Effects of the N-terminal Domains of Myosin Binding Protein-C in an in Vitro Motility Assay

EVIDENCE FOR LONG-LIVED CROSS-BRIDGES*

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Myosin binding protein-C (MyBP-C) is a thick-filament protein whose precise function within the sarcomere is not known. However, recent evidence from cMyBP-C knock-out mice that lack MyBP-C in the heart suggest that cMyBP-C normally slows cross-bridge cycling rates and reduces myocardial power output. To investigate possible mechanisms by which cMyBP-C limits cross-bridge cycling kinetics we assessed effects of recombinant N-terminal domains of MyBP-C on the ability of heavy meromyosin (HMM) to support movement of actin filaments using in vitro motility assays. Here we show that N-terminal domains of cMyBP-C containing the MyBP-C “motif,” a sequence of ~110 amino acids, which is conserved across all MyBP-C isoforms, reduced actin filament velocity under conditions where filaments are maximally activated (i.e. either in the absence of thin filament regulatory proteins or in the presence of troponin and tropomyosin and high [Ca2+]i). By contrast, under conditions where thin filament sliding speed is submaximal [i.e. in the presence of troponin and tropomyosin and low [Ca2+]i), proteins containing the motif increased filament speed. Recombinant N-terminal proteins also bound to F-actin and inhibited acto-HMM ATPase rates in solution. The results suggest that N-terminal domains of MyBP-C slow cross-bridge cycling kinetics by reducing rates of cross-bridge detachment.

Myosin binding protein-C (MyBP-C) is a sarcomeric protein associated with the thick filaments of vertebrate striated muscle (1). Although the precise function of MyBP-C within the sarcomere is not well understood, evidence from MyBP-C knock-out mice that lack cardiac MyBP-C (2) indicate cMyBP-C slows cross-bridge cycling and rates of force development, especially at submaximal [Ca2+]i (3–5). The idea that MyBP-C limits cross-bridge kinetics was initially proposed by Hofmann et al. (6) who suggested that MyBP-C acts as an internal load within the sarcomere based on their observations that partial extraction of MyBP-C from skeletal fibers reversibly accelerated a low velocity phase of shortening at submaximal Ca2+ activation (7).

Although the exact structural arrangement of MyBP-C within the sarcomere is not known, MyBP-C could contribute to an internal load by tethering myosin heads to the thick filament and thereby limiting the extension of attached myosin heads as shortening proceeds (6). Consistent with this idea, Calaghan et al. (8) proposed that simultaneous binding of MyBP-C to two positions on myosin, i.e. to myosin S2 (near the S1/S2 junction) and to the light meromyosin segment of myosin rod, could restrict the extension of myosin heads away from the thick filament. The net effect might be to limit myosin interactions with actin. However, a recombinant MyBP-C protein containing only the C1C2 domains and thus a single S2 binding site increased Ca2+ sensitivity of force in myocytes from cMyBP-C knock-out mice (9). Because effects of C1C2 did not depend on a second myosin binding site, the results implied that the C1C2 domains could affect actomyosin interactions independent of tethering myosin heads to thick filaments.

