Myosin Binding Protein C Interaction with Actin

CHARACTERIZATION AND MAPPING OF THE BINDING SITE

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Myosin binding protein C (MyBPC) is a multidomain protein associated with the thick filaments of striated muscle. Although both structural and regulatory roles have been proposed for MyBPC, its interactions with other sarcomeric proteins remain obscure. The current study was designed to examine the actin-binding properties of MyBPC and to define MyBPC domain regions involved in actin interaction. Here, we have expressed full-length mouse cardiac MyBPC (cMyBPC) in a baculovirus system and shown that purified cMyBPC binds actin filaments with an affinity of 4.3 \( \times 10^8 \) M\(^{-1}\) and a 1:1 molar ratio with regard to an actin protomer. The actin binding by cMyBPC is independent of protein phosphorylation status and is not significantly affected by the presence of tropomyosin and troponin on the actin filament. In addition, cMyBPC-actin interaction is not modulated by calmodulin.

To determine the region of cMyBPC that is responsible for its interaction with actin, we have expressed and characterized five recombinant proteins encoding fragments of the cMyBPC sequence. Recombinant N-terminal fragments such as C0–C1, C0–C4, and C0–C5 cosediment with actin in a linear, nonsaturable manner. At the same time, MyBPC fragments lacking either the C0–C1 or C0–C4 region bind F-actin with essentially the same properties as full-length protein. Together, our results indicate that cMyBPC interacts with actin via a single, moderate affinity site localized to the C-terminal region of the protein. In contrast, certain basic regions of the N-terminal domains of MyBPC may act as small polycations and therefore bind actin via nonspecific electrostatic interactions.

MyBPC is a myosin-associated protein found in the crossbridge-bearing zone (C region) of A bands in striated muscle. MyBPC is expressed as three isoforms, two skeletal isoforms (fast and slow) and one cardiac isoform encoded by three distinct genes. The isoforms all tend to be specific for different muscle fiber types, with the cardiac isoform found only in the heart (1–3). The two skeletal isoforms of MyBPC are composed of seven immunoglobulin I (Ig I) and three fibronectin (Fn) type III domains, whereas cardiac MyBPC has an extra Ig I motif (designated C0) at the N terminus (see Fig. 1A).

MyBPC contributes to thick filament structure via interactions of its C-terminal domains C7–C10 with light meromyosin and with titin (4–6).

MyBPC is also believed to be a factor in the regulation of contraction via the phosphorylation-dependent binding of its N-terminal C1–C2 region to the myosin S2 (7–9). When dephosphorylated, the C1–C2 region interacts with myosin. Upon phosphorylation, MyBPC dissociates from S2, which is thought to increase the probability of myosin head interactions with actin. The phosphorylation sites are located in the MyBPC motif, which is a linker connecting the C1 and C2 domains. The number of phosphorylation sites appears to differ between species, but the cardiac isoforms consistently have more sites (usually three).

Interestingly, yeast two-hybrid assay identified interactions between domains C5 and C8, and between domains C7 and C10 of MyBPC (10). An interaction between domains C6 and C9 was also predicted to occur. As a result, a model of three MyBPC molecules binding serially to one another and forming a collar around the myosin thick filament was developed. According to this model, domains C0–C4 extend into the interfilament space and interact with myosin S2 (10). An alternative model of MyBPC has the C-terminal domains C7–C10 running parallel to the myosin rod and the N terminus extending perpendicularly toward the thin filaments (11). This model takes into account the fact that MyBPC, which binds to myosin, has a greater periodicity than the myosin repeat along the thick filament as assessed in x-ray diffraction patterns. The model explains the observed spacing by allowing the N terminus of MyBPC to interact with actin.

Earlier in vitro studies of native MyBPC purified from skeletal and cardiac muscles also suggested a MyBPC-actin interaction, with apparent inhibition by EGTA and restoration by Ca\(^{2+}\) in regulated but not unregulated thin filaments (12, 13). However, evidence for such an interaction in the intact filament lattice is lacking. Moreover, the location of such a binding site on MyBPC has not yet been determined clearly. From structural modeling and sequence analysis, the putative actin-binding site was proposed to be on the proline-alanine-rich linker that is located just before the C1 domain of MyBPC (11). In experiments on skinned trabeculae, maximal force was reduced by the addition of a high concentration of a C0-proline-alanine-rich fragment that was shown to co-immunoprecipitate and co-sediment.
with actin. The effect was only seen when endogenous MyBPC was phosphorylated (i.e. when C1–C2 would not be bound to myosin S2), leading to the conclusion that the C0-proline-alanine-rich region not only interacts with actin but that this interaction is physiologically relevant (14). On the other hand, actin-binding analysis of various combinations of N-terminal domains of MyBPC suggested that MyBPC may interact with actin via multiple binding sites located in its motif and in the C1 region (15). In addition, neutron contrast variation analysis provided evidence that a C0–C2-actin interaction occurs via the C0–C1 end of the molecule, with the MyBPC motif and C2 domains projected out into solution (16).

