Isoform Transitions of the Myosin Binding Protein C Family in Developing Human and Mouse Muscles

Lack of Isoform Transcomplementation in Cardiac Muscle

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Abstract—Mutations in the gene for the cardiac isoform of myosin binding protein C (MyBP-C) have been identified as the cause of chromosome 11–associated autosomal-dominant familial hypertrophic cardiomyopathy (FHC). Most mutations produce a truncated polypeptide that lacks the sarcomeric binding region. We have now investigated the expression pattern of the cardiac and skeletal isoforms of cMyBP-C in mice and humans by in situ hybridization and immunofluorescence microscopy using specific antibodies and probes. We demonstrate that the cardiac isoform is expressed only in cardiac muscle throughout development. The slow and fast isoforms of MyBP-C remain specific for skeletal muscle, where they can be coexpressed. Immunological evidence also suggests that an embryonic isoform of MyBP-C precedes the expression of slow MyBP-C in developing skeletal muscle. This suggests that transcomplementation of MyBP-C isoforms is possible in skeletal but not cardiac muscle. **(*Circ Res*. 1998;82:124-129.)**

Key Words: familial hypertrophic cardiomyopathy ■ myosin binding protein C ■ C-protein isoforms ■ embryonic expression patterns ■ cardiac muscle

Myosin filaments in striated muscle are assembled by the polymerization of myosin in association with a family of MyBPs and titin. These associated proteins[1] all share the molecular building plan of the immunoglobulin superfamily.[2–5] Titin is thought to fulfill the role of a molecular ruler,[6] and MyBP-C might play a role as a spatially defined regulatory protein.[5,7–9] Myosin, MyBP-C, and titin form a stable ternary complex where MyBP-C is arranged regularly in 9 of the 11 thick-filament stripes.[1,10–12]

Mutations in the gene for cMyBP-C are the cause of the chromosome 11–associated form of FHC. Most mutations identified so far lead to truncated proteins lacking the thick-filament binding region of cMyBP-C.[13–16] Protein analysis of cardiac biopsies from a patient with a cMyBP-C–associated FHC has indicated that the truncated protein is not present in detectable amounts.[16] A possible explanation for the cellular events in cMyBP-C–associated FHC could therefore be a haplophenotype caused by an altered protein stoichiometry. This would suggest that the missing allele cannot be complemented by any of the other known MyBP-C isoforms.

Skeletal muscle contains at least two isoforms of MyBP-C, expressed predominantly either in slow or fast muscle fibers.[12,17,18] However, these isoforms can be coexpressed in single myofibrils in varying stoichiometries leading to diverse arrangements of the characteristic sarcomeric stripes.[12] Therefore, skeletal muscle has a great potential to adapt in a flexible way to alterations in MyBP-C isoform stoichiometry by the modification of coexpression ratios. So far, it is unclear whether the same applies for cardiac muscle, a question of pressing importance for a more detailed understanding of the cMyBP-C–associated FHCs. Therefore, in the present study, we examine the expression patterns of all known MyBP-C isoforms in mammalian skeletal and cardiac muscle to elucidate this question and suggest that the cardiac isoform of MyBP-C cannot be transcomplemented by skeletal MyBP-Cs.

Materials and Methods

Antibodies

Monoclonal antibodies were raised against the recombinant N-termini of the slow and fast MyBP-C isoforms: Original λ phage isolates containing cDNAs coding for human slow MyBP-C and fast MyBP-C[4,19] were used as templates for PCR amplification[20] of domains 1 and 2, encompassing the MyBP-C motif. PCR products were cloned into a pET23a vector (Novagen), providing the resulting proteins with an additional histidine tag at their amino termini. Since this tag is recognized by a monoclonal anti-His tag antibody (DIANOVA), expression in *Escherichia coli* BL21(DE3)pLysS can be monitored by immunoblot analysis. Purification of the soluble recombinant proteins on Ni-NTA agarose columns based on their oligohistidine tags followed standard protocols (Qiagen).