The current experiments were performed to investigate mechanisms by which N-terminal domains of MyBP-C influence myosin contractile properties and whether these effects depend on organization of myosin into thick filaments. Results from in vitro motility assays demonstrate that organized thick filaments are not required for recombinant proteins containing N-terminal domains of MyBP-C to affect mechanical properties of myosin and further suggest that effects of MyBP-C to slow cross-bridge kinetics may be due to slowing of cross-bridge detachment rates.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant MyBP-C N-terminal Proteins—N-terminal cMyBP-C proteins containing the C0C1, C0C2, C1C2, and C2C4 domains (Fig. 1) were cloned by PCR amplification of a full-length mouse cMyBP-C cDNA sequence (GenBank accession number AF097333) using primers shown in Table 1. Upstream and downstream primers contained restriction site sequences for Ndel and HindIII, respectively. The resulting PCR products were cloned into the Zero-Blunt vector (Invitrogen) and gel-purified following Ndel/HindIII digestion. For expression in bacteria gel-purified inserts were subcloned into the pQE-2 expression vector (Qia-gen) in frame with a His6-tag at the N terminus of the clone sequence to facilitate purification. Protein expression in M15 (Qiagen) transformants was induced by IPTG and the expressed proteins were purified under native conditions using...
In vitro Motility Assays—Skeletal muscle myosin was prepared from back muscle of male New Zealand White rabbits according to Margossian and Lowey (10) and stored at −20 °C in 50% glycerol for up to 4 months. Heavy meromyosin (HMM) was prepared by mild chymotryptic digestion (12.5 μg/ml) of myosin as described (11) and used within 1 week. At the beginning of each day of experiments ATP-insensitive heads were removed from an aliquot of HMM by ultracentrifugation as described previously (12). F-actin was prepared from rabbit back and leg muscle ether powder (13) according to Pardee and Spudich (14) and stored on ice for up to 2 months. Rhodamine-phalloidin (RhPh; Molecular Probes, Eugene, OR) labeled F-actin was prepared according to Kron et al. (11). Before use, RhPh F-actin was diluted 1:200 in AB buffer plus 5 mM DTT. Cardiac tropinin (cTn) and tropomyosin (cTm) were isolated by foam adhesive strips. Total chamber volume was typically 50–70 μl. The lower slide surface was coated with 0.1% nitrocellulose in amylacetate (Sigma). Experimental procedure was similar to Gordon et al. (12). Briefly, HMM (0.35–0.55 mg/ml) was added to the flow cell for 3 min. Nonspecific protein binding to the surface was blocked with bovine serum albumin (BSA) added at 0.5 mg/ml in AB. The chamber was rinsed with AB, and non-labeled sheared F-actin was added to the chamber for 1 min followed by wash with AB and 0.5 mM ATP in AB. RhPh F-actin was then added for 1 min followed by infusion of the motility buffer. For unregulated assays, the motility buffer consisted of AB buffer with 2 mM ATP, 18 μg/ml catalase, 0.1 mg/ml glucose oxidase, 3 mg/ml D-glucose, and 40 mM DTT to minimize photooxidation and photobleaching (11). For regulated assays, RhPh actin filaments were followed with AB containing 100 nm each cTn and cTm. RhPh actin and regulatory proteins were reconstituted for 6 min before the motility buffer was added (16). For regulated assays the motility buffer consisted of (in mmol/liter): 25 imidazole, 2 MgATP, 1 EGTA, 1 free Mg2+, and 100 nm each cTn and cTm. CaCl2 was added to set the free Ca2+ concentration at whole values of pCa from 9.0 to pCa 5.0, and ionic strength was adjusted to 50 mM with KCl according to calculations performed using custom software and the National Institute of Standards and Technology Critically Selected Stability Constants of Metal Complexes Data Base. Intermediate pCa values were achieved by mixing the closest whole point pCa solutions (i.e. pCa 6.0 and 7.0 to achieve 6.5). Antioxidants were added as above to minimize photobleaching. C1C2 in AB or an equivalent volume of distilled water as control was added to achieve the desired concentration in motility buffers. All motility assays were performed at 23 ± 0.3 °C. Temperature was controlled by a circulating water bath with a programmable set point that maintained the desired chamber temperature via a copper coil wrapped around the 100X objective. The temperature of the fluid inside the assay chamber was checked with a thermocouple device after each experiment.

Data Acquisition and Analysis—After infusion with motility buffer, flow cells were transferred to the stage of an inverted microscope where RhPh labeled filaments were visualized by epifluorescence excitation with a 100-watt mercury lamp (Zeiss) and recorded using a SIT camera connected to a VCR. At least six different fields from each flow cell were recorded for 30 s. Each field was then digitized with a sampling rate of 10 frames per second and analyzed using ExpertVision software (Motion Analysis System, Santa Rosa, CA). Prior to analysis, the data were smoothed using a five point moving average filter to yield an apparent sampling rate of 2 fps. To eliminate erratically moving filaments due to digitizing jitter, all paths with mean ± S.D. speed >0.5 were excluded from analysis (17). Due to digitizing errors under rigor conditions, the system measured an apparent sliding speed of 0.2 μm/s ("rigor speed") and 10% of the recorded filaments moving. These values were taken as the lower resolution limit and filaments moving with rates equal or lower then 0.2 μm/s were considered stopped.