Here, we expressed full-length mouse cardiac MyBPC (cMyBPC) and its fragments in a baculovirus expression system. We further characterized the actin binding properties of each recombinant protein and demonstrated that cMyBPC interacts with F-actin and reconstituted thin filaments in a saturable manner via a single moderate affinity site localized to the C-terminal half of the protein; no region spanning domains C0 to C4 is responsible for MyBPC binding to actin in vitro. Regarding regulation of CMyBPC binding to actin, we observed no effect on binding due to the protein phosphorylation state or the presence of calmodulin. The presence of tropomyosin on the actin filament caused only a slight decrease in the binding of cMyBPC to actin, indicating that both proteins can interact with the same actin filament simultaneously.

**EXPERIMENTAL PROCEDURES**

**Proteins**—DNA fragments encoding full-length or truncated MyBPC proteins were PCR-amplified from a clone containing the full-length mouse cardiac myosin binding protein C sequence (courtesy of Dr. P. A. Powers, University of Wisconsin, Madison, WI). The sequence verified PCR products were cloned into pFastBac1 transfer plasmid (Invitrogen) so that all expressed proteins contained the N-terminal FLAG tag epitope (MDYKDDDDKH) followed by a native MyBPC sequence (courtesy of Dr. P. A. Powers, University of Wisconsin, Madison, WI). The sequence verified PCR products were cloned into pFastBac1 transfer plasmid (Invitrogen). The refolded proteins were further purified on nickel-nitritotriacetic acid agarose (Qiagen) under native conditions per the manufacturer’s instructions.

Cardiac troponin and tropomyosin were purified from bovine heart according to Greaser and Gergely (18, 19). The protein concentrations were determined by Bio-Rad DC protein assay kit using bovine serum albumin as a standard. Lyophilized rabbit skeletal and bovine cardiac muscle actins and bovine brain calmodulin were purchased from Cytoskeleton, Inc. and Calbiochem, respectively, and handled according to the manufacturer’s instructions.

**Size Exclusion Chromatography**—0.9 mg of cMyBPC was loaded onto a 2.5 × 50-cm Sephacryl-300 column. The column was eluted at 0.5 ml/min in 50 mM NaH2PO4, 0.15 M NaCl, pH 7.4. The Stokes’ radius for cMyBPC was determined from a calibration curve of the protein standards ovalbumin (43 kDa, 30.5 Å), aldolase (158 kDa, 48.1 Å), catalase (232 kDa, 52.2 Å), and ferritin (440 kDa, 61 Å) as described in the Amersham Biosciences Gel Filtration Calibration Kit instruction manual.

**In Vitro Phosphorylation and Dephosphorylation of cMyBPC**—Purified cMyBPC was phosphorylated with the catalytic subunit of protein kinase A (PKA, Sigma). 0.01 units of PKA was used per 1 μg of cMyBPC in 10 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 2 mM MgCl2, 0.1 mM ATP, and 0.2 mM dithiothreitol. Dephosphorylation of recombinant cMyBPC was achieved by incubation with λ-phosphatase (gift of Dr. V. Klenchin, University of Wisconsin, Madison), an Mn2+-dependent nonspecific protein phosphatase with activity toward serine, threonine, and tyrosine residues (20). Dephosphorylation was carried out in 10 mM Tris-HCl, pH 8.0, 0.1 M NaCl, and 2 mM MnCl2 at 30 °C for 30 min. 0.1 μg of λ-phosphatase was used per 1 μg of cMyBPC. Phosphorylation and dephosphorylation status of cMyBPC was assessed by ProQ Diamond phosphoprotein staining followed by Sypro Ruby protein staining (Invitrogen).

