Modulation of Myosin Filament Organization by C-Protein Family Members

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We have analyzed the interactions between two types of sarcomeric proteins: myosin heavy chain (MyHC) and members of an abundant thick filament-associated protein family (myosin-binding protein; MyBP). Previous work has demonstrated that when MyHC is transiently transfected into mammalian nonmuscle COS cells, the expressed protein forms spindle-shaped structures consisting of bundles of myosin thick filaments. Co-expression of MyHC and MyBP-C or -H modulates the MyHC structures, resulting in dramatically longer cables consisting of myosin and MyBP encircling the nucleus. Immunoelectron microscopy indicates that these cable structures are more uniform in diameter than the spindle structures consisting solely of MyHC, and that the myosin filaments are compacted in the presence of MyBP. Deletion analysis of MyBP-H indicates that cable formation is dependent on the carboxy terminal 24 amino acids. Neither the MyHC spindles nor the MyHC/MyBP cables associate with the endogenous actin cytoskeleton of the COS cell. While there is no apparent co-localization between these structures and the microtubule network, colchicine treatment of the cells promotes the formation of longer assemblages, suggesting that cytoskeletal architecture may physically impede or regulate polymer formation/extension. The data presented here contribute to a greater understanding of the interactions between the MyBP family and MyHC, and provide additional evidence for functional homology between MyBP-C and MyBP-H.

INTRODUCTION

The muscle sarcomere is a complex and highly ordered array of numerous proteins, the precise organization and alignment of which are essential for proper muscle function. One of the major components of the sarcomere is the thick filament, which interacts with the thin filaments to generate the force resulting in muscle contraction. Although myosin is the predominant component of the thick filament, several other thick filament-associated proteins have been described. These proteins were initially described as contaminants of conventional myosin preparations, but later came to be recognized as proteins with specific

myosin binding properties (Starr and Offer, 1971). These proteins are quite abundant, representing up to 4% of the mass of the myofibril (Starr and Offer, 1971; Offer *et al.*, 1973; Yamamoto, 1984; Bahler *et al.*, 1985a). These include C-protein (MyBP-C), H-protein (86-kDa protein; MyBP-H), and X-protein (MyBP-X) (Offer *et al.*, 1973; Starr and Offer, 1983; Yamamoto, 1984; Bahler *et al.*, 1985a). Based on their similar amino acid composition and sarcomeric localization, these three proteins have been classified as a family of myosin-binding proteins (MyBP) (Einheber and Fischman, 1990; Vaughan *et al.*, 1993a,b; Weber *et al.*, 1993). Members of this family contain repeated motifs with homology to the Ig C2 (Ig C2) repeat and the fibronectin type III (Fn III) repeat, as do other MyBPs such as titin, twitchin, and skelemin (Einheber and Fischman, 1990). These proteins are found in a series of trans-

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verse stripes spaced 43 nm apart within the inner two-thirds of the A-band of striated muscle in mammals, birds, and frogs (Craig, 1977; Dennis *et al.*, 1984; Bahler *et al.*, 1985b; Bennett *et al.*, 1986).

Fast-skeletal isoforms have been identified for MyBP-C and MyBP-H, and slow-skeletal, cardiac, and several developmentally regulated isoforms of MyBP-C have been isolated (Jeacocke and England, 1980; Callaway and Bechtel, 1981; Reinach *et al.*, 1982, 1983; Obinata *et al.*, 1984; Starr *et al.*, 1985; Takano-Ohmuro *et al.*, 1989). The fast-skeletal isoform of MyBP-H is also expressed in the conduction system of cardiac tissue (Mikawa and Fischman, unpublished observation). No C-protein family members have been detected in smooth- or non-muscle tissue, although smooth muscle myosin light chain kinase and telokin contain related Ig C2 and Fn III motifs, similar in sequence to the MyBPs (Olson *et al.*, 1990; Holden *et al.*, 1992).