A mean speed value for each flow cell was calculated by combining the data from all of the accepted filament paths. Reported average mean speed ± S.D. values for each type of experimental condition were calculated as weighted mean ± S.D. values,

\[
\bar{\mu} = \frac{\sum n_i \mu_i}{\sum n_i}
\]

\[
\sigma^2 = \frac{\sum n_i (\mu_i - \bar{\mu})^2}{\sum n_i^2 - \overline{\mu^2}}
\]

where \( n_i \) is the number of slides, \( n \) is the number of accepted filament paths per slide, \( \mu_i \) is the mean filament velocity for this slide, \( \bar{\mu} \) is a weighted average, and \( \sigma \) is the weighted S.D. Statistical differences (p < 0.01) between weighted mean values were evaluated using one-way analysis of variance followed by Bonferroni post hoc comparisons.

Binding of C1C2 to RhPh Actin in Motility Flow Cells—Binding of C1C2 to labeled actin was assessed in motility flow cells using solutions as used for in vitro motility assays. Three different flow cell loading sequences were used: 1) BSA (0.5 mg/ml, 3 min) → AB wash → motility buffer plus C1C2 and RhPh-actin

| TABLE 1 | Primers used to generate recombinant N-terminal domains of cMyBP-C |
|---|---|
| cMyBP-C domains | Primer pairs (upstream primer/downstream primer) |
| C0C1 | 5'-GGTACCATATGCCTGCCAGCAGGAAAG-3'/5'-CTAAGCTTATGTCAGCTCACGACGGACGG-3' |
| C0C2 | 5'-GGTACCATATGCCTGCCAGCAGGAAAG-3'/5'-CTAAGCTTATGTCAGCTCACGACGGACGG-3' |
| C1C2 | 5'-GGTACCATATGCCTGCCAGCAGGAAAG-3'/5'-CTAAGCTTATGTCAGCTCACGACGGACGG-3' |
| C2C4 | 5'-GGTACCATATGCCTGCCAGCAGGAAAG-3'/5'-CTAAGCTTATGTCAGCTCACGACGGACGG-3' |
filaments (sequence similar to that used during in vitro motility experiments), 2) C1C2 in AB (3 min) → AB wash → BSA (0.5 mg/ml, 1 min) → AB wash → RhPh-actin filaments → motility buffer, 3) BSA (0.5 mg/ml, 3 min) → AB wash → RhPh-actin filaments (1 min) → motility buffer. Sequence 3 served as a negative control. Images of the flow cell surfaces were recorded periodically for up to 60 min after loading. For experiments to assess ability of C1C2 to bind actin in the presence of regulatory proteins, flow cells were treated sequentially with C1C2 in AB (or AB buffer alone as control) followed by BSA. Flow cells were then rinsed in AB buffer and labeled F-actin was added. Finally, motility buffers containing 100 nm each bovine cardiac tropo-
nin and tropomyosin were added. Motility buffers contained either low (pCa 9.0) or high (pCa 5.0) calcium.

Cosedimentation Binding Assays—F-actin was prepared as described for in vitro motility experiments and dialyzed into AB buffer plus 1 mM ATP prior to use. Reconstituted actin filaments were prepared by incubation of F-actin overnight at 4 °C with purified cardiac troponin and tropomyosin in a 4:1:1 ratio of actin to regulatory proteins (12). N-terminal cMyBP-C-proteins were dialyzed into AB and spun at 100,000 rpm in an Optima TL centrifuge to pellet insoluble material immediately prior to use in binding assays. F-actin or reconstituted thin filaments and recombinant proteins were then mixed at varying molar ratios and incubated for 30 min (22 or 4 °C). In some experiments KCl was added to increase ionic strength. Samples were centrifuged at 100,000 × g for 30 min. Supernatants were decanted and mixed with an equal volume of urea-thiourea sample buffer (18). Pellets were washed once with 50 μl of AB and resuspended in 100 μl of a 1:1 mix of urea-thiourea sample buffer and AB. Equal volumes of supernatant and pellet were analyzed by SDS-PAGE (18).