**Actin Binding Analysis**—The actin binding properties of MyBPC proteins were measured using the previously described high speed co-sedimentation assay used for studies of dystrophin binding to actin (21). Briefly, increasing amounts of purified protein were incubated with 5 μM muscle F-actin in 10 mM Tris-HCl, pH 8.0, 0.1 M NaCl, and 2 mM EGTA, 2 mM MgCl2, 0.1 mM ATP, and 0.2 mM dithiothreitol and subjected to centrifugation at 100,000 × g for 20 min. The amounts of free and bound protein were determined densitometrically from Coomassie Blue-stained gels of resulting supernatants and F-actin pellet fractions. Binding data were fitted to a hyperbolic binding equation by nonlinear regression analysis (22). Similarly, the binding of thin filaments by MyBPC proteins was analyzed in the presence or absence of Ca2+. Thin filaments were assembled by incubating F-actin with troponin and tropomyosin at a 4:1:1 molar ratio with respect to actin (23). Incubation was performed overnight at 4 °C.

**RESULTS**

To characterize the actin binding properties of MyBPC, we expressed full-length cMyBPC in a baculovirus expression
system. We also generated truncated proteins encoding various domains of cMyBPC (Fig. 1A). The domain assignments were performed based on the alignment of MyBPC isoforms from different species (24). Protein C0–C1 included the proline-alanine-rich domain that was suggested to interact with actin based on a structural modeling (11). To evaluate whether this region is indispensable for actin binding, we also generated C0–C1 MyBPC protein from which the C0, C1, and proline-alanine-rich domains were deleted (Fig. 1A). To remove all of the domains of MyBPC that were previously shown to interact with actin (15, 16), we produced a C5–C10 protein. Fragment C0–C4 was designed to prevent possible interactions between domains C5 and C8, and domains C7 and C10 (10). These interactions, if strong enough, would result in protein multimerization and therefore complicate quantitative analysis of cMyBPC binding to actin. Finally, a C0–C5 protein represents the largest N-terminal region of MyBPC that is known to be free from interaction with myosin rod and with titin. All the constructs were designed with the FLAG epitope on the amino termini and purified on anti-FLAG M2-agarose (Fig. 1B). All recombinant proteins were highly soluble and migrated on SDS-PAGE according to their predicted molecular weights (Table 1). In all cases, protein purity and quantity were more than sufficient to analyze its actin binding properties.

To gain insight into the multimerization/aggregation state of the purified full-length protein, we analyzed cMyBPC by size exclusion chromatography (Fig. 2). cMyBPC eluted from the column as a single symmetrical peak along with the globular protein standard catalase (232 kDa), a Stokes radius of 51 Å for cMyBPC (12) was estimated based on following protein standards: ovalbumin (43 kDa, 30.5 Å), aldolase (158 kDa, 48.1 Å), catalase (232 kDa, 52.2 Å), and ferritin (440 kDa, 61 Å).

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### Table 1

| Protein                | C0–C1 | C0–C4 | C0–C5 | ΔC0–C1 | C5–C10 | cMyBPC |
|------------------------|-------|-------|-------|--------|--------|--------|
| Predicted $M_r$ (kDa)  | 28.224| 70.786| 85.391| 115.002| 71.032 | 141.815|
| Denatured $M_r$ (kDa) | 34.105| 74.540| 92.047| 114.211| 71.433 | 139.714|

FIGURE 1. Expression and purification of MyBPC proteins. Shown in A is the general domain organization of cardiac full-length myosin binding protein C (cMyBPC) and cMyBPC fragments (C0–C1, C0–C4, C0–C5, ΔC0–C1, and C5–C10) expressed in the baculovirus expression system. Arrows connect the putative binding domains with the interacting protein as reported in studies indicated by the superscript. B, MyBPC proteins were designed with an N-terminal FLAG epitope and purified from insect cell lysates on anti-FLAG M2-agarose. cMyBPC purification is shown as an example. Infected insect cells (Sup) solubilized in 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, anti-FLAG M2-agarose void, and purified cMyBPC eluted with an excess of FLAG peptide as 3-ml fractions were subjected to SDS-PAGE and stained with Coomassie Blue. The molecular weight standards (× 10 $^{-2}$) are indicated on the left.

