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Direct visualization of myosin-binding protein C bridging myosin and actin filaments in intact muscle

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Myosin-binding protein C (MyBP-C; C-protein) is a 130 kDa thick filament protein playing an essential role in muscle contraction, and MyBP-C mutations cause heart and skeletal muscle disease in millions worldwide. Despite its discovery 40 years ago, the mechanism of MyBP-C function remains unknown. In vitro studies suggest that MyBP-C could regulate contraction in a unique way—by bridging thick and thin filaments—but there has been no evidence for this in vivo. Here we use electron tomography of exceptionally well preserved muscle to demonstrate that MyBP-C does indeed bind to actin in intact muscle. This binding implies a physical mechanism for communicating the relative sliding between thick and thin filaments that does not involve myosin and which could modulate the contractile process.

C-protein | sarcomere structure | thick filament structure | cardiac muscle regulation | cardiac disease

Myosin-binding protein C (MyBP-C; C-protein) is a 130 kDa thick filament protein in vertebrate striated muscle located on seven to nine stripes 43 nm apart in each half A-band (1–4). Skeletal MyBP-C is a rod-shaped molecule, ~40 nm long and often bent near its center (3). The molecule is composed of a chain of ten globular, 4 nm-diameter, 10-kDa domains (C1 to C10) from the immunoglobulin (Ig) and fibronectin 3 (FN3) families, with an additional, MyBP-C-specific motif (also called the M-domain) between C1 and C2 (3, 5). The cardiac isoform, cMyBP-C, has an additional Ig domain, C0, at the N terminus (6, 7), and is phosphorylatable at four sites on the M-domain (2, 6, 8). MyBP-C is essential for the normal functioning of striated muscles in humans. Phosphorylation of the cardiac isoform plays a key role in the enhancement of cardiac function that results from β-adrenergic stimulation (2, 3, 6), and mutations in this isoform are a major cause of familial hypertrophic cardiomyopathy, affecting 60 million people worldwide (2, 9). Similarly, mutations in skeletal MyBP-C cause distal arthrogryposis, a disease characterized by joint contractures and abnormal muscle development (10).

The structural arrangement of MyBP-C in the sarcomere is poorly understood, although it is likely to play a crucial role in MyBP-C’s function. MyBP-C is bound to the thick filament via its C-terminal region (C8–C10), which interacts with the myosin tail and with titin (3); its N-terminal region can also bind reversibly to subfragment 2 of myosin (3, 4, 11, 12). Surprisingly, in vitro this myosin-binding protein can also bind to actin, via its N-terminal region (C0–C2) (13–18). However, there has been no evidence that this binding occurs physiologically. Electron microscopy of isolated thick filaments has revealed the likely location of the C-terminal domains on the thick filament surface (19), but the putative actin-binding N-terminal region was not visualized, possibly due to its disordering when thick filaments are removed from the native filament lattice. Here we use electron tomography of exceptionally well preserved muscle sections to demonstrate that MyBP-C binds to actin in intact muscle. This physical bridge between thick and thin filaments, independent of myosin heads, may play a key role in modulating muscle contraction in vivo.

Results

Electron Tomography of Rapidly-Frozen/Freeze-Substituted Frog Muscle. Thin (~100 nm) longitudinal sections (containing about 2.5 unit cells of the A-band lattice in depth) were cut from rapidly-frozen, freeze-substituted frog sartorius muscle embossed in epoxy resin (Fig. 14). This preparative technique is known to provide the best preservation of muscle ultrastructure available (20). Clear transverse stripes (numbered 1 to 11) are seen at 43 nm intervals on each side of the M-band (M), giving rise to corresponding peaks in the profile plot of these images (Fig. 1A). These stripes are known to represent nonmyosin proteins, primarily MyBP-C on stripes 5–11 (the C-zone) (21, 22), and other proteins on stripes 1–4 in the proximal (P-) zone (23). There are no nonmyosin stripes in the distal (D-) zone (23, 24). Two finer lines are seen between each pair of stripes. These lines arise from the arrangement of crowns of myosin heads at a mean axial spacing of 14.3 nm (25). The averaged Fourier transform of 23 half A-bands (Fig. 1A, inset) shows layer lines at orders of 43 nm, implying good preservation of the near-helical arrangement of the heads (20), with intensity visible as far out as the sixth order, corresponding to an axial resolution of 7 nm (see also Fig. S1). “Forbidden” meridional reflections, at the first, second, and fourth orders of 43 nm come from the nonmyosin stripes and perturbations in the myosin helix (24–26).