ATPase Assays—The ATPase activities of HMM and actin-activated HMM were determined as described previously (19). Brieﬂy, HMM was dialyzed into an ATPase reaction buffer consisting of: 0.1 mM K-EGTA, 10 mM imidazole, 2 mM MgCl₂, and 1 mM DTT and diluted to a final concentration of 1 μM. 1 μM ATP was then added to start each reaction. Reactions were terminated at desired time points by the addition of stop solution (3.3% SDS, 0.12 mM Na-EDTA, pH to 7.4). Phosphate production was determined via colorimetric assay as described by White (20). For actin-activated HMM ATPase assays, HMM in ATPase buffer was diluted to 0.1 μM and combined with 5 μM F-actin. All assays were performed at 22 °C.

RESULTS

C1C2 Inhibits F-actin Filament Sliding in Unregulated Motility Assays—We had previously shown that a recombinant protein containing the C1 through C2 domains of cMyBP-C (i.e. C1C2, Fig. 1) increased Ca²⁺ sensitivity of tension in permeabilized myocytes (9). To investigate whether C1C2 could also affect actomyosin interactions in the absence of myosin organization into thick filaments, effects of C1C2 were assessed using in vitro motility assays. Fig. 2 shows results from motility assays using the HMM subfragment of myosin to propel RhPh-labeled F-actin filaments over nitrocellulose-coated slides. Under control conditions in the absence of thin filament regulatory proteins, movement of labeled F-actin was constitutively “on” and insensitive to Ca²⁺ (12). This is demonstrated by the high rates (>2 μm/s) and uniform filament speeds obtained in motility buffers even in the absence of Ca²⁺ (e.g. in AB motility buffer). However, addition of C1C2 to the motility buffers reduced filament sliding in a dose-dependent manner with movement completely stopped (indistinguishable from rigor) at 2 μM C1C2.

Dual Effects of C1C2 on Reconstituted Thin Filament Motility—We next investigated whether the presence of thin filament regulatory proteins affected the ability of C1C2 to inhibit F-actin motility. For these experiments, Ca²⁺-sensitive activation of filament motility was reconstituted by including purified whole cardiac troponin and tropomyosin in motility buffers as described previously (16). As shown in Fig. 3 under control conditions (0 μM C1C2), addition of regulatory proteins restored Ca²⁺-activation of motility, with movement stopped and indistinguishable from rigor at low Ca²⁺ (pCa 9.0), but fully activated in the presence of high Ca²⁺ (pCa 5.0). Under the latter Ca²⁺-activated conditions at pCa 5.0, C1C2 was again effective at inhibiting sliding movement in a dose-dependent manner, although a greater amount of C1C2 was required to completely stop movement in the presence of thin filament regulatory proteins than in their absence (compare with Fig. 2).

By contrast, in the presence of low Ca²⁺ (pCa 9.0) and under regulated assay conditions where filament movement is normally stopped, addition of C1C2 activated motility. As shown in Fig. 3, C1C2 activated regulated filament movement at pCa 9.0 in a dose-dependent manner with effects most pronounced between 1–2 μM C1C2. There was a close correspondence...
between the increase in filament sliding velocity and the percentage of motile filaments, indicating that C1C2 increased movement of the majority of filaments and did not affect a subpopulation of filaments. However, activating effects were biphasic, and concentrations of C1C2 exceeding 2 μM again inhibited motility. Summary data for C1C2 effects on regulated proteins to restore Ca$^{2+}$-dependent activation as described under “Experimental Procedures.” Bars represent mean ± S.D. A, filament speed (solid bars) and percentage motile filaments (hatched bars) in pCa 9.0 buffer. B, filament speed (solid bars) and percentage motile filaments (hatched bars) in pCa 5.0 buffer. Asterisks indicate significant difference from control (0 μM C1C2).