FIGURE 2. Determination of the self-association/aggregation state of MyBPC expressed in the baculovirus system. 900 µg of purified cMyBPC were analyzed by gel filtration on Sephacryl S-300. A, cMyBPC eluted as a single symmetrical peak along with the globular protein standard catalase (232 kDa), a Stokes radius of 51 Å for cMyBPC (12) was estimated based on following protein standards: ovalbumin (43 kDa, 30.5 Å), aldolase (158 kDa, 48.1 Å), catalase (232 kDa, 52.2 Å), and ferritin (440 kDa, 61 Å).
servations, along with the published molecular dimensions for MyBPC purified from heart (25), strongly suggest that cMyBPC purified from infected insect cell lysates is an elongated monomer.

We further used the co-sedimentation assay to investigate whether baculovirus-expressed MyBPC constructs interacted with actin. In this assay, F-actin can be sedimented at 100,000 × g due to its filamentous nature, whereas nonfilamentous or noninteracting proteins remain in the supernatant. As shown in Fig. 3, all purified MyBPC proteins remained in supernatant fractions in the absence of actin. When skeletal muscle F-actin was added, virtually no C0–C1 was found in the pellet, whereas C0–C4, C0–C5, ΔC0–C1, C5–C10, and cMyBPC proteins consistently co-sedimented with actin to various extents. Of these, only cMyBPC, C0–C1, and C5–C10 bound actin in a saturable manner with K\textsubscript{d} values of 4.3 ± 1.1 μM, 1.3 ± 0.3 μM, and 4.2 ± 1.5 μM, respectively (Fig. 4B and Table 2). The B\textsubscript{max} values for cMyBPC (1.1 ± 0.1), ΔC0–C1 (1.1 ± 0.1), and C5–C10 (1.0 ± 0.2) indicate the proteins bound to actin in a 1:1 molar ratio. In contrast, the co-sedimentation of C0–C1, C0–C4, and C0–C5 with actin was nonsaturable in the range of concentrations from 0.5 to 40 μM (Fig. 4A). Nonsaturable co-sedimentation in the range of concentrations from 0.5 to 90 μM was also observed for the His-tagged C0–C1 fragment of MyBPC expressed in E. coli (supplemental Fig. S1A). Similar results were obtained when MyBPC proteins were co-sedimented with bovine cardiac muscle actin (data not shown). Because it was shown that the amounts of C1–C2 fragment and native MyBPC found in the pellet with F-actin were reduced by increased ionic strength (26, 27), we assessed whether the cMyBPC-actin interaction was sensitive to increasing NaCl concentrations ranging from 0.1 to 0.5 M. As shown in Fig. 4C, the binding of cMyBPC to F-actin is not strongly affected by rising ionic strength, exhibiting an IC\textsubscript{50} of 0.5 M.

Previously, we demonstrated that >90% of cMyBPC expressed in baculovirus is represented by phosphorylated species (28). Qualitatively, the phosphorylation pattern of recombinant cMyBPC (Ser-273, Ser-282, and Ser-302) is remarkably
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**TABLE 2**

| Protein      | cMyBPC | ΔC0–C1 | C5–C10 |
|--------------|--------|--------|--------|
| $B_{max}$, μM | 1.1 ± 0.1 | 1.1 ± 0.1 | 1.0 ± 0.2 |
| $K_p$, μM     | 4.3 ± 1.1 | 1.3 ± 0.3 | 4.2 ± 1.5 |

**FIGURE 5.** Actin binding characteristics of phosphorlated and dephosphorylated cMyBPC. In A, purified cMyBPC was treated with PKA or λ-phosphatase and subjected to gel electrophoresis. The resulting SDS-PAGE was stained with Pro-Q Diamond phosphoprotein stain (PQ) and subsequently with SYPRO Ruby total protein stain (SR). Absence of Pro-Q Diamond staining indicates loss of all phosphates (λ-phosphatase (PPase)-treated cMyBPC). The greater intensity of Pro-Q Diamond staining corresponds to PKA-treated cMyBPC and indicates that the phosphorylation state of the protein was enhanced. B, increasing amounts of cMyBPC treated with λ-phosphatase (solid line) or PKA (dashed line) were co-sedimented with 5 μM F-actin. The amounts of free and bound protein were determined densitometrically from Coomassie Blue-stained SDS-PAGE loaded with equal volumes of resulting supernatants and pellets. Symbols denote data from two independent side-by-side experiments. Nonlinear regression analysis represented by lines yielded $K_v$ values of 3.1 ± 1.1 and 4.0 ± 1 μM for λ-phosphatase (solid line) and PKA (dashed line)-treated cMyBPC, respectively.