To obtain three-dimensional information on the organization of MyBP-C, three dual-axis tomographic tilt series were obtained from these sections. The images in each series were aligned, and


The authors declare no conflict of interest.

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Data deposition: The density map of the thick filament tomogram has been deposited in the Electron Microscopy Data Bank, http://www.ebi.ac.uk/pdbe/EMDB/ (Accession number: EMD-5805).

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three-dimensional tomograms were computed by back-projection (Movie S1 shows one of the aligned tilt series, and Movie S2 shows two-dimensional slices of a tomogram). From these individual tomograms, 207 thick filaments were extracted and used to calculate a single, averaged in situ thick filament three-dimensional reconstruction. The zone used for averaging extended to a radius of about 45 nm, which includes nearest neighbor thin and thick filaments.

The Thick Filament Tomogram and Its Interpretation. The thick filament reconstruction was made by separately averaging tomograms of each distinct region of the A-band: the P-, C-, and D-zones (Fig. 2). This procedure provided a higher quality average of each region than averaging over the thick filament as a whole. The most striking feature of the tomogram is the presence of clear transverse density every 43 nm in the C-zone (S5–S11 in Fig. 2), at the same levels as the stripes observed in the raw images (Fig. 1). These densities are sharply defined axially (4–8 nm, thus maximally one to two MyBP-C domains wide) and extend to high radius (>25 nm from the thick filament center). As in the original micrographs, the P-zone shows strong transverse density only at stripe 3, and nonmyosin densities are absent from the D-zone. Between adjacent MyBP-C densities are two clearly defined additional features. These features correspond to the finer lines seen in the micrographs, and represent helically organized crowns of myosin heads. There are three crowns in each repeat. We define crown 1 as the crown at the level of the stripe, while crowns 2 and 3 (c2 and c3) lie between the stripes. This interpretation of the major densities in the reconstruction is supported by thin cross-sections of the tomogram taken every 1.2 nm along the filament axis (Fig. S2).

While the single contour level used to depict the thick filament in Fig. 2 clearly reveals the MyBP-C and myosin head densities, it does not show their relative strengths. A gray-level depiction of the tomogram reveals that the high-radius density (MyBP-C) is weak compared to the c2 and c3 crowns of heads, consistent with the relative masses of heads (6 heads \( \times \) 130 kDa) and MyBP-C (3 MyBP-Cs \( \times \) 130 kDa) at each level, together with the diffuse region over which MyBP-C density extends (Fig. 2 inset, Movie S3). The gray-level depiction also suggests that the high density features at low radius in the stripes represent myosin heads (crown 1), as these appear similar to, and lie on the same helical path as, crown 2 and 3 heads. This interpretation of the stripes (S5–S11) is supported by fitting crown 1 heads from regions of the filament where the stripe density is weak or absent (and thus the head density is unambiguous) to the C-zone reconstruction. A segment comprising stripe 3 (crowns 2 and 3) and stripe 4 (crown 1), where the stripe density is very weak, was extracted from the P-zone and fitted to the C-zone at stripes 8 and 9 (Fig. 3A and B). Crowns 2 and 3 of the segment fitted well with the underlying C-zone crowns. Most importantly, using this fitting, crown 1 heads (yellow, blue, Fig. 3D and E) fitted well into the high density, low radius features concluded above to be myosin heads. A similar result was obtained by fitting crown 1 from the D-zone. We conclude that the high-radius cloud of density in the stripes is MyBP-C (purple, Fig. 3A–E), while the low radius, high-density features are myosin heads.