Specific monoclonal antibodies were obtained from female Balb/c mice that had been immunized by a standard immunization scheme[21,22] using purified recombinant proteins. Hybridomas were made monoclonal by limiting dilution and characterized further by immunofluorescence and immunoelectron microscopy[21,23] and by Western blotting. This yielded the following monoclonal antibodies: α-sMyBP-C (reactive only with slow MyBP-C) and α-fMyBP-C

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Selected Abbreviations and Acronyms

cMyBP-C = cardiac isoform of MyBP-C
FHC = familial hypertrophic cardiomyopathy
gd (with number) = gestation day
MHC = myosin heavy chain
MyBP = myosin binding protein
MyBP-C = myosin binding protein C
PCR = polymerase chain reaction

(specific for fast MyBP-C). Antibodies were used as undiluted hybridoma supernatants.

Polyclonal antibodies were raised in rabbits against recombinant fragments of human cMyBP-C (region C0-C1[24]) and domains 1 and 2 of human slow MyBP-C. Sera were used at dilutions of 1:500 for indirect immunofluorescence and at 1:2000 for Western blot analysis. The preimmune sera were found to be unreactive against their respective antigens in immunofluorescence and Western blotting. The monoclonal antibodies I18/23 and S54/6 against the cardiac and ubiquitous isoforms of titin were reported previously.[25] Antibodies directed against sarcomeric myosin, actin, titin, and desmin were also described before.[26]

Probes

For in situ hybridization, the following probes were used: cMyBP-C, a fragment comprising base pairs 33 to 785 of human cMyBP-C (corresponding to the cardiac-specific region C0-C1[24]); slow MyBP-C, complete cDNA[4]; fast MyBP-C, a fragment comprising base pairs 1270 to 3560 of the coding sequence amplified by reverse-transcriptase PCR using human skeletal muscle cDNA (Clontech) as a template (primer sequences were designed from the sequence published[19]); and MHC probes complementary to the 3′ untranslated regions of MHC-β/slow, MHC-2A, and MHC-2X transcripts.[27] For in situ hybridization, antisense probes of these fragments were used. Sense probes of the same regions were tested by Northern hybridization on human multitissue blots (Clontech) and found to be negative, whereas the antisense probes detect their respective message (data not shown).

Embryos

Mouse embryos were from spontaneous matings of Balb/c mice. The presence of a vaginal plug indicating a successful mating was regarded as gd 0. Pregnant mice were killed by cervical dislocation, and their uterine horns were removed and dissected. Further treatment of embryos was as described.[28]

Human tissues were obtained from 11-week-old and 19-week-old human fetuses from induced or spontaneous abortions and from adult human skeletal muscle by needle biopsy in normal volunteers.[29]

In Situ Hybridization and Immunocytochemistry

Cryosections of human fetal and adult muscle samples (see above) were processed for in situ hybridization and immunoperoxidase cytochemistry as previously described.[30] Final concentration of ^{35}S-labeled cRNA probes was 25 000 to 50 000 cpm/μL; slides were processed for autoradiography using Kodak NTB-2 emulsion and exposed for 3 to 15 days (generally, 7 days). The distribution of MyBP-C transcripts and isoproteins in the different fiber types present in adult human muscle was determined using the DNA probes and antibodies specific for the MyBP-C and MHC isoforms described above. Preparation of mouse embryos for immunofluorescence microscopy was as described.[26]

Miscellaneous Methods

Protein extraction and blotting procedures were performed by standard procedures. Mouse embryo carcasses were dissected by decapitation and evisceration of freshly removed embryos in ice-cold Ringer's solution under a binocular microscope at ×20 magnification. The tissues, consisting only of the rump and thus largely of somitic tissue, were transferred into liquid nitrogen immediately after dissection. The tissues were pulverized under liquid nitrogen in a mortar, transferred immediately into sample buffer,[31] and subsequently analyzed on 8% SDS-PAGE. A standard high molecular weight marker (Bio-Rad) was used for reference. All DNA cloning steps followed standard protocols.[32]