**FIGURE 6.** Lack of effect of calcium, calmodulin, and tropomyosin on cMyBPC binding to F-actin. In A, 5 μM cMyBPC was co-sedimented with 5 μM F-actin in the presence or absence of 5 μM calmodulin (CaM) and 0.2 mM CaCl$_2$. The binding data (S.D., $n = 3$) were normalized against the amount of actin pelleted and expressed as the percentage of cMyBPC co-sedimented with F-actin in a buffer containing 1 mM EGTA where 100% equals 0.49 ± 0.02 mol of cMyBPC bound per mol of actin. cMyBPC was treated with PKA (1) or λ-phosphatase (2) prior to cosedimentation assays. In B, increasing amounts of cMyBPC were sedimented side-by-side with F-actin alone (open bars) or F-actin saturated with tropomyosin (filled bars). Actin and tropomyosin were present at 2.4 and 0.6 μM, respectively. The amount of cMyBPC bound to F-actin in the presence of tropomyosin was expressed as the percentage of cMyBPC cosedimented with actin in the absence of tropomyosin. The data represent the average of two independent side-by-side experiments.

Similar to that described for native protein (8), however, purified recombinant cMyBPC was heterogeneous, as it was comprised of mono-, bis-, and tris-phosphorylated protein products. To maximize the ratio of these phosphorylated underprocessed protein products, we performed in vitro phosphorylation of purified cMyBPC using the catalytic subunit of PKA. Also, to release phosphate groups from cMyBPC, it was treated with λ-phosphatase. The phosphorylation state of PKA and λ-phosphatase treated cMyBPC was analyzed on Pro Q Diamond-stained SDS-PAGE (Fig. 5A). Both dephosphorylated cMyBPC and cMyBPC with enhanced phosphorylation state were compared for their abilities to interact with actin. As shown in Fig. 5B, actin binding activities of PKA and λ-phosphatase-treated cMyBPC were similar to one another and to those measured for heterogeneously phosphorylated preparation of the recombinant protein (Fig. 4B).

Besides PKA, MyBPC can be phosphorylated by Ca$^{2+}$-calmodulin-activated kinase, and Ca$^{2+}$-calmodulin-activated kinase was shown to co-purify with MyBPC from cardiac muscle (29). It is therefore not inconceivable that the MyBPC-actin interaction could be regulated by calmodulin. As shown in Fig. 6A, no effect of calmodulin on cMyBPC cosedimentation with actin was observed in the presence of either 1 mM EGTA or 0.2 mM CaCl$_2$. It is also clear from Fig. 6A that cMyBPC binding to actin was not sensitive to the presence of Ca$^{2+}$ or EGTA. Furthermore, virtually no calmodulin co-sedimented with phosphorylated or unphosphorylated cMyBPC and actin, indicating that there is no interaction between the proteins (data not shown).

Because in muscle fibers, actin is the major component of the thin filaments, it became relevant to determine whether the MyBPC-actin interaction is regulated by the presence of other thin filament proteins such as tropomyosin and tropinin. Recent studies also suggested that tropomyosin and the N-terminal domains of MyBPC share binding sites on actin filaments, and therefore, MyBPC might compete with tropomyosin and displace it from F-actin (15, 16). With regard to competitive binding of tropomyosin and MyBPC to F-actin, actin was first saturated with tropomyosin and the binding of increasing amounts of cMyBPC to tropomyosin-actin complex was assayed. As shown in Fig. 6B, saturation of actin filaments with tropomyosin caused only a slight decrease in the binding of cMyBPC to actin. The interaction of cMyBPC with actin was not prevented, even when F-actin was preincubated with tropomyosin at molar ratios as high as 1:1 prior to...
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Prior to actin binding analysis, we assessed the multimerization/aggregation state of cMyBPC. Size exclusion chromatography yielded a Stokes’ radius of 51 Å for recombinant cMyBPC, which is in a good agreement with the previously published value of 50.7 Å for native MyBPC purified from cardiac muscle (25). There are two possible explanations for a Stokes’ radius of 51 Å, which is larger than expected from a globular protein of 142 kDa. First, the protein exists in an oligomeric form, such as dimers or trimers formed via interactions of domains C5/C8 and C7/C10 of adjacent MyBPC monomers. However, dimers (284 kDa) and trimers (426 kDa) would be expected to migrate through the gel filtration column faster than the protein standard catalase (232 kDa). Furthermore, the oligomeric protein is likely to elute from the column as one broad peak or as multiple peaks, corresponding to multimers of different size. Instead, cMyBPC eluted from the gel filtration column as a single sharp peak (Fig. 2A). The alternative and more likely explanation for the observed Stokes’ radius is that cMyBPC purified from infected insect cell lysates is an elongated monomer. Indeed, hydrodynamic analysis of native MyBPC from skeletal (30) and cardiac (12) muscles suggested that both proteins were asymmetric molecules. Electron microscopy images of MyBPC purified from cardiac muscle revealed predominantly V-shaped particles in which the V-arm was ~22 nm long and 4 nm wide (25). At the same time, it was not determined whether the MyBPC particles represented monomers or dimers. According to structural analysis, the average length of MyBPC Ig I domains is ~3.3 nm, and the width is ~2.2 nm (31–33), whereas the Fn type III domains are 5 nm long and 2.5 nm wide (34). The width of MyBPC molecule is thus no smaller than 2.2–2.5 nm. The length of MyBPC monomer composed of eight Ig I and three Fn type III domains is ~40 nm long. This rough estimate fits with double the length of the V-arm (2 × 22 nm) of the MyBPC particles on electron microscopy images (25).