The role of the MyBPs in muscle formation and contraction has not yet been determined. However, considerable biochemical analysis has been carried out on MyBP-C from chicken fast-skeletal muscle, and a variety of rabbit skeletal muscles. Using chicken fast-skeletal MyBP-C, it has been determined that the protein binds directly to the myosin molecule through a domain within the carboxyl terminal Ig C2 repeat (Okagaki *et al.*, 1993). MyBP-C binds to the light meromyosin region of the myosin molecule, and, to a lesser extent, subfragment-2 (Moos *et al.*, 1975; Starr and Offer, 1978). Subfragment-1 does not bind MyBP-C (Starr and Offer, 1978). In vitro biochemical studies have shown that the addition of purified rabbit skeletal muscle MyBP-C to synthetic thick filaments decreases the critical concentration of myosin required for filament formation, and produces filaments of greater length and more uniform diameter than those formed in the absence of MyBP-C (Koretz, 1979; Davis, 1988). Additional studies have shown that MyBP-C binds to purified actin, regulated actin filaments, and the actin-containing I-bands of myofibrils (Moos *et al.*, 1978; Moos, 1981; Yamamoto, 1986), and can alter actin-activated myosin ATPase activity (Moos and Feng, 1980). Changes in actin-activated myosin ATPase activity have also been shown to occur in vitro with rabbit skeletal MyBP-H (Yamamoto, 1984). At low ionic strength, both MyBPs inhibit actin-activated myosin ATPase of skeletal muscle MyHC, while at higher ionic strength, MyBP-C mildly activates ATPase activity (Moos and Feng, 1980; Yamamoto, 1984). Partial extraction of MyBP-C from rabbit skeletal muscle leads to an increased rate of contraction, and increases the Ca^{++} sensitivity of the force-velocity curve (Hofmann *et al.*, 1991).

Many properties of thick filament assembly, sarcomere content, and sarcomeric organization have been established. However, an understanding of the function of many sarcomeric proteins has been hindered by the inability to study the physiologic interactions of these molecules outside of the complex milieu of the intact sarcomere. We have used a system in which components of the sarcomere are transfected into mammalian (COS) nonmuscle cells to allow analysis of the interactions of a variety of sarcomeric proteins. The absence of endogenous muscle proteins in COS cells allows the cellular distribution of sarcomeric proteins, alone or in pairwise combinations, to be assessed. When sarcomeric myosin is expressed in COS cells, it assembles into bundles of thick filaments (Moncman *et al.*, 1993; Vikstrom *et al.*, 1993). The experiments described below have allowed us to address the role of MyBPs in modulating thick filament formation and organization. It has been suggested that the binding proteins interact with myosin after myofilaments have formed (Craig, 1977), and that the 43 nm spacing seen for the binding proteins results from the spacing of their binding sites within the organized thick filament. The fact that MyBP-C is one of the last sarcomeric proteins to be expressed in myoblast differentiation is consistent with a passive, rather than an active role in sarcomere formation. However, the possibility exists that these proteins may play a more active role in sarcomere assembly, and may recruit or organize thick filaments into the pre-existing thin filament network of the premyofibril (Rhee *et al.*, 1994). The data reported here support the idea of a more active role. Rather than simply binding to existing myosin structures in transfected COS cells, the binding proteins alter their distribution, and cause them to form thinner, more uniform structures. A similar effect has been seen in in vitro studies (Koretz, 1979; Moos *et al.*, 1975; Yamamoto, 1988). It has also been shown that MyBP-C lowers the critical concentration of myosin required for polymerization (Davis, 1988). Taken together, these data suggest that MyBPs may help regulate the number and/or the organization of myosin molecules within the thick filament.

The COS cell transfection system allows us to analyze the interactions between constituents of the sarcomere and to define the domains of the proteins required for these interactions. While in vitro biochemical systems have been used to ask similar questions, the COS cell transfection system also allows us to determine whether interactions between components of the sarcomere can modulate their organization and distribution. In this report we demonstrate the ability of MyBPs not only to bind to MyHC, but to modulate the distribution of MyHC and to identify the portion of MyBP-H required for this interaction.