Results

Characterization of Antibodies

Tissue specificity of all the antibodies used in the present study was first controlled by immunofluorescence microscopy using frozen sections of mouse and baboon tissues (Bios). Western blots on total extracts of murine skeletal and cardiac muscle extracts confirmed their reactivity with specific MyBP-C isoforms. Finally, immunoelectron microscopy using Triton-extracted fiber preparations of rat slow and fast skeletal muscles and cardiac ventricle revealed the sarcomeric localization described in earlier studies (results not shown). The polyclonal antibody α-panC was raised against the recombinant MyBP-C motif of human slow MyBP-C. This protein motif is found only in members of the MyBP-C family, where it is highly conserved.[5] The antibody α-panC was found to react with MyBP-C of all muscle types, including cardiac muscle (results not shown).

Expression of MyBP-C Isoforms in Embryonic and Adult Mouse Tissues

In skeletal muscle, the isoforms of MyBP-C clearly appeared in a developmentally regulated pattern demonstrating a successive expression of the slow and fast isoforms of MyBP-C (Fig 1). Expression of the slow isoform of MyBP-C succeeded the expression of titin and sarcomeric myosin by 4 or 5 days, ie, at approximately gd 14, a time when primary myotubes have begun to form. Thus, slow or fast MyBP-C and titin/myosin are not coexpressed during early stages of sarcomere formation in mononuclear myoblasts. The fast isoform of MyBP-C was not detected until 4 days later and was then coexpressed in some muscles together with the slow isoform. Cardiac tissue was never stained by antibodies against the slow or fast skeletal isoforms of MyBP-C.

Antibodies directed against the cardiac isoform of MyBP-C showed strong staining of the developing heart from gd 8 onward and coincided with the staining for cardiac titin (I18/23[25]). However, the cMyBP-C antibody failed to detect any protein in somite-derived muscles at any developmental stage. An interesting aspect was revealed when the analysis of embryo sections was performed with the pan-MyBP-C–reactive antibody α-panC: strong staining was observed in somites from gd 9.5 onward. Cardiac muscle was stained as well. This somitic expression pattern immediately followed the staining for titin and coincided with the staining for the sarcomeric isoforms of MHC and actin (data not shown) (Fig 1).

Western blot analysis of mouse embryos of gd 10.5 using the antibodies against the cardiac (region C0-C1) and slow MyBP-C isoforms and the pan-reactive antibody α-panC demonstrated a band reactive with the panC antibody of an approximate molecular weight of 150 kD in embryo carcasses, containing only somitic tissues. In the same somitic specimens, there is no detectable expression of cMyBP-C in whole embryo bodies that also contain the heart (Fig 2). Reaction