Taking into account the linkers between the domains, the proline-alanine-rich region and the MyBPC motif, the dimensions of V-shaped molecules therefore correspond to MyBPC monomers. Although in vitro results are consistent with the purified full-length cMyBPC being an asymmetric monomer...
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in solution, the possibility that in vivo the protein trimerizes into a collar around the thick filament cannot be ruled out.

Actin binding analysis demonstrated that recombinant full-length MyBPC binds actin with an affinity of 4.3 ± 1.1 μM and 1:1 stoichiometry. Saturable binding for the full-length MyBPC was shown previously when all three isoforms of the protein purified from muscle tissue were shown to co-sediment with F-actin equally (12, 27). In the case of actin binding by native MyBPC isoforms, the data generally yielded nonlinear Scatchard plots such that a reliable binding constant for MyBPC-actin interaction was not determined. As an indication of binding affinity, it was noted that the free MyBPC concentration at half-maximal binding in 0.1 nM KCl was in the range of 20 μM (12, 27). Here, the affinity for MyBPC-actin interaction was accurately determined for the first time. Notably, it is about the same order as the one crudely estimated for the native protein. Although moderate (~5 μM), this affinity is more than sufficient to yield significant interactions considering the in vivo concentrations of actomyosin-interacting proteins.

The analysis of MyBPC fragments with regard to their actin binding properties yielded several important findings. First, the N-terminal fragments such as C0–C1, C0–C4, and C0–C5 all co-sediment with actin in a linear, nonspecific manner (Fig. 4A). The C0–C1 protein exhibits only very weak actin binding activity that does not saturate at concentrations as high as 40 μM. Although the binding of the larger N-terminal fragments to actin was also found to be nearly linear, C0–C4 and C0–C5 both co-sediment with F-actin more readily than the C0–C1 protein. Second, the δC0–C1 fragment that lacks the C0–C1 region binds F-actin at least as tightly as the full-length MyBPC. The Kd values even suggest that δC0–C1 has an overall greater affinity (1.3 ± 0.3 μM) for actin than cMyBPC (4.3 ± 1.1 μM) (Fig. 4B and Table 2). A potential explanation for the ~3-fold higher affinity of δC0–C1 is that the deletion of C1 and C0 domains may cause the opening of the V-shaped MyBPC molecule and lead to better accessibility to the actin-binding region. The opening and closing of the V-arms of MyBPC may, therefore, regulate its interaction with actin. However, a Stokes’ radius of 48 Å observed for δC0–C1 by size exclusion chromatography argues against this possibility. In fact, this is smaller than the Stokes’ radius of 51 Å for the full-length protein (supplemental Fig. S2), which indicates that the deletion of the C0–C1 region does not result in extension of the MyBPC molecule. Overall, the higher affinity of δC0–C1 for actin demonstrates that the C0–C1 sequence is not necessary for MyBPC interaction with actin in vitro. Third, the C5–C10 protein corresponding to the isolated C terminus of cMyBPC binds actin with properties almost indistinguishable from those measured for the full-length protein (Fig. 4B and Table 2). Fourth, the Bmax values for cMyBPC (1.1 ± 0.1), δC0–C1 (1.1 ± 0.1), and C5–C10 (1.0 ± 0.2) binding to actin (Fig. 4B and Table 2) imply that one molecule of protein binds just one monomer on the actin filament. Taken together, these findings lead to the conclusions; 1) MyBPC interacts with actin via a single actin-binding site localized to the C6–C10 domains of the protein; 2) weak actin binding by C0–C4 or C0–C5 does not contribute much to the overall affinity and stoichiometry measured for MyBPC-actin interaction; 3) C0–C1 protein and therefore the proline-alanine-rich region of MyBPC have no appreciable affinity for F-actin.