MATERIALS AND METHODS

Expression cDNA Cloning

Full length chicken fast skeletal MyBP-C and MyBP-H cDNAs (Einheber and Fischman, 1990; Okagaki *et al.*, 1993; Vaughan *et al.*, 1993a) were subcloned into the *EcoRI* site of the pMT21 expression vector (Kaufman *et al.*, 1989) as follows: the 2066-bp MyBP-H cDNA insert was excised from pBluescript (p86–5') with *EcoRI*, and ligated into the unique *EcoRI* site of pMT21. The 3488-bp MyBP-C cDNA insert was excised from pBluescript with *EcoRI* and *NdeI*, blunted with Klenow polymerase and 0.25 mM dNTPs (United States Biochemical, Cleveland, OH), and ligated into the similarly blunted *EcoRI* site of pMT21. The MyBP-H deletions pMT/HA and pMT/HX were created by digesting p86–5' with *EcoRI* and *AclI* (pMT/HA) or *EcoRI* and *XmnI* (pMT/HX). The 1802- and 1391-bp fragments generated in this manner were filled-in as described above and ligated into the filled-in *EcoRI* site of pMT21. The MyBP-H deletion pMT/HD was created by digesting p86–5' with *NaeI* and *BglII*, releasing a 408-bp fragment. The remaining coding sequences were filled-in as described, and closed with T4 DNA ligase. This deletion, pH/D, was then excised from pBluescript with *EcoRI*, and ligated into the *EcoRI* site of pMT21.

A full-length perinatal myosin heavy chain expression clone was constructed as follows. Sequences encoding nucleotides 1–2644 (Karsch-Mizrachi *et al.*, 1990) were excised from pGem1 (Promega, Madison, WI) with *BamHI* and *EcoRI*. This fragment was inserted into the pMT21 vector that had been digested with *EcoRI* and *PstI*, using a *BamHI*–*PstI* adaptor (New England Biolabs, Beverly, MA). This construct was designated pSS1. Sequences encoding nucleotides 2645–3381 of human perinatal MyHC (Feghali and Leinwand, 1989) were excised from pGem1 with *EcoRI*. This fragment was ligated into *EcoRI*-digested pSS1, resulting in the full length cDNA. Unless otherwise indicated, all enzymes were obtained from New England Biolabs.

Cell Culture

COS-7 cells were obtained from Dr. Margaret Kielian (Albert Einstein College of Medicine) and maintained in DMEM with 10% fetal calf serum (HyClone, Logan, UT), 2 mM L-glutamine, penicillin G at 50 U/ml, and streptomycin at 50 µg/ml. Transient transfections were performed using a previously described DEAE-dextran protocol (Levy-Mintz and Kielian, 1992; Vikstrom *et al.*, 1994). Colchicine treatment was for 4 h with colchicine (Sigma, St. Louis, MO) at a final concentration of 10 µg/ml in culture medium.

Immunoblotting

Protein expression of transfected cells was analyzed by Western blotting 36–48 h post-transfection. One hundred-millimeter dishes were washed three times with 5 ml of phosphate-buffered saline (PBS) containing 171 mM NaCl, 6 mM Na₂HPO₄, 6 mM KH₂PO₄, and 3 mM KCl, and lysed with 500 µl of Laemmli sample buffer (Laemmli, 1970). Ten microliters of each sample was electrophoresed on 8% SDS-PA gels, transferred to 0.2 µm nitrocellulose (Schleicher and Schuell, Keene, NH) using a previously described protocol (Towbin *et al.*, 1979), and blocked with 5% nonfat dry milk in PBS. Monoclonal antibody NA4 (Bourke *et al.*, 1991) was used to detect MyHC, and a polyclonal serum, γ-serum was used to detect MyBPs (Einheber and Fischman, 1990). Secondary antibodies were goat anti-mouse or goat anti-rabbit horseradish peroxidase (Bio-Rad, Richmond, CA). Immunoreactive proteins were visualized using ECL chemiluminescence (Amersham, Arlington Heights, IL). Blocking and primary antibody incubations were performed at 37°C for 1 h or at 16°C overnight. Secondary antibody incubations were performed at 37°C for 1 h.

Immunocytochemistry

Coverslips were fixed in 3.7% formaldehyde in PBS for 5 min, permeabilized in ice-cold acetone for 2 min, and blocked in 10% normal goat serum (NGS) in PBS for 30 min at 37°C. Primary antibodies were IgG monoclonal antibody F59 (Miller *et al.*, 1989), IgM monoclonal antibody N3.36 (Silberstein *et al.*, 1986), and γ-serum. Secondary antibodies were goat anti-mouse fluorescein, goat anti-rabbit rhodamine, IgM-specific goat anti-mouse fluorescein, and IgG-specific goat anti-mouse Texas Red (Jackson ImmunoResearch, West Grove, PA) diluted 1:40 in 10% NGS in PBS. Actin was visualized with rhodamine phalloidin (Molecular Probes, Eugene, OR) diluted 1:20 in 10% NGS in PBS. Microtubules were visualized using an anti-β tubulin IgG monoclonal antibody (Amersham). Antibody incubations were performed for 30 min at 37°C.