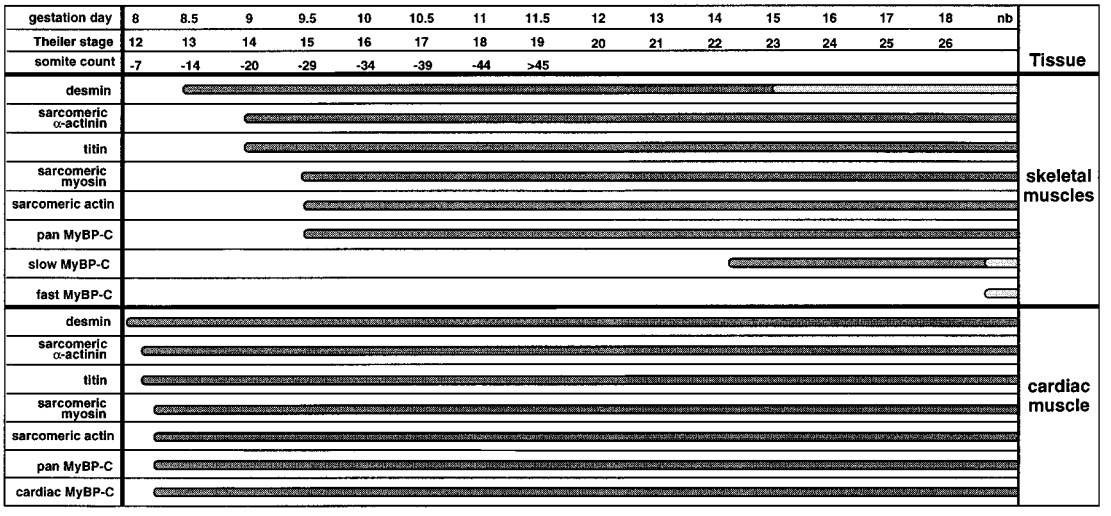

Figure 1. Temporal expression patterns of myofibrillar proteins in mouse myogenesis. Expression as monitored by immunofluorescence microscopy is shown as darkly shaded bar; lightly shaded bars reflect redistribution of certain proteins during myotube formation or fiber maturation. The upper half of the figure shows expression patterns in body muscles of somitic origin; the lower half shows expression patterns in cardiac muscle. Gestation day and developmental stages of embryos according to Theiler (1972) are indicated at the top. Note that slow MyBP-C is expressed at a relatively late developmental stage in skeletal muscles, whereas a new embryonic isoform is detectable from the first stages of sarcomerogenesis. Similarly, cMyBP-C expression coincides with the expression of titin MHC in cardiac muscle.

with the slow MyBP-C antibody is also negative in these early embryos (Fig 2). We conclude that an immunologically distinct MyBP-C isoform is coexpressed in early embryonic somites with titin and precedes the expression of the slow or fast isoforms of MyBP-C in skeletal muscle. The cardiac isoform remains cardiac specific.

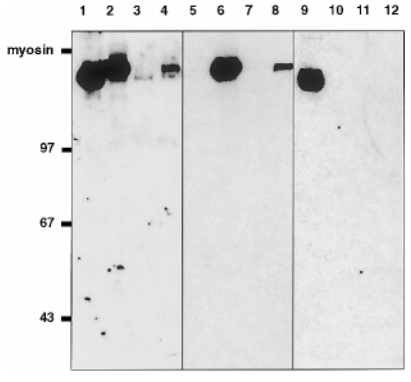

Figure 2. Expression of MyBP-C isoforms in gd-10.5 mouse embryos and adult mouse muscles by Western blot analysis. Embryonic adult tissue samples were separated on 8% SDS-PAGE gels, blotted, and probed as described in "Materials and Methods." The pan-MyBP-C antibody (α-panC) detects the cardiac and skeletal isoforms in adult muscle. The cardiac isoform of 165 kD is detected in embryo rumps; additionally, a 150-kD protein is detected as the sole protein species in embryo carcasses which identifies its somitic origin (lanes 1 to 4). The reactivity with the anti-cardiac antibody (α-C0-C1) is specific for adult heart muscle and detects the cardiac protein in whole embryo rumps but not in somitic tissue (lanes 5 to 8). The anti–slow antibody (α-sMyBP-C) reacts specifically with adult back muscle (lanes 9 to 12). Lanes are as follows: 1, 5, and 9, adult mouse back muscle; 2, 6, and 10, adult heart muscle; 3, 7, and 11, gd-10.5 embryo carcasses; and 4, 8, and 12, gd-10.5 embryo rumps (without heads). At the left are protein size markers in kilodaltons. Bar=40 μm.

Expression of MyBP-C Isoforms in Human Embryonic and Adult Tissues

The expression of the cardiac and skeletal isoforms of MyBP-C was analyzed in early human fetus tissues from weeks 11 and 19 by in situ hybridization. Figs 3 and 4 demonstrate that in week-11 fetal muscles, the transcript of cMyBP-C was detected only in cardiac muscle but not in skeletal muscle. The fast MyBP-C was still absent in skeletal muscle at this stage but was detected in week-19 fetal skeletal muscles. The cardiac isoform of MyBP-C remained cardiac specific. The successive

