Published in final edited form as:

J Struct Biol. 2011 April ; 174(1): 44–51. doi:10.1016/j.jsb.2010.12.003.

Binding of the N-terminal Fragment C0–C2 of Cardiac MyBP-C to Cardiac F-actin

Robert W. Kensler¹, Justin F. Shaffer^{2,3}, and Samantha P. Harris²

¹University of Puerto Rico, San Juan, PR

²University of California, Davis, Davis, CA

³University of Washington, Seattle, WA, USA

Abstract

Cardiac myosin binding protein C (cMyBP-C), a major accessory protein of cardiac thick filaments, is thought to play a key role in the regulation of myocardial contraction. Although current models for the function of the protein focus on its binding to myosin S2, other evidence suggests that it may also bind to F-actin. We have previously shown that the N-terminal fragment C0–C2 of cardiac myosin binding protein-C (cMyBP-C) bundles actin, providing evidence for interaction of cMyBP-C and actin. In this paper we directly examined the interaction between C0–C2 and F-actin at physiological ionic strength and pH by negative staining and electron microscopy. We incubated C0–C2 (5 – 30 μ M, in a buffer containing in mM: 180 KCl, 1 MgCl₂, 1 EDTA, 1 DTT, 20 imidazole, at pH 7.4) with F-actin (5 μ M) for 30 min and examined negatively-stained samples of the solution by electron microscopy (EM). Examination of EM images revealed that C0–C2 bound to F-actin to form long helically-ordered complexes. Fourier transforms indicated that C0–C2 binds with the helical periodicity of actin with strong 1st and 6th layer lines. The results provide direct evidence that the N-terminus of cMyBP-C can bind to F-actin in a periodic complex. This interaction of cMyBP-C with F-actin supports the possibility that binding of cMyBP-C to F-actin may play a role in the regulation of cardiac contraction

Keywords

cardiac myosin binding protein C; cMyBP-C; F-actin; electron microscopy

1. Introduction

Mutations in the thick filament associated protein myosin-binding protein-c (cMyBP-C) are a major cause of familial hypertrophic cardiomyopathy (reviewed by Marian and Roberts, 2001). This finding, plus evidence that cMyBP-C plays both structural and regulatory roles in the contraction of cardiac muscle (Jeacocke and England, 1980; Hartzell and Titus, 1982;

© 2010 Elsevier Inc. All rights reserved.

Please send all editorial correspondence to: Robert W. Kensler, Department of Anatomy, University of Puerto Rico Medical School, Medical Sciences Campus, PO Box 365067, San Juan, Puerto Rico 00936-5067, Phone: +1 (787) 758-2525 Extension 1507, Fax: +1 (787) 767-0788, robert.kensler@upr.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Garvey et al., 1988; Schlender and Bean, 1991; Weisberg and Winegrad, 1996, 1998; Tong et al., 2008), emphasizes the importance of its functional role in contraction.

cMyBP-C, like most of the other accessory myosin binding proteins, belongs to the immunoglobulin superfamily and contains repeating immunoglobulin C2 (IgC2) domains and fibronectin-like type III (FnIII) motifs (Einheber and Fischman, 1990; Furst et al., 1992; Vaughan et al., 1993). Although similar to the skeletal muscle isomer, cMyBP-C has 11 fibronectin and Ig domains, rather than the 10 domains seen in the skeletal muscle isomer (Gautel et al., 1995; Yasuda et al., 1995; Kasahara et al., 1994). The extra domain C0 is located at the N-terminus, with subsequent domains labeled sequentially, with C10 at the C-terminus. The region between C1 and C2 near the N-terminus constitutes the MyBP-C motif (m-domain) and is common to all MyBP-C isomers.