Electron Microscopy

Cells were grown and transfected as described above. Dishes were rinsed with cold PBS, three times for 5 min, and fixation was done with cold 4% paraformaldehyde in PBS for 15–30 min at 0°C. Dishes were again rinsed with PBS, as above, and cells were permeabilized with 1% Triton X-100 in PBS for 1 min at 4°C. Samples were again rinsed with PBS, and free aldehydes were reduced with 0.05% sodium borohydride in PBS for 30–60 min at 4°C. Cells were again rinsed with PBS, and then blocked in 2% normal goat serum in PBS for 1 h at 25°C. Dishes were washed with 1% bovine serum albumin (BSA), 0.05% Tween-20 in PBS (BSA/Tween/PBS), three times for 5 min. Primary antibody was diluted to 1 µg/ml in 1% BSA, 0.05% Tween-20, and incubated with samples for 5–6 h at 4°C. Primary antibodies were monoclonal antibody NA4 against MyHC and γ-serum. Cells were gently washed with BSA/Tween/PBS, three times for 5 min, and incubated with secondary antibody for 12–16 h at 4°C. Secondary antibodies, 5-nm gold-conjugated goat anti-mouse IgG (Amersham), and 10-nm gold-conjugated goat anti-rabbit IgG (E-Y Labs, Amersham) were diluted 1:10 in BSA/Tween/PBS. Samples were then washed with 0.05% Tween-20 in PBS, five times for 10 min at 25°C, and then with PBS, three times for 5 min. Dishes were then washed in 0.1 M sodium cacodylate pH 7.4, and post-fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate. COS cell monolayers were embedded, stained, and sectioned as previously described (Luft, 1961; Sabatini *et al.*, 1963). Serial sections were cut parallel to the plane of the substratum, and examined in a JEOL 1200CX electron microscope at an accelerating voltage of 80 kV.

RESULTS

Expression of MyHC and MyBP in COS Cells

Sarcomeric MyHC assembles into arrays of thick filaments when expressed in nonmuscle cells (Moncman *et al.*, 1993; Vikstrom *et al.*, 1993). Because MyBPs have been postulated to play a role in the organization of thick filaments, we investigated the interactions of these two sarcomeric components through transfection of nonmuscle cells. cDNA expression constructs encoding either human perinatal or rat α cardiac MyHC and MyBPs were transiently transfected into COS-1 cells, either singly or in pairwise combinations. The constructs used in these studies are shown in Figure 1, and express full-length human perinatal MyHC, full-length chicken fast-skeletal MyBP-C, and MyBP-H, and also include several MyBP-H deletion mutants used for functional domain mapping that will be described below. The transfection efficiency generally ranged from 2–20%, and in co-transfections, ap-

post-translationally modified form of the protein, as the same cDNA expressed in COS cells under the control of the cytomegalovirus promoter produces

Figure 2. Electrophoretic mobilities of expressed proteins as determined by Western blotting. Aliquots of whole cell lysates from cells transfected with individual expression constructs were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with the sarcomeric myosin-specific antibody F59 (lane 1) or with γ -serum recognizing both MyBP-C and MyBP-H (lanes 2–6). Lane 1, pMT/P; lane 2, pMT/C; lane 3, pMT/H; lane 4, pMT/HA; lane 5, pMT/HX; and lane 6, pMT/HD. Molecular weight markers are indicated by arrows.

only the 86-kDa protein. The MyHC/MyBP-H structures formed by pMT/H, with multiple immunoreactive bands, and VR/H, which produces a single immunoreactive band of 86 kDa, appear identical (Seiler and Leinwand, unpublished observations), indicating that the additional immunoreactive species do not contribute to the MyBP/MyHC interaction.