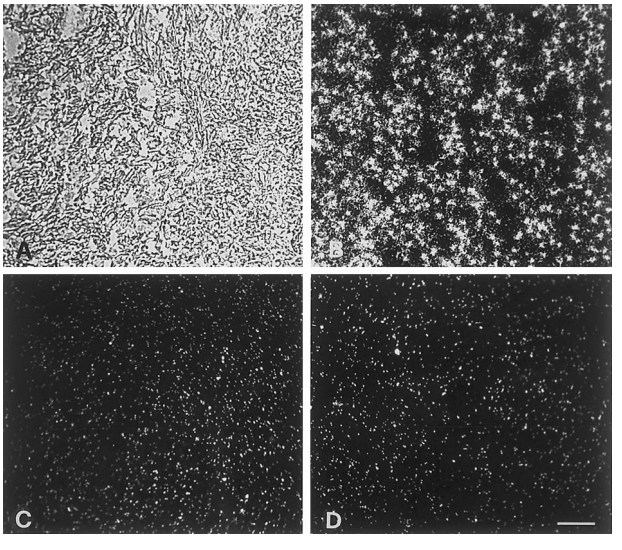

Figure 3. Expression of MyBP-C genes in cardiac muscle from human 11-week fetus. A, Cryosection of human fetal left ventricle (phase-contrast microscopy). B through D, Serial sections of human fetal ventricle hybridized with probes specific for cMyBP-C (B), slow MyBP-C (C), and fast MyBP-C (D) transcripts. Note that only the cMyBP-C probe gives a positive reaction. Bar=40 μm.

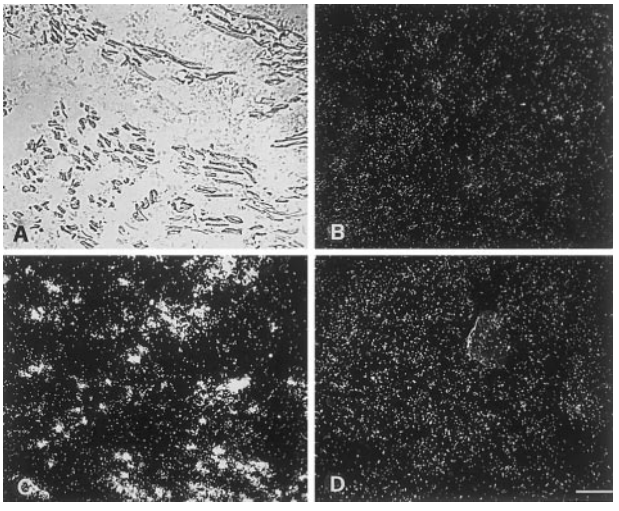

Figure 4. Expression of MyBP-C genes in skeletal muscle from human 11-week fetus. A, Cryosection of thigh skeletal muscle (phase-contrast microscopy). B through D, Serial sections of human fetal skeletal muscle hybridized with probes specific for the cMyBP-C (B), slow MyBP-C (C), and fast MyBP-C (D) transcripts. Note that skeletal muscle fibers react with slow MyBP-C but not with fast MyBP-C or with the cMyBP-C probes. Bar=40 μm.

expression of slow, followed by fast, MyBP-C is therefore common to both mouse and human muscle development (Figs 4 and 5).

In adult human muscle, the transcripts of the slow and fast isoforms of MyBP-C were expressed in variable patterns. The fast MyBP-C isoform was specific for fast type 2 fibers, whereas slow MyBP-C was coexpressed with fast MyBP-C in type 2 fibers and expressed solely in slow type 1 fibers (Fig 6). The distribution of the fast MyBP-C transcript and isoprotein was apparently identical in type 2A and type 2X fibers, identified