![Fig. 1. Observation of MyBP-C in sectioned muscle.](A) Electron micrograph of frog sartorius muscle A-band prepared by fast-freezing/freeze-substitution (20). Transverse stripes of 43 nm periodicity (numbered 1–11) are due to MyBP-C and other nonmyosin proteins, and fine lines of 14.3 nm repeat are due to myosin heads. For ease of description, the numbering at 43 nm intervals is continued beyond stripe 11, although there are no nonmyosin proteins at these positions. Layer lines in the Fourier transform (inset; third and sixth marked) indicate good preservation of myosin head helical order. See also Fig. S1. (B) Mean profile plot of several boxed regions similar to that in (A). M, M-band; stripe 1 to 5, P-zone; stripe 5 to 11, C-zone; and stripe 11 to edge of A-band, D-zone. (Scale bar, 200 nm).
The tomogram reveals in addition that the distal MyBP-C density (furthest from the thick filament backbone) is annular and coincides with the positions of the thin filaments (white in Fig. 3). The density appears to curve round to contact either the closest or next-closest thin filament to its origin on the thick filament, without any particular preference (Fig. 3 C–E). These varying contacts may reflect different accessibility of specific MyBP-C binding sites on actin at different axial levels, due to the unequal thick and thin filament helical repeats. These different modes of contact are consistent with the known flexibility of the MyBP-C molecule (3), which may be an essential feature of its structure, enabling it to bind to variably placed actin-binding sites. Assuming that the MyBP-C molecules at each 43 nm level and their actin-binding partners have specific binding sites for each other, and that the actin filaments in each half sarcomere are in helical register (27), calculations suggest every MyBP-C molecule should be able to bind to actin at each level where there is filament overlap, independent of sarcomere length (Fig. S3).

The distribution of actin filaments around the myosin filament suggests that at any level there would generally be a mixture of MyBP-C molecules moving to the left or the right to find the most accessible actin-binding site. However, the resolution of the tomogram and the averaging procedures used have not allowed us to test this prediction, and the paths shown (white, Fig. 3 B–D) and the schematic drawing in Fig. 3F represent only a guide as to possible conformations.

**Fitting the Tomogram to a Reconstruction of Isolated Thick Filaments.** While the tomogram shows broadly how MyBP-C is organized in the filament lattice of intact muscle, the resolution is limited to ~7 nm, and details of MyBP-C’s binding to the thick filament are unclear. Conversely, reconstruction of isolated, negatively stained cardiac thick filaments has revealed clear density that is thought to represent MyBP-C C-terminal domains on the thick filament surface; however, projecting regions are absent from the reconstruction, probably due to disorder in the absence of the filament.
lattice (19). To obtain a more complete picture of MyBP-C organization, we have combined these complementary approaches. The higher resolution (3–4 nm) isolated filament reconstruction was fitted into the tomogram of the in situ filament by matching the positions of the highest density regions of each map, which correspond to the myosin heads. Although the two reconstructions are based on very different methods of specimen preparation and on different muscle types, there was good agreement in the position of the myosin heads in the two maps (Fig. 4, Fig. S4; Movie S4). Crucially, the fitting shows that the axial position of the MyBP-C stripe of the tomogram coincides with that of the putative MyBP-C domains observed in the isolated filament (orange in Fig. 4); this coincidence is a result of matching head densities and was not a criterion used for the fitting. This fitting suggests that MyBP-C both binds to and projects from the filament at crown 1.

Discussion

Visualization of MyBP-C in Intact Muscle. The three-dimensional organization of MyBP-C in the sarcomere has remained obscure ever since it was first shown to be responsible for the 43 nm-spaced stripes in vertebrate muscle A-bands over 30 yr ago (21). While MyBP-C’s narrow axial distribution was inferred from the narrowness of the stripes, its radial extent was unknown, and the possibility that it might interact with actin in vivo (as suggested by in vitro observations) remained an unresolved hypothesis. Our use of fast-freezing, freeze-substitution, and electron tomography has enabled us to reveal the three-dimensional arrangement of MyBP-C in situ. The averaged tomogram confirms that MyBP-C lies in narrow planes perpendicular to the thick filament and, crucially, that a major portion is at high radius, beyond the myosin crowns and in contact with actin. We conclude that MyBP-C interacts with actin in vivo, and further, because our studies are of relaxed muscle, that it can do so when tropomyosin is in the blocking position on the thin filament (28). While this finding contrasts with an early in vitro finding suggesting that MyBP-C could only bind to activated thin filaments (29), it is consistent with subsequent studies showing binding at low as well as high Ca$^{2+}$ (16, 30). Although the tomogram clearly demonstrates MyBP-C binding to actin, we cannot entirely rule out the possibility that binding to neighboring thick filaments might also occur. However, MyBP-C is only just long enough to span between thick