The MyBP-C motif in cardiac MyBP-C differs from skeletal MyBP-C, however, in that the cardiac sequence has an additional 9 amino acids (LAGGGRRIS) and 4 phosphorylation sites in this region (Jia et al., 2010), rather than the single phosphorylation site in the skeletal muscle isomers. cMyBP-C differs also in the presence of a 28-amino acid insertion within the C5 domain. cMyBP-C has 2 binding sites for myosin; the C1–C2 region binds to the S2 region of myosin (Gruen and Gautel, 1999) and a binding site for the LMM region of myosin is located in C10 (Okagaki et al., 1993; Alyonycheva et al., 1997). A binding site for titin is also present at the C-terminus and may involve domains C8 to C10 (Freiburg and Gautel, 1996).

The presence of the additional phosphorylation sites in cMyBP-C is significant since these are the sites phosphorylated by cAMP-dependent protein kinase A in response to β -adrenergic agonists (Hartzell and Titus, 1982; Hartzell and Glass, 1984; Schlender et al., 1987; Gautel et al., 1995; Gruen and Gautel, 1999; McClellan et al., 2001). Phosphorylation may also occur by an endogenous calcium/calmodulin-dependent kinase that is closely associated with cMyBP-C (Schlender and Bean, 1990; Hartzell and glass, 1984); suggesting that phosphorylation of these sites may be important to a regulatory role for cMyBP-C. Further evidence for a regulatory role for cMyBP-C was demonstrated by experiments showing that ablation or extraction of cMyBP-C increases Ca^{+2} sensitivity, shortening velocity, and rates of tension redevelopment (k_{tr}) in permeabilized muscle fibers (Hofmann et al., 1991; Korte et al., 2003; Stelzer, 2006). cMyBP-C knockout mouse hearts also have abbreviated systolic ejection times, reduced ejection volumes, and decreased rates of relaxation compared to wildtype hearts (Harris et al., 2002; Palmer et al., 2004a,b,c). Taken together, these data support the idea that cMyBP-C normally constrains the interaction of myosin heads with actin and suggests that ablation of cMyBP-C causes an acceleration of crossbridge cycling mechanics and the shortened time of ejection that contributes to reduced stroke volume by the heart.

Although many of these studies were interpreted as consistent with a model in which the regulatory role of cMyBP-C is mediated through a phosphorylation-dependent tethering of the S2 region of myosin to the filament backbone (Weisberg and Winegrad, 1996, 1998; Calaghan et al., 2000), it is unclear that this is the entire mechanism. Kunst et al. (2000) and Harris et al. (2004) have demonstrated that a fragment of cMyBP-C containing only C1–C2 (C1-m-C2) with the associated phosphorylation sites and the S2-binding site is sufficient to affect myosin contractile function. Since the fragment does not contain the C10 domain required for binding of the molecule to the light meromyosin portion of myosin, the results suggest that cMyBP-C may affect myosin kinetics separate from a tether to the thick filament backbone. Also consistent with this conclusion, results from in vitro motility assays have shown that the C1–C2 fragment can affect the ability of heavy meromyosin (which lacks the myosin rod) to promote actin movement (Razumova et al., 2006). In another model

Ababou et al. (2008) proposed that binding of the C0–C2 N-terminal region of cMyBP-C near to the S1–S2 hinge area of myosin could affect myosin head orientation and kinetics independent of a tethering mechanism. However, observations that the N-terminus of cMyBP-C can influence actomyosin interactions in ATPase or motility assays, even in the absence of myosin S2 (i.e., when only actin and myosin S1 are present), suggest that interactions with myosin S2 alone cannot account for all of the functional effects of the N-terminus of cMyBP-C (Shaffer et al., 2009). Thus, while the evidence is clear that the N-terminal C0–C2 region of cMyBP-C is necessary for the regulatory role of cMyBP-C, the mechanism(s) by which cMyBP-C exerts its effect is less certain.

Several investigations have also provided evidence that C0–C2 and C1–C2 may bind F-actin in addition to binding the S2 region of myosin (Kulikovskaya et al., 2003, Razumova et al., 2006). In support of this idea, using cosedimentation binding assays we demonstrated that the N-terminal fragments C0–C2, C1–C2, and C1-m of cMyBP-C can bind to F-actin specifically at physiological pH (7.4) and ionic strength (Shaffer et al., 2009). In addition, solution turbidity and EM analyses showed that several of these fragments, including C0–C2 can bundle F-actin filaments.

