle [28]. The unique localization of MyBP-C slow variant 1 is further supported by our ultrastructural studies, shown in Figure 4. Immunolabeling of ultrathin cryosections prepared from adult mouse FDB muscle with antibodies to the unique COOH-terminus of variant 1 also demonstrated that it preferentially concentrates at the edges of the sarcomeric *M*-band (Figures 4(a)–4(b), arrowheads). Thus, it appears that the different MyBP-C slow isoforms have distinct distributions in skeletal myofibers, with variants 2–4 localizing at the *A*-band and variant 1 at the *M*-band.

At the *M*-band, MyBP-C slow variant 1 interacts with obscurin and four and a half lim protein 1 (FHL1) [28, 69]. The second Ig domain of obscurin and the last Ig domain of MyBP-C slow variant 1 are both necessary and sufficient to support their interaction, however, binding is enhanced significantly in the presence of the novel 26 amino acids at the COOH-terminus of variant 1. Overexpression of the second Ig domain of obscurin in primary cultures of skeletal myotubes inhibited the integration of MyBP-C slow variant 1 at the *M*-band and severely disrupted the formation of *M*- and *A*-bands. These findings suggested that variant 1 might contribute to the assembly and integrity of these structures via its interaction with obscurin and possibly other proteins. Similarly, McGrath et al. also demonstrated

that overexpression or downregulation of FHL1, which is localized at the A-I junction and the M-band, in adult mouse soleus muscle resulted in impaired thick filament assembly, which was accompanied by reduced sarcomeric incorporation of different forms of MyBP-C slow, including variant 1 [69].

3.4. MyBP-C Slow in Muscle Disease. Mutations within the cardiac isoform of MyBP-C cause familial hypertrophic cardiomyopathy (reviewed in [70, 71]). To date, there are no muscle diseases causally associated with mutations in the transcripts of the skeletal forms of MyBP-C. However, changes in the expression levels of the skeletal isoforms have been reported in hypertrophic and dystrophic skeletal muscles. The expression levels of MyBP-C slow were significantly increased in rat plantaris muscle induced to hypertrophy by surgical ablation of the neighboring soleus and gastrocnemius muscles, whereas the amounts of MyBP-C fast were dramatically decreased [72]. Likewise, the protein levels of MyBP-C slow were increased in both chicken and mouse dystrophic skeletal muscles [20, 24]. Further research is required in order to understand the molecular mechanisms that lead to the differential regulation of the skeletal forms of MyBP-C in these models, and their potentially unique roles in regulating the formation and activity of contractile structures.

4. Summary and Future Perspectives

MyBP-C slow comprises a subfamily of at least four isoforms that result from complex alternative splicing of the single MyBP-C slow gene. The presence of the four MyBP-C slow variants is not restricted to slow-twitch muscles, as select isoforms are abundantly expressed in fast-twitch muscles, too, where they may coexist with MyBP-C fast. More importantly, the four isoforms of MyBP-C slow may be coexpressed in the same muscle, fiber or sarcomere, where they may exhibit unique topographies concentrating either at A-bands (variants 2, 3, and 4) or M-bands (variant 1). Previous and current findings strongly favor a structural role for MyBP-C slow at the sarcomeric A- and M-bands, and point to a regulatory role on muscle contraction. Consequently, the detailed characterization of the biochemical and biophysical properties of the four MyBP-C slow variants, and the development of the appropriate molecular tools and animal models are imperative in order to study the cell biology and functional properties of this intricate subfamily of proteins.

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