Fig. 4. Fitting of isolated filament reconstruction into in situ tomogram. Mouse isolated filament reconstruction (gray surface rendering) (19) is docked into the frog tomogram (purple mesh). Four crowns of myosin heads are shown, including crown 1 lying under MyBP-C in stripe 7. Asymmetric myosin head dimer atomic structures are fitted into crowns 1 and 3 (circled) of the isolated filament reconstruction (19). The MyBP-C disk at stripe 7 coincides axially with three globular domains, (orange) in the isolated filament reconstruction, thought to be the C-terminal myosin/titin-binding domains (19). The mesh densities in the tomogram lying at high radius (outside the isolated filament reconstruction) at c2 and c3 are actin filaments (discontinuous at the threshold used). Fig. S4 provides additional details.
filaments and could do so only by projecting radially out towards its thick filament neighbor (Fig. S5). A straight, radial path towards neighboring thick filaments is inconsistent with the bent/curved path leading to actin that we see.

Relation to In Vitro Observations. Our tomogram strongly supports and complements previous observations of MyBP-C binding to actin. These earlier studies, based on centrifugation assays (14–16, 30–32), light and electron microscopy (14, 16, 29, 30, 33–35), neutron scattering data (36), and motility assays (15, 32, 37, 38), studied binding to F-actin (14–16, 31, 32) and to regulated thin filaments (14, 15, 32–34), and used both tissue-prepared protein (14, 29, 30, 32) and expressed whole MyBP-C or N-terminal fragments (15, 16, 31). While these in vitro studies clearly demonstrated MyBP-C's ability to interact with F-actin and thin filaments, and the consequent functional effects (14, 15, 18, 32, 37–39), our work demonstrates that this binding is relevant in vivo. The in vitro studies show that both skeletal (14, 29, 30) and cardiac (15, 16, 18, 31, 32) MyBP-C can bind to actin. For our experiments we used frog skeletal muscle, whose excellent order and orientation made it possible to obtain the accurate longitudinal sections needed in this work (20). MyBP-C is similar in overall domain structure in skeletal and cardiac muscles, the main differences being the presence in cardiac MyBP-C of an additional N-terminal domain (C0, which may (18) or may not (16) contribute significantly to actin binding) and of phosphorylation sites on the M-domain (2, 3, 5). It therefore appears likely that in situ binding of MyBP-C to thin filaments in cardiac muscle could be similar to that described here for skeletal muscle. While our tomogram nicely reveals the extension of MyBP-C from the thick filament to the vicinity of the thin filaments, it does not provide molecular detail on the interaction with actin. Such details are now emerging from neutron scattering and EM studies of F-actin decorated with MyBP-C (33–36).

Implications of MyBP-C Linking Myosin and Actin Filaments. The in vitro studies suggest that binding to actin occurs primarily through the C1 and M-domains (near the N terminus), the latter being phosphorylation-dependent in cardiac muscle (16). A recent report concludes that such N-terminal binding is weak and occurs through nonspecific, electrostatic interactions; surprisingly, it also demonstrates that in vitro, specific binding can occur via the C-terminal (myosin-binding) half of the molecule (31). How the myosin-binding end could also bind to actin in intact muscle is not clear, and the in vivo relevance of this finding remains in question (31) (but see Fig. S5). Combination of our tomographic data from in situ thick filaments with our earlier reconstruction of isolated filaments (19) suggests a simple model for MyBP-C organization in the relaxed state, in which the C-terminal three or four domains bind longitudinally along the thick filament surface (19) while the N-terminal half extends out towards the thin filaments (Figs. 3 and 4). This arrangement argues against a collar model for MyBP-C (3, 40). With the N terminus close to actin, even weak, nonspecific interactions may become significant and have profound physiological consequences, by modulating filament lattice stability and sensing and modulating filament sliding. Such interactions could be strong enough to stabilize the filament lattice (possibly enhancing the efficiency of contraction) but weak enough to permit sliding during stretch or contraction (15). Weakening of actin binding by phosphorylation of the M-domain that occurs in cardiac MyBP-C (16) could contribute to the enhanced contractility of the heart that occurs in response to β-adrenergic stimulation (2, 3). Binding to actin could further explain a long-standing enigma: how MyBP-C, present in a restricted zone of the thick filament, and only at every third level of myosin heads within that zone, can have an effect on thick-thin filament sliding as a whole (41). Clearly MyBP-C cannot interact with all myosin heads. Direct connection to actin (16, 41) could explain effects at the whole filament level straightforwardly.