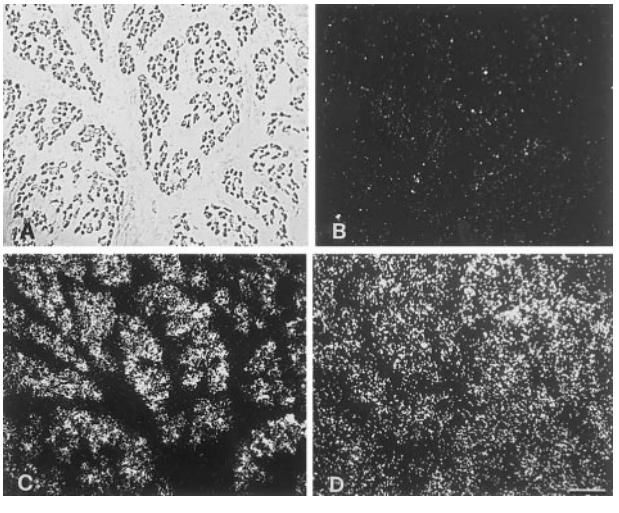

Figure 5. Expression of MyBP-C genes in skeletal muscle from human 19-week fetus. A, Cryosection of gastrocnemius muscle (phase-contrast microscopy). B through D, Serial sections of fetal gastrocnemius muscle hybridized with probes specific for the cMyBP-C (B), slow MyBP-C (C), and fast MyBP-C (D) transcripts. Note that both the slow and fast MyBP-C probes are reactive, whereas the cMyBP-C probe is unreactive with skeletal muscle fibers. Bar=40 μm.

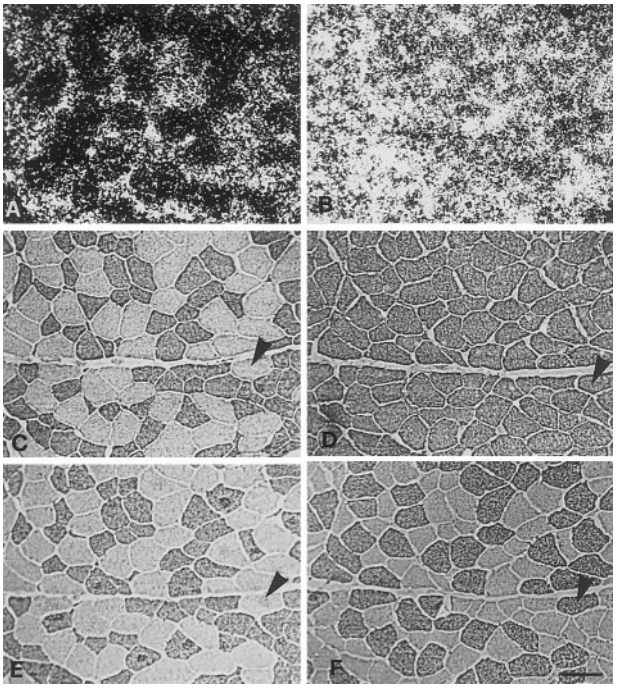

Figure 6. Expression of MyBP-C genes in human adult skeletal muscle. A and B, Cryosection of vastus lateralis muscle hybridized with probes specific for the fast MyBP-C (A) and slow MyBP-C (B) transcripts. C through E, Serial sections of the same muscle incubated with antibodies specific for fast MyBP-C (C), slow MyBP-C (D), fast MHC (E), and slow MHC-β (F). Note that fast MyBP-C transcripts (A) and protein (C) are present in the fast type 2 fibers, which are selectively labeled by the fast specific myosin antibody (E), but not in the slow type 1 fibers, which are selectively labeled by the slow specific myosin antibody (F). In contrast, slow MyBP-C transcripts (B) and protein (D) are present in both type 1 and type 2 fibers. A single fiber has been marked by arrowheads in the serial sections.

by the presence of MHC-2A and MHC-2X transcripts (not shown).

Although very low levels of transcript or protein could remain undetected in the present study, our data show that the expression of skeletal isoforms of MyBP-C in cardiac muscle and the cardiac isoform in skeletal muscle does not reach the levels of the respective proper tissue-specific isoform. Thus, although coexpression of MyBP-C isoforms seems possible in skeletal muscles, the cardiac isoform of MyBP-C was never observed in conjunction with any of the other MyBP-C isoforms in either cardiac or skeletal muscle.