Distribution of Sarcomeric Proteins

The intracellular distribution of the expressed proteins was examined by indirect immunofluorescence. Human perinatal MyHC forms discrete spindle-shaped structures that are distributed throughout the cytoplasm of the cell (Figure 3a). The same distribution pattern is seen when rat α - or β -cardiac MyHC is expressed in COS cells (Vikstrom *et al.*, 1993), and occurs even when protein expression levels vary over 100-fold (Seiler and Leinwand, unpublished observations). These structures fail to form when a construct lacking the myosin rod is expressed, indicating that the structures represent assemblages of myosin, and not aggregates of protein (Vikstrom *et al.*, 1993). Approximately 1% of cells transfected with MyHC constructs demonstrate a staining pattern in which no structures are visible. It is not clear whether this results from a failure of the myosin to form spindle-like structures or from extremely high levels of expression in which the entire cytoplasm is packed with structures. Both MyBP-C and MyBP-H remain diffusely distributed throughout the cytoplasm (Figure 3, b and c, respectively). MyBPs have a dramatic effect on myosin distribution when co-expressed. Rather than forming spindle-like structures, virtually all of the MyHC expressed in co-transfected cells forms extremely long cables, the majority of which encircle the nucleus (Figure 3d). A portion of the expressed binding proteins co-localizes with these myosin structures, while the rest remains cytoplasmic (Figure 3e). In cells expressing higher levels of MyBP, the visualization of co-localization with MyHC is sometimes obscured by the large amounts of binding protein present in the cytoplasm (note the cell marked by the arrow in Figure 3e). In the co-transfection experiments, there was always significantly more MyBP protein expressed than MyHC protein, despite the use of identical expression vectors. The stoichiometry of MyBP:MyHC in co-transfected COS cells does not reflect the stoichiometry between these proteins found in muscle, where the ratio of MyBP:MyHC is 1:8 for MyBP-C and 1:1 for MyBP-H. To determine whether the ratio of MyBP:MyHC had any obvious effect on the structures formed, MyHC protein levels were reduced 100-fold by using a weaker promoter (RSV). Our unpublished observations indicate that there is no apparent difference in the structures formed.

It had previously been shown by electron microscopy that sarcomeric MyHC expressed in COS cells assembles into arrays of thick filaments (Moncman *et al.*, 1993; Vikstrom *et al.*, 1993). To examine the organization of the cable structures, immunogold electron microscopy was carried out. MyHC expressed in COS cells forms bundles of myosin positive structures, as determined by immunogold labeling (Figure 4a and b). Within these structures, individual myosin filaments are visible (Figure 4b). On rare occasions, these bundles formed higher ordered structures, as evidenced by a 20- to 30-nm repeat (our unpublished observations). However, the majority of the structures did not demonstrate this periodicity. Neither MyBP-C nor MyBP-H, when expressed singly, formed any detectable ordered structures (Figure 4c). Examination of the myosin cables formed in cells expressing both MyHC and MyBP was performed using 5-nm immunogold particles to detect MyHC, and 10-nm immunogold particles to detect MyBP-C. As shown in Figure 4, d–h, these structures appear to be thinner, and individual myosin filaments are no longer visible. Comparison of Figure 4b to 4e–4h indicates that while individual myosin filaments (shown as bracketed region) are visible within the structures containing only MyHC, they cannot be detected in structures containing both MyHC and MyBP. Because the sections examined can cut across any bundle longitudinally, tangentially, or in cross-section, it is difficult to determine the absolute lengths and widths using electron microscopy. However, even with the limitations of such measurements, there was a remarkable effect on the uniformity of structure width when a number of structures were measured, with the range decreasing from 0.8 μ m to 3.5 μ m for MyHC alone and from 0.4 μ m to 0.8 μ m for MyHC and MyBP. The average width of structures containing only myosin (P) was $1.87 \pm 0.12 \mu$ m, while the average width for structures containing MyHC and MyBP-C (P/C) or -H (P/H) was $0.53 \pm 0.04 \mu$ m and $0.49 \pm 0.04 \mu$ m (Figure 5). These data suggest that the presence of MyBP leads to compaction of myosin filaments, and provide evidence that the MyBPs within the sarcomere may act to bundle myosin filaments together, and that this compaction may account for the decreased width of structures containing both MyBP and MyHC.