Our structural observations support the view that the N-terminal end of MyBP-C can modulate contraction of striated muscle. The N terminus can interact in vitro with both myosin S2 (11, 12) and actin (14, 16). While our tomogram reveals clear interaction with actin in situ, detail is not sufficient to reveal any putative binding to S2. However, the tomogram shows clear myosin head density close to the filament backbone in relaxed muscle, and it is difficult to envisage how the N-terminal domains that bind to actin could simultaneously attach to S2 on either the same or a neighboring thick filament (Fig. S5). Our studies do not address what happens during contraction. Weakening of the 44.2 nm MyBP-C X-ray reflection on contraction suggests that MyBP-C may become much more mobile in this state (26). One possibility is that binding to S2 occurs transiently by switching with actin during filament sliding.

Materials and Methods

Specimen Preparation. Frog sartorius muscle was skinned in 1% triton in a relaxing solution, rapidly-frozen/freeze-substituted, embedded, and sectioned on a diamond knife. The preparation of this sample is described in our earlier study (20) in which we demonstrate the exceptional preservation of fine structure made possible by these techniques.

Electron Tomography. Images of three dual-axis tilt series of well oriented, thin (−100 nm), longitudinal sections similar to Fig. 1A were collected as described in Wü et al., (42). Briefly, grids were transferred into a Gatan model 670 Ultrahigh tilt analytical holder (Gatan), examined in an FEI CM300-FEG electron microscope (FEI Company) at 300 kV, and imaged with a TVIPS TemCam F224 2 k × 2 k CCD camera (TVIPS GmbH). EM-MENU software (TVIPS) was used for automated tilt series data collection in low dose mode. Each tilt series of ~100 images was collected from −70° to +70° using cosine rule increments. For each dual-axis dataset, one series was collected while tilting about an axis parallel to the filaments. The second tilt series was obtained after manually rotating the specimen grid ~90° and relocating the same region. Section shrinkage that occurs during electron irradiation (mainly in the depth of the section), and which could thus cause changes during collection of the tomographic series, was minimized by preirradiation (43, 44). Section shrinkage at a set electron dose has a rapid phase and then a slow phase where the section is relatively stable. Following the rapid initial shrinkage induced by the preirradiation, imaging in low dose mode in the stable phase avoided any further significant shrinkage during tomographic data collection. We noted from the tomograms that the shrinkage observed had no significant effect on the thick filaments themselves. However, it did affect the interfilament spacing as judged from the cross-sections of the reconstructions, which showed variable distance between the filaments.

The two orthogonal tilt series from each area were aligned using the PROTOmO marker-free tilt series alignment package (45). The tomograms from the two series were then merged by patch correlation using IMOD (46). In total, three dual-axis tomograms were generated in this way from three different areas in the same EM grid.

Subvolume Averaging. Longitudinal sections of striated muscle show transverse stripes numbered 1 to 11. These stripes appear different along the thick filament. Therefore we chose the whole thick filament in half an A-band as the individual unit for subvolume averaging. To obtain the final thick filament reconstruction shown, cylindrical volumes that included single thick filaments and the surrounding six actin filaments and myosin filaments (radius of 45 nm) were extracted from the tomograms and iteratively aligned in three-dimensions and then averaged. For each tomogram, one thick filament was selected as a reference. All the other thick filaments were identified by computing a cross-correlation map between the reference and the entire tomogram. In total, 207 (67, 72, 68) filaments were selected from the three tomograms. The initial alignment was based on the center of the cross-correlation peak that identified each filament within each tomogram. After taking into account the azimuthal rotation of filaments, all the filaments within each tomogram were aligned and averaged to generate a three-dimensional structure of the thick filament, which includes transverse stripes numbered 1 to 11. Once the structure of the thick filament was convincingly ascertained, 3-fold symmetry was imposed for subsequent rounds of refinement. Three real-space maps were designed separately for local
refinement in order to improve the structural details of the three distinct regions of the A-band: the P-, C-, and D-zones. Surface renderings were carried out using UCSF Chimera.

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