**TABLE 2**

Regulated F-actin summary data

Values represent means ± S.D.

| Motility buffer | [C1C2] μM | Rate μM/s | Fraction moving | No. slides | No. paths |
|-----------------|-----------|-----------|-----------------|------------|-----------|
| pCa 9.0         | 0         | 0.2 ± 0.1 | 0.1 ± 0.1       | 16         | 2536      |
|                 | 0.5       | 0.2 ± 0.1 | 0.14 ± 0.04     | 8          | 2246      |
|                 | 1.0       | 1.2 ± 0.4*| 0.5 ± 0.2*      | 9          | 2059      |
|                 | 2.0       | 1.3 ± 0.3*| 0.3 ± 0.1*      | 6          | 1038      |
|                 | 3.0       | 0.9 ± 0.1*| 0.6 ± 0.1*      | 4          | 1571      |
|                 | 5.0       | 0.3 ± 0.2 | 0.20 ± 0.03     | 4          | 549       |

| pCa 5.0         | 0         | 0.5 ± 0.8 | 0.9 ± 0.1       | 17         | 5560      |
|                 | 0.5       | 4.1 ± 0.3 | 0.87 ± 0.05     | 7          | 3912      |
|                 | 1.0       | 2.8 ± 0.7*| 0.87 ± 0.09     | 6          | 1816      |
|                 | 2.0       | 2.6 ± 0.7*| 0.92 ± 0.09     | 4          | 682       |
|                 | 3.0       | 1.2 ± 0.5*| 0.7 ± 0.2       | 5          | 1296      |
|                 | 5.0       | 0.4 ± 1.0*| 0.20 ± 0.06*    | 5          | 701       |

*Significantly different from control ($p < 0.01$).

Effects of C2C4 were also investigated. C2C4 is similar to C0C2 in that it contains 3 Ig-like domains, but unlike C0C2 it lacks the MyBP-C motif. As shown in Fig. 7, C2C4 had no effect on Ca dependence of sliding velocity.
on unregulated filament motility and did not activate motility at pCa 9.0. Thus, combinations of 2 or 3 Ig-like domains alone (e.g. C0C1 and C2C4) are not sufficient to inhibit filament sliding under unregulated conditions or to activate filament motility at submaximal Ca\textsuperscript{2+} under regulated conditions. Instead, the MyBP-C motif, which is present in both C1C2 and C0C2 appears specifically required for these effects. However, although C2C4 did not affect filament sliding under unregulated assay conditions, sliding velocity was significantly slowed under regulated conditions at high Ca\textsuperscript{2+} (Fig. 7). These results suggest that C2C4 or other Ig-like domains may interact with thin filament regulatory proteins to slow regulated sliding at high Ca\textsuperscript{2+}.

**C1C2 Binds Actin**—Although myosin S2 is the sole binding partner identified thus far for the C1C2 domains (21, 22), we next investigated whether the C1C2 fragment could cross-link labeled F-actin filaments to the motility surface and thereby account for effects of C1C2 to slow filament movement. Similar tethering effects were reported for recombinant titin class I and II motifs, which inhibited filament movement in motility assays by cross-linking actin to glass slides (23). We therefore assessed the ability of C1C2 to cross-link F-actin by determining whether labeled filaments were retained on motility flow cell surfaces under conditions similar to those used for motility experiments except with HMM omitted. As shown in Fig. 8 when nitrocellulose-covered slides were coated first with BSA followed by a mixture of C1C2 and RhPh labeled F-actin (according to the order of addition of experimental buffers), actin filaments were not retained on the slide. This result argues strongly against the idea that C1C2 slows filament motility by cross-linking actin to the motility surface. However, somewhat surprisingly and in contrast to another report (22), we found that C1C2 binds to F-actin under conditions of our motility

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**FIGURE 6.** Velocity-pCa relationships in the presence of C0C2. A, reconstituted filament velocity was measured as a function of pCa in the presence of 1 \( \mu M \) C0C2 (dashed line). For comparison, the control curve (solid line) obtained in the absence of added recombinant protein was redrawn from Fig. 4. B, percentage of motile filaments under control conditions (solid line redrawn from Fig. 4) and in the presence of 1 \( \mu M \) C0C2 (dashed). Symbols represent mean ± S.E.
assays. For these experiments, we added C1C2 to flow cells prior to addition of BSA to facilitate nonspecific binding of C1C2 to the nitrocellulose surface. BSA was then added to block all remaining sites on the slide. Under these conditions labeled F-actin filaments were retained on the flow cell surface, suggesting that C1C2 binds readily to F-actin. Moreover, C1C2 interactions with actin persisted even in the presence of tropinin and tropomyosin and calcium (Fig. 8). Slides coated with BSA alone served as negative controls and did not retain F-actin filaments (data not shown).