Functional Domain Mapping of MyBP-H

Biochemical data have localized the MyHC binding domain to the carboxy-terminal 14 kDa of MyBP-C (Okagaki *et al.*, 1993). The C-terminal 40 kDa of MyBP-C and MyBP-H show 77% homology to each other (50% identity, and an additional 27% of conserved amino acids) (Vaughan *et al.*, 1993a; Okagaki *et al.*, 1993). Several MyBP-H deletions were generated

Figure 3. Cellular localization of expressed proteins. Indirect immunofluorescence of sarcomeric proteins expressed in COS cells. Cells were transfected singly with pMT/P (a), pMT/C (b), pMT/H (c), or co-transfected with pMT/P and pMT/C (d and e). Indirect immunofluorescence was performed to visualize MyHC (a and d) using antibody F59, and MyBP (b, c, and e) using γ -serum. MyHC forms spindle-like structures (a), while the binding proteins remain diffusely distributed (b and c). Co-expression of MyHC and MyBP leads to the formation of cables of myosin (d and e). Some of the binding proteins co-localize with these cables (e) while the rest remains diffusely distributed throughout the cytoplasm.

Figure 4. Ultrastructural analysis of expressed proteins. Immunogold electron microscopy of expressed proteins was performed on COS cells transfected with pMT/P (a and b), pMT/C (c), or pMT/P and pMT/C (d–h). Monoclonal antibody NA4 was used to detect MyHC (a, b, and d–h); γ -serum was used to detect MyBP-C (c–h). Secondary antibodies were 5-nm gold-conjugated goat anti-mouse IgG, and 10-nm gold-conjugated goat anti-rabbit IgG. Thin sections were examined under the electron microscope at an accelerating voltage of 80 kV.

Figure 4 (cont.)

(Figure 4 cont.) Five-nanometer gold particles (small arrows) are localized within myosin containing structures (b and e–h). When MyBP-C is expressed alone, the 10-nm gold particles (large arrows) are distributed randomly throughout the cell (c). When MyHC and MyBP-C are co-expressed, 10-nm and 5-nm gold particles co-localize (e–h). Bracketed region in panel b shows an individual filament.

Figure 5. Width variations of structures. Measurements were taken from negatives of electron micrographs of thin sections. P, structures consisting solely of MyHC. P/C and P/H indicate structures containing MyHC and MyBP-C or MyBP-H respectively. N values indicate the number of structures examined for each sample. Co-expression of MyHC with MyBPs produces a dramatic decrease in structure diameter.

and analyzed in co-transfection experiments for their ability to organize co-expressed MyHC into cables. The results are shown in Figure 6. pMT/HA, lacking 24 amino acids at the carboxy terminus of the molecule, and pMT/HX, lacking 161 amino acids at the carboxy terminus, do not form ropes when co-expressed with MyHC (Figure 6, a–d). In these cells, myosin distribution is unchanged, and the truncated binding protein is distributed throughout the cytoplasm, showing no association apparent with MyHC. In contrast, pMT/HD, expressing a MyBP-H lacking 136 amino acids within the amino-terminal portion of the molecule is still capable of promoting cable formation (Figure 6, e–f). For each construct, at least 100 co-transfected cells were examined. The percentage of cells with the indicated phenotype is listed below each panel. In all cases, failure to show the indicated phenotype resulted from a diffuse staining for MyHC (see above).

Expressed Proteins and the Endogenous Cytoskeleton

Within the sarcomere, MyHC interacts with the thin filaments, and MyBP-C has been shown to bind to skeletal actin in vitro (Moos *et al.*, 1978; Yamamoto, 1986), and to the I bands of skeletal muscle myofibrils (Moos, 1981). To determine whether these proteins are capable of interacting with the nonmuscle actin cytoskeleton of the transfected COS cells, double-label immunofluorescence was utilized to visualize transfected proteins and endogenous actin. MyHC (Figure 7a, large arrow; Figure 7b) shows no alignment along the COS cell actin filaments when expressed alone. MyBP (Figure 7, c and d) does appear to show some association with the actin cytoskeleton. Cable formation does not alter this interaction with nonmuscle actin (Figure 7a, small arrow; Figure 7b).