Discussion

We have used in situ hybridization and immunofluorescence analysis to investigate the developmental tissue distribution of the known isoforms of MyBP-C in two mammalian species, mice and humans. Our data demonstrate a successive expression of the skeletal muscle–specific isoforms in an ordered way that resembles the conserved program of myogenesis observed also, although on a shorter time scale, in cultured avian skeletal muscle cells.[33] Our data further demonstrate that in two evolutionarily diverged mammals, the cardiac isoform of MyBP-C is not expressed in skeletal muscle throughout development. Our data demonstrate that this is in contrast to the situation in birds, where a splice variant of cMyBP-C is

transiently expressed in embryonic skeletal muscle.[34,35] The embryonic splice variant of cMyBP-C expressed in avian skeletal muscles lacks the cardiac-specific phosphorylation site.[5,35] It was shown that the deletion of this phosphorylation loop in the human cardiac isoform renders the protein essentially insensitive to cAMP-dependent protein kinase A.[5] Although birds appear to lack a specific embryonic isoform of skeletal MyBP-C, the embryonic skeletal splice variant of the cMyBP-C gene is therefore similar in its regulatory properties to the genuine skeletal isoforms.

Most cardiac sarcomeric proteins are also expressed in skeletal muscle. Available evidence indicates that in mammals, only two myofibrillar protein isoforms, cardiac troponin I and atrial myosin light chain 2, are exclusively expressed in cardiac muscle.[36] Some cardiac isoforms, such as cardiac troponin T, are transiently expressed in developing skeletal muscle both in birds[37] and in mammals.[38] This is also observed for cMyBP-C in birds[34,35,39] but not in mammals.

In mammalian cardiac muscle, the cMyBP-C transcript and protein are detected early on, and the expression coincides with that of sarcomeric MHC in the earliest detectable stages (Fig 1). Therefore, during cardiac myofibrillogenesis, both proteins are tightly linked and might be coassembled with titin and myosin. Again, the isoform specificity of MyBP-C remains tissue specific, and neither skeletal isoform is detected in cardiac muscle throughout development (Figs 1 and 3). Both muscle types are therefore following a defined expression program that allows no switching of isoforms between skeletal and cardiac muscle.

Our results show that slow MyBP-C is first expressed in developing skeletal muscle both in mice and humans and that fast MyBP-C is detected only at later developmental stages. This predominance of a slow isoform in developing muscle has been observed with other myofibrillar protein gene families. For example, the slow troponin I gene is predominantly expressed during development, and there is a subsequent switch to fast troponin I in fast muscles.[40]

In addition, our results indicate that an embryonic MyBP-C isoform, immunologically distinct from the fast, slow, and cardiac isoforms, is present in mouse skeletal muscle at very early developmental stages. Previous biochemical observations were restricted to avian muscle and suggested the existence of such an embryonic MyBP-C isoform possibly related to the cardiac isoform.[33–35] Interestingly, the detection of the mammalian embryonic isoform coincides with the expression of titin and MHC (Fig 6), suggesting that similar to the nearly synchronous coexpression of cMyBP-C and titin/myosin in the heart, a corresponding situation is found in skeletal muscle. Although one would not have assumed that the assembly mechanisms of cardiac and skeletal thick filaments would be distinctly different, the observation of the large time lag of >5 days between the expression of titin or myosin and slow MyBP-C in mice might have led to a model of sequential sarcomeric assembly of titin, myosin, and MyBP-C rather than the coassembly that our data strongly suggest.

The expression of cMyBP-C is therefore tightly restricted to cardiac muscle, which does not express the slow or fast skeletal isoforms of MyBP-C under normal circumstances. Unlike in skeletal muscle, which expresses up to three isoforms of MyBP-C in addition to MyBP-H, in cardiac muscle a transcomplementation of isoforms therefore seems to be impossible. This could plausibly explain why a dominant-negative phenotype is observed in cardiac muscle in mutations of the cMyBP-C gene, whereas no skeletal myopathies have been associated with a member of the MyBP-C family. Clearly, it will now be interesting to assay diseased myocardium from patients with MyBP-C–associated FHC for the presence of skeletal isoforms of MyBP-C in order to determine whether the expression barrier of skeletal MyBP-Cs could be overcome under pathological conditions.

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