A number of studies are consistent with our conclusions. For example, nonsaturable co-sedimentation with actin was demonstrated for the C1–C2 region of MyBPC in the range of concentrations from 0 to 25 μM (26). In a different study, the shorter fragment containing the C0 domain and the proline-alanine-rich linker was shown to co-sediment with actin less strongly than the C0–C2 protein (14). In the case of C0–C2, the co-sedimentation assay was performed using only four different concentrations of the recombinant protein in the range 0.1–0.9 μM. Because the stoichiometry of protein to actin in the pellets with 0.6 or 0.9 μM C0–C2 was the same, the assumption was made that the binding reached saturation (14). Notably, when MyBPC N-terminal fragments such as C0–C1 and C0–C2 were expressed in myocytes, their localization was restricted to the A-band, and there was no evidence for C0–C1 or C0–C2 binding to the proteins of the I-band (35). Alone, this observation does not exclude the possibility that the region spanning domain C0 to domain C2 binds to actin. However, it was also shown that exogenous C0–C1 activated Ca2+-independent cross-bridge cycling in skinned myocyte preparations. Based on muscle-type specificity and the rate of the activating effect, as well as C0–C1 localization to the A-band, Herron et al. (35) concluded that C0–C1 and particularly its proline-alanine-rich region bind myosin rather than actin.

Our findings, however, differ from a recent study that reported saturable binding of the C0–C2 and C1–C2 regions of MyBPC to actin (15). In that study, various combinations of the N-terminal domains were probed for their interactions with actin, and as a result, the actin binding sites were assigned to the MyBPC motif and C1 domain. Remarkably, neutron contrast variation data did not depict binding of the MyBPC motif to actin and demonstrated that C0–C2-actin interaction occurs via the C0–C1 end of the molecule, with motif C2 domains projected out into solution (16).

At first glance, the reason for the conflicting results (15) might be related to differences in the recombinant expression systems used in the two studies. In this work, we have chosen baculovirus protein expression over E. coli employed in all the previous analyses of actin binding by MyBPC fragments. Unlike E. coli, insect cells allow proper folding and post-translational processing of many more eukaryotic polypeptides and therefore give a better chance of obtaining soluble and biologically active proteins. In experiments performed in skinned myocardium from hearts of mice expressing (wild type) or lacking (cMyBPC−/−) MyBPC, we have verified the proper functioning of baculovirus produced cMyBPC (36). Indeed, the reconstitution of cMyBP-C−/− myocardium with purified cMyBPC restored the calcium sensitivity of force (pCa50) and rate of force redevelopment (krec) to the values observed in wild type myocardium. However, a FLAG-tagged C0–C1 fragment of MyBPC expressed in baculovirus has actin-binding characteristics identical to those measured for His-tagged C0–C1 protein expressed in E. coli (supplemental Fig. S1B).
This indicates that the differences in tag or the expression system cannot account for the disagreements between our results and those of others (15).

Another possible explanation is that the differences in results are related to differences in the analysis of actin binding data. Notably, we used a conventional approach for the analysis of actin binding data, in that the amounts of free and bound protein were determined densitometrically from Coomassie Blue-stained gels of resulting supernatants and pellet fractions, whereas the earlier study (15) analyzed the pellet fractions only. Remarkably, when this group used conventional actin binding data analysis, the co-sedimentation of the C1–C2 region with actin was linear in the range of concentrations from 0 to 25 \textmu M (26). This is in agreement with our observation of linear co-sedimentation of the His-tagged C1–C2 region of MyBPC with actin (supplemental Fig. S1C).