To confirm the ability of C1C2 to bind actin and further characterize the interaction, we performed high speed cosedimentation assays with C1C2 and F-actin filaments. As shown in Fig. 9, C1C2 was soluble and found only in supernatant fractions following centrifugation in the absence of actin. However, when F-actin was included in the assays C1C2 was found in both supernatant and pellet fractions, consistent with C1C2 binding to and pelleting with F-actin. In similar experiments C1C2 was also found in pellet fractions when reconstituted thin filaments containing tropinin and tropomyosin were used instead of F-actin (Fig. 9).

We next assessed whether interactions of C1C2 with F-actin were sensitive to ionic strength, since initial cosedimentation experiments were performed under low ionic strength conditions similar to those used for in vitro motility assays. As shown in Fig. 9, increasing ionic strength reduced the amount of C1C2 found in the pellet with F-actin. However, binding was still evident even at physiological ionic strengths (~0.2 M KCl), indicating a persistent interaction of C1C2 with actin. A binding constant for the C1C2-actin interaction was not obtained, however, since binding was linear and not saturable for concentrations of C1C2 up to 20 μM (Fig. 9). Taken together these data suggest that interactions of C1C2 with F-actin may persist at physiological ionic strength but appear mediated primarily by relatively weak electrostatic interactions.

**C1C2 Inhibits Acto-HMM ATPase Rates in Solution**—The finding that C1C2 binds to actin and/or myosin S2 (21) in solution prompted us to investigate whether HMM or acto-HMM ATPase rates were affected by C1C2. As shown in Table 3, 10 μM C1C2 had no effect on HMM ATPase rates but significantly inhibited actin-activated HMM ATPase rates. In addition, C0C2 inhibited actin-activated ATPase rates to a similar extent as C1C2, but C0C1 did not. These results suggest that C1C2 and C0C2, which contain the MyBP-C motif, specifically inhibit actomyosin ATPase rates without affecting intrinsic myosin ATPase activity.
The major findings from this study are that the C1 through C2 domains of cMyBP-C influence actomyosin interactions independent of myosin organization into thick filaments and that these domains bind to actin. The former result establishes that release of myosin S1 heads away from the thick filament is not required for cMyBP-C to affect myosin contractile properties and that the N terminus of cMyBP-C is sufficient to slow filament sliding velocities in vitro. The latter suggests that interactions of cMyBP-C with actin may be important in slowing cross-bridge detachment rates in vitro and shortening velocities in sarcomeres.

The finding that the C1C2 domains of cMyBP-C are able to influence actin motility without inclusion of HMM molecules in thick filaments is consistent with observations that native MyBP-C affects ATPase activity of acto-S1 in solution (24). However, MyBP-C is present in sarcomeres at a limited molar ratio with respect to myosin, and its localization is restricted to the middle one-third of thick filament A-bands (25). The latter observations, along with findings that MyBP-C affects thick filament assembly and stability in vitro (26), led to the idea that MyBP-C influences contraction primarily by modulating characteristics of the thick filament. In support of this idea, phosphorylation of MyBP-C increases apparent cross-bridge extension away from thick filaments (27). Since movement of cross-bridge heads away from thick filaments and toward thin filaments could accelerate the kinetics of actomyosin interactions by bringing actin and myosin closer together, modulation of cross-bridge extension could provide an effective means of regulating contraction. The current results do not exclude this possibility, but they do suggest that MyBP-C is able to exert more direct effects on actomyosin interactions regardless of myosin organization into thick filaments.