To assess the potential interaction of the transfected proteins with other proteins present in COS cells, the role of microtubules in the cellular distribution of MyHC containing structures was examined. As shown in Figure 8, cable structures composed of MyHC and MyBP-C do not associate with the endogenous microtubule network (Figure 8, a and b). Transfected cell monolayers were treated with a final concentration of 10 μ g/ml of colchicine for 4 h, resulting in collapse of the microtubule network as determined by indirect immunofluorescence using an anti- β tubulin antibody (Figure 8d). While the myosin containing structures are stable in the absence of microtubules, they appear longer than those seen in untreated cells (Figure 8c). In colchicine-treated cells the cables extend further into the periphery of the cell rather than being confined predominantly to the perinuclear region of the cytoplasm (Figure 8, compare a and c). These results indicate that microtubules, and possibly other cellular constituents, limit the maximum attainable cable length by physically impeding further elongation.

DISCUSSION

The MyHC structures formed in COS cells consist of numerous thick filaments, and therefore, at an average of 1.87 μ m, are about 100 times larger than the diameter of native (0.012 μ m) or synthetic thick filaments (0.02–0.03 μ m). However, the changes that are seen in these structures upon the addition of MyBPs are the same as those seen in synthetic thick filaments when MyBPs are added (Huxley, 1963; Koretz, 1979; Yamamoto, 1988). These changes include decreased filament diameter, as well as compaction and disordering of myosin within the filament (Koretz, 1979; Yamamoto, 1988). In our studies, addition of MyBPs causes a decrease in the width of the bundles and results in structures of more uniform width, while the width of structures composed of MyHC alone varies over a much wider range. The widths of these MyBP/MyHC

Figure 6. Effect of mutant MyBPs on MyHC distribution. COS cells were co-transfected with pMT/P and pMT/HA (a and b), pMT/HX (c and d), or pMT/HD (e and f). MyHC was detected with F59 (a, c, and e); MyBPs were detected with γ -serum (b, d, and f). Co-expression of MyHC with pMT/HA or pMT/HX produced myosin structures that were identical to those formed by myosin alone, while co-expression with pMT/HD produced cable-like structures that were indistinguishable from those formed with full length MyBPs. The numbers below each micrograph indicate the percentage of co-transfected cells exhibiting the depicted phenotype. For each MyBP-H deletion construct, over 100 transfected cells were examined.

structures ($0.4\text{--}0.8\ \mu\text{m}$) are still about 50 times wider than synthetic filaments formed in the presence of MyBP ($0.013\text{--}0.026\ \mu\text{m}$) (Koretz, 1979; Yamamoto, 1988). While the absolute filament diameter is clearly different between structures formed in *in vitro* biochemical systems and those produced in COS cells, the impact of the addition of MyBPs to both types of structures is the same, i.e., decreased filament width. This report also demonstrates that the presence of MyBP-C or -H within the cable structures leads to a compaction of myosin filaments. Although individual filaments are detectable within structures composed solely of MyHC, single filaments cannot be seen when MyBP is also present in the structures. The decreased structure diameter and the compaction of MyHC filaments in structures containing MyBP and MyHC are clear evidence that the MyBPs regulate the organization of MyHC within these structures, and suggest that the MyBPs may have an active role in myosin organization during myofibrillogenesis.

The data reported here also provide insight into the functional domains of MyBPs. *In vitro* biochemical data have shown that the myosin binding do-

main is located within the carboxy terminus of MyBP-C (Okagaki *et al.*, 1993), and preliminary data for MyBP-H indicate a similar location for the myosin binding domain (Alyonycheva, Mikawa, and Fischman, unpublished observation). Deletion analysis of MyBP-H presented here supports these data. Removal of the last 24 amino acids is sufficient to eliminate the ability of this protein to alter the distribution of MyHC. Deletion of 136 amino acids within the amino terminus of the protein does not impede the ability of this molecule to promote cable formation. These experiments define a domain at the carboxyl terminus of MyBP-H that is required for this interaction with MyHC. As previously reported, both MyBP-C and MyBP-H contain repeated motifs with homology to the Ig C2 repeat and the Fn III repeat (Einheber and Fischman, 1990; Vaughan *et al.*, 1993; Weber *et al.*, 1993). These two MyBPs exhibit 60% amino acid homology (42% identity with an additional 18%, conserved amino acids). This increases to 77% homology (50% identity with an additional 27%, conserved amino acids) when the last three domains are compared, and 85% when the last Ig C2 repeat is examined. A comparison of