Increased ionic strength and pH, as well as phosphorylation, reduced C1–C2 co-sedimentation with actin (supplemental Fig. S1C) (15, 26). At the same time, the binding of cMyBPC was neither significantly reduced in the near physiologic range of NaCl concentration between 0.1 and 0.2 M (Fig. 4C) nor affected by the phosphorylation status of the protein (Fig. 5). Although controversial at first glance, together, these findings provide further support for the conclusion that in vitro actin binding by cMyBPC is independent from its N-terminal domains. Phosphorylation may modulate C1–C2 actin binding properties through changes in protein conformation or net charge. Indeed, addition of negatively charged phosphate groups to the protein may be expected to reduce its interaction with the negatively charged actin filaments. According to the program DNAStar, the predicted isoelectric point (pI) of the MyBPC region spanning domain C1 to domain C2 is 8.3. The basic nature of the C1–C2 region and the reduced binding at increased ionic strength or pH suggest a simple electrostatic mechanism of C1-C2-actin interaction. The actin bundling activity demonstrated for the C1–C2 protein (15, 37) is consistent with such a suggestion. Indeed, in a manner analogous to the basic protein calponin (38), polycationic C1–C2 fragment may cross-link F-actin by reducing the electrostatic repulsion between the negatively charged actin filaments. Interestingly, the phosphorylation of C1–C2 protein reversed its actin bundling activity (15). Similarly, electron microscopy analysis demonstrated the periodicity of C0–C2 attachment to actin filaments (37). Both neuron contrast variation and electron microscopic data analysis do not yet imply that interactions are saturable. Although no current representation of the MyBPC C terminus interaction with actin is available at the micromolecular level, the above studies (16, 37) clearly support the concept that weak electrostatic attachment of multiple N termini from different MyBPC molecules may provide binding strong enough for the regulation of myosin-S1 interaction with actin. Importantly, we found that cMyBPC and its C-terminal fragment (C5–C10) bind to both F-actin and thin filaments in a saturable manner (Figs. 4 and 7). Indeed, the relevance of MyBPC binding to actin depends on whether such a binding can occur in the presence of other thin filament proteins, particularly tropomyosin and troponin. C0–C5, the N-terminal fragment of MyBPC, also interacts with thin filaments. However, as in the case with F-actin, this interaction was found to be nonsaturable. Although C0–C5 binding is not much affected by Ca\textsuperscript{2+}, the presence of calcium ions causes a slight increase in cMyBPC and C5–C10 co-sedimentation with thin filaments. It is known that in the presence of Ca\textsuperscript{2+}, the position of tropomyosin in the groove of the double-stranded actin filaments is shifted, so the actin is available for other interactions. In this context, there are two possible explanations for the observed effect shown in Fig. 7. First, there is more than one actin-binding site on MyBPC, and the presence of Ca\textsuperscript{2+} makes actin available for the interactions with these multiple sites. Second, in a manner analogous to the binding of the myosin head, the binding of MyBPC to actin fully displaces tropomyosin and allows additional MyBPC to bind. However, our data do not support either conclusion. Indeed, Ca\textsuperscript{2+} does not significantly affect the stoichiometry of binding (Fig. 7 and Table 3). Besides, the interaction of cMyBPC with actin is not significantly affected by the presence of tropomyosin. Interestingly, in the absence of Ca\textsuperscript{2+}, MyBPC cosedimentation with thin filaments was reduced by \textasciitilde25\%, which is comparable with the decrease in cMyBPC binding to F-actin presaturated with tropomyosin (Fig. 6B). Moreover, the binding of cMyBPC, C0–C5, and C5–C10 to thin filaments in the presence of Ca\textsuperscript{2+} is quite similar to that of F-actin alone (Figs. 4 and 7 and Tables 2 and 3). Although the observed effect of tropomyosin on cMyBPC-actin interaction is not significant, tropomyosin may cause some shielding of binding sites, so they are not so easily accessible by MyBPC. As a result, the interaction between MyBPC and actin may be delayed. Even though tropomyosin and Ca\textsuperscript{2+} do not turn the MyBPC-actin interaction on and off, they may regulate the rate of binding in vivo.

Paradoxically, the specific and relatively strong actin binding by the C6–C10 region of MyBPC might not be physiologically relevant, as the primary myosin and titin binding sites...
Interactions of the N-terminal domains of MyBPC may act as small polyvalent actin binders. In contrast, certain basic regions of the C-terminal half of the protein to reach from the thick filament to bind actin. Clearly, more structural, physiological and biochemical studies are required to refine our understanding of MyBPC–actin interaction.

In conclusion, our results demonstrate that cMyBPC interacts with actin in a reconstituted thin filament in a saturable manner via a single moderate affinity site localized to the C-terminal half of the protein. In contrast, certain basic regions of the N-terminal domains of MyBPC may act as small polymers and therefore bind actin via nonspecific electrostatic interactions.

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