The ability of the N-terminal domains of cMyBP-C to slow filament sliding speeds in motility assays and to inhibit actomyosin ATPase rates in solution is consistent with a primary effect of C1C2 to slow cross-bridge detachment rates (28). A decrease in cross-bridge detachment rates can also account for the activating effects of C1C2 in regulated assays (Fig. 4), since a concomitant increase in the proportion of attached cross-bridges could promote activation of the thin filament through cooperative cross-bridge binding (29). Accordingly, effects of C1C2 on

![Figure 9](image_url)

**TABLE 3**

Summary data for effects of cMyBP-C domains on ATPase activities

| cMyBP-C Domains | HMM | Acto-HMM |
|-----------------|-----|----------|
| None            | 0.04 ± 0.01 (5) | 4.35 ± 1.21 (20) |
| C0C1            | ND  | 2.96 ± 0.73 (5) |
| C0C2            | ND  | 0.99 ± 0.38 (5) |
| C1C2            | 0.04 ± 0.01 (4) | 1.40 ± 0.99 (7) |

* Significantly different from control (p < 0.05).
filament motility may be similar to effects of increasing the proportion of strongly bound cycling cross-bridges by increasing [ADP] (30). In this regard, increasing [ADP] also facilitated motility at low Ca\textsuperscript{2+} (increased the pCa\textsubscript{50} of the thin filament) and slowed sliding rates at maximal activation, presumably by limiting cross-bridge detachment rates (30).

An alternative explanation for the ability of C1C2 to activate filament sliding is that C1C2 binds to and activates the thin filament in a manner analogous to chemically modified non-cycling cross-bridges (31). However, it seems unlikely that thin filament activation by itself could account for both the activating and inhibitory effects of C1C2. For instance, Nem-S1, a strong binding derivative of S1, was effective at activating filament sliding at low Ca\textsuperscript{2+} but did not create a drag that slowed filament motility at high Ca\textsuperscript{2+} (31, 32). Similarly, binding of C1C2 to actin could compete with S1 and reduce filament sliding speed by reducing the number of S1 heads interacting with the thin filament. This possibility also seems remote, however, since filament velocity is largely independent of the number of cycling heads and declines only at very low head densities (33).

Moreover, competition of C1C2 with S1 for actin binding might be expected to reduce binding of actin filaments to the coverslip surface as was reported for Nem-S1 (34). However, under conditions where C1C2 completely stopped filament motility (e.g. under unregulated assay conditions at 2 µM C1C2) actin filaments appeared firmly anchored to the coverslip surface and Brownian motion was stopped. Thus, while we cannot completely eliminate the possibility that C1C2 functions as an antagonist of S1 binding, the simplest explanation to account for the current data is that C1C2 slows cross-bridge detachment rates.

Slowing of cross-bridge detachment rates could also provide an explanation for previous observations that C1C2 increased the Ca\textsuperscript{2+} sensitivity of tension in permeabilized cardiac myocytes and skeletal fibers (9, 35). For instance, augmentation of force by C1C2 could either be a direct result of an increase in the time that cross-bridges spend in force generating states or an indirect result of increased activation of the thin filament as discussed above. However, in contrast to the activating effects of C1C2 on sliding velocity, neither we (9) nor others (36) found that C1C2 activated force in myocytes in the absence of Ca\textsuperscript{2+}. A potential explanation may be related to intrinsic differences between force and velocity measurements and their relative dependencies on cross-bridge number (i.e. whereas force is directly proportional to the total number of cross-bridges, velocity is not). Thus, activation of filament sliding speed through cooperative cross-bridge mechanisms may be more readily detected in motility assays compared with an analogous activation of force in myocytes because the former measurement requires relatively few cross-bridges (above a critical number) to reach maximal velocity. Alternatively, differences between cardiac and skeletal protein isoforms could account for differences between the ability of C1C2 to activate filament movement in \textit{in vitro} motility assays and force in myocytes, since skeletal actin and myosin isoforms were used in the current experiments. However, the activating (and inhibitory) effects of C1C2 were not specific to cardiac troponin and tropomyosin isoforms, since effects were still evident when skeletal regulatory proteins were substituted for cardiac in \textit{in vitro} motility assays (data not shown). More puzzling is the related observation that while C0C1 was effective at activating force in permeabilized myocytes (36), it had no effect on the speed of filament movement in motility assays. The activating effects of C0C1 on force may therefore depend on sarcomere lattice structure or on other protein interactions not present in motility assays. In this regard, concentrations of C0C1 required to activate force in myocytes (effects were maximal at 80 µM) also exceeded those used in the present experiments.