Figure 7. Expressed sarcomeric proteins do not associate with the endogenous COS cell actin cytoskeleton. Cells were transfected with pMT/P (a, large arrow; and b), pMT/C (c and d), or co-transfected with both proteins (a, small arrow; and b). Expressed proteins were detected by immunofluorescence to visualize MyHC (a, F59) or MyBP (c, γ -serum), and the actin cytoskeleton was visualized using rhodamine-phalloidin (b and d). Neither MyHC nor MyBP co-localize with the endogenous COS cell cytoskeleton.

numerous proteins that are known to bind MyHC including titin, twitchin, skelemin, projectin, M-protein, and myomesin reveals that many of them contain Ig C2 and Fn III repeats (Vaughan *et al.*, 1992), suggesting that sequences within these motifs contain the myosin contact sites. However, because these proteins contain multiple repeats, it is difficult to tell which repeat actually interacts with MyHC. Between MyBP-C and MyBP-H, the greatest degree of homology is found within the last Ig C2 repeat, and in both proteins, the carboxy-terminal Ig C2 is the only repeat that contains the 14 conserved amino acids found in all Ig C2 domains (Williams, 1987; Einheber and Fischman, 1990). Other Ig C2-like domains in both MyBP-C and -H lack some of these conserved residues. These data suggest that the myosin binding domain is within this region. Our data support this. Comparison of this domain among several proteins including titin, twitchin, and skelamin, has shown three amino acid residues that are absolutely conserved. Experiments are underway to mutate these amino acids in MyBP-C, and to deter-

mine whether or not these point mutants are still functional. Function will be assessed both by *in vitro* binding assays as well as in the COS cell transfection system. In this manner, it should be possible to determine which amino acids compose the binding site. Additionally, this system, in conjunction with biochemical analysis, should be useful in defining which parts of the MyHC molecule interact with the various MyBPs. Previous work with MyBP-C has shown that this protein binds both within LMM and S2 (Moos *et al.*, 1975; Starr and Offer, 1978). Recent observations indicate that most of the binding of MyBP-C occurs within the amino-terminal one-third of LMM (Labedova and Fischman, unpublished observation). It should be noted that a MyHC mutant lacking 168 amino acids at the S2/LMM junction (residues 972-1139) is capable of forming cables when co-expressed with either MyBP-C or MyBP-H (Seiler and Leinwand, unpublished results).

Of particular interest in these studies is the ability of proteins from different species and different muscle

Figure 8. Effect of colchicine on MyHC distribution. Cells were co-transfected with pMT/P and pMT/C (a–d). Untreated cells are shown in a and b; colchicine-treated cells are shown in c and d. MyHC (a and c) and MyBP (b and d) were detected by indirect immunofluorescence, using N3.36 (a) and γ -serum (b). Microtubules were detected by indirect immunofluorescence using a commercially available anti- β tubulin antibody (Amersham).

types to interact with one another. The MyBPs have been isolated from chicken, yet they are capable of interacting with MyHC from both human (this report) and rat (Seiler and Leinwand, unpublished observation). Additionally, both MyBPs are fast-skeletal isoforms, yet both interact with perinatal MyHC as well as with cardiac MyHC (Seiler and Leinwand, unpublished observation). It has also been shown that chicken fast-skeletal MyBP-C and MyBP-H are both capable of being incorporated into the sarcomeres of rat cardiocytes (Seiler and Leinwand, unpublished observation). This is of particular interest, as MyBP-H is not expressed in the myocardium. These data indicate the high degree of conservation of these proteins between muscle type and across species. We have not been able to detect any interaction between the MyBPs and the endogenous COS cell nonmuscle MyHC, demonstrating that, in spite of regions of homology between sarcomeric and nonmuscle MyHC, there is still an isoform specificity inherent in the MyHC/MyBP interaction.

We have demonstrated that MyBP-C and MyBP-H alter the organization of MyHC, and we have defined the myosin binding domain of MyBP-H. Our data suggest that the MyBPs help to regulate the organization of myosin within the thick filament, and indicate that, for MyBP-H, this interaction requires the carboxyl terminal 24 amino acids. Future experiments will be directed toward defining the regions of the myosin molecule with which the MyBPs interact, as well as analyzing the impact of MyBP point mutations on myosin organization.

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