The idea that the N terminus of cMyBP-C slows filament sliding by slowing cross-bridge detachment rates agrees well with previous observations that partial extraction of MyBP-C from skeletal fibers (6) or knock-out of cardiac MyBP-C significantly sped shortening velocities at submaximal Ca\textsuperscript{2+} (5). Based on their results, Hofmann \textit{et al} (6) suggested that MyBP-C contributes to an internal load that opposes shortening in sarcomeres. In particular they suggested that MyBP-C constitutes an activation-dependent load which contributes to the biphasic shortening behavior observed in fibers activated at submaximal [Ca\textsuperscript{2+}] (7). Although the molecular basis for slowed shortening at low Ca\textsuperscript{2+} has not been fully resolved, Moss (7) suggested that a population of slowly cycling cross-bridges could give rise to a drag force that limits shortening velocity (28). Such slowly cycling cross-bridges might originate in transition regions of the thin filament between areas that are fully activated and neighboring regions that have become de-activated as shortening proceeds (7, 37). The current results suggest that slowly cycling cross-bridges could also arise in regions occupied by MyBP-C.

The molecular mechanism(s) by which C1C2 affects cross-bridge interactions with actin is presently unknown. However, C1C2 is known to bind myosin S2 (21) and results from the current study suggest that C1C2 also interacts with actin. Therefore, interactions of C1C2 with either or both actin and myosin could potentially affect cross-bridge interactions. For instance, by simultaneously binding to both actin and myosin S2, an actin-C1C2-myosin S2 complex could cross-link thick and thin filaments and thereby create a drag force that opposes shortening. Alternatively, distinct from constituting a mechanical load \textit{per se}, interactions of C1C2 with either or both actin and myosin S2 could influence discrete steps in the cross-bridge cycle and directly promote or prolong strong cross-bridge binding. In this regard, the effects of the N terminus of MyBP-C may be similar to effects of either caldesmon or calponin in smooth muscle which also bind actin and S2 and slow filament motility \textit{in vitro} (38). Both caldesmon and calponin apparently affect weak to strong cross-bridge transitions, albeit through distinct mechanisms (38 – 40).

Interactions of MyBP-C with actin have been proposed previously based on observations that native MyBP-C binds to actin (24, 41, 42) and on model fits of structural data which suggest systematic interactions of the N terminus of MyBP-C with actin (43). Since binding interactions of both native MyBP-C and C1C2 are sensitive to ionic strength, C1C2 may contain all or part of the actin binding site of native MyBP-C. It is notable, however, that a Pro-Ala-rich region located at the N terminus of skeletal MyBP-C (or between domains C0 and C1

Dual Effects of C1C2 in an \textit{in Vitro} Motility Assay
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of cMyBP-C, which was identified as a putative actin binding domain (43) is absent in the C1C2 protein used here. The current results therefore suggest that other residues must also be able to contribute to actin binding. In this regard, analysis of the C1C2 sequence predicts that the MyBP-C motif is highly basic (pI ~9.2) and would thus carry a net positive charge at physiologic pH. Basic residues could potentially interact with actin in a manner similar to the way in which basic repeats of dystrophin bind actin (44). Such electrostatic interactions might be of sufficient strength to stabilize actomyosin interactions and/or to slow cross-bridge detachment rates but labile enough to permit filament sliding during passive stretch or active contraction (43).

In summary, N-terminal domains of MyBP-C that contain the MyBP-C motif are effective at inhibiting acto-HMM interactions in solution and slowing filament sliding velocities in vitro. The results are consistent with an effect of MyBP-C to slow cross-bridge detachment rates and thereby prolong the lifetime of attached cross-bridge states. The presence of MyBP-C at discrete positions within the A-band, corresponding roughly to every third crown of myosin heads (43), may therefore provide specific nucleation sites for the origin of long-lived cross-bridges which in turn would be expected to influence cooperative activation and relaxation kinetics throughout the sarcomere (29, 45).

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