Further evidence that the N-terminal region of cMyBP-C can bind to actin was also provided by the neutron scattering studies of Whitten et al. (2008). These studies demonstrated that the C0–C2 fragment can bind to G-actin and promote its polymerization to form long macromolecular F-actin complexes. However, conclusions regarding the three-dimensional structure of the C0–C2/F-actin complex were limited because the neutron scattering data could only provide useful information regarding the cross-sectional (2-D) shape of the complexes rather than the whole three dimensional structure. Furthermore, modeling of the two-dimensional data to obtain a 3-D structure relied on the use of a rigid body model of the cMyBP-C N-terminus (Jeffries et al., 2008) and so potential changes in the conformation of these domains upon binding actin were not considered. Finally, because the neutron scattering studies were performed at relatively low ionic strength and at pH 8.0, it is unclear whether the modeled interactions occur under the conditions of physiological ionic strength and pH used in the experiments reported in Shaffer et al. (2009).

Although evidence for the binding of fragments of the N-terminus region of cMyBP-C to actin has been shown indirectly through binding assays and through modeling of neutron scattering data, the binding of these fragments to actin has not been examined directly by electron microscopy. Therefore, in the current study we used electron microscopy to examine the ability of the C0–C2 fragment of cMyBP-C to bind to F-actin. The results demonstrate that the N-terminal region C0–C2 of cMyBP-C binds to F-actin in a regular, periodic manner under conditions of normal physiological ionic strength and pH. A preliminary abstract of the results was presented as a poster (Kensler et al., 2010).

2. Materials and methods

2.1 Protein expression and purification

The recombinant murine C0–C2 N-terminal fragment of cMyBP-C was cloned and purified as previously described (Razumova et al., 2006, 2008; Shaffer et al., 2009). At least two separate preparations of recombinant C0–C2 were used for the experimental data sets. Bovine cardiac actin was prepared from ether powder as described (Pardee and Spudich, 1982; Shaffer et al., 2009). F-actin was maintained in a storage buffer (in mmol/L: 50 KCl, 1 MgCl₂, 2 Tris-HCl, pH 8.0, 0.2 CaCl₂, 0.5 BME, 1 ATP, and 0.02% sodium azide) and kept at 4°C until use. Recombinant C0–C2 was phosphorylated as described for the C1–C2 fragment in Shaffer et al. (2009). Phosphorylation status was assessed as in that study by ProQ Diamond staining followed by Sypro Red staining (Invitrogen, Carlsbad, CA).

2.2 Electron microscopy

Recombinant C0–C2 and bovine cardiac F-actin were mixed, as previously described (Shaffer et al., 2009), to give final concentrations of 5 – 30 μ M C0–C2, 5 μ M F-actin, 1 mM ATP, and 1 mM DTT in a total final volume of 50 μ l of cosedimentation buffer (in mmol/L: 20 imidazole, pH 7.4, 180 KCl, 1 MgCl₂, 1 EGTA, 1 DTT). Samples were incubated at room temperature for 30 min and 5 μ l aliquots of each sample were applied to a freshly evaporated carbon film supported by holey Formvar covered grids. After adsorption to the carbon film (30 sec), excess sample was rinsed off with 8 drops of either 100 mM ammonium acetate or the cosedimentation buffer. The rinse was followed by application of 8 drops of 1% uranyl acetate for negative staining. Dried grids were examined and photographed at 80kV in a JEOL-1200EXII electron microscope equipped with a 2k $\times$ 2k AMT HR60 High resolution Digital Camera (AMT, Danvers, MA) as previously described (Kensler et al., 2002, 2005a,b). Magnification was calculated using the average axial helical periodicity of F-actin as 36 nm.

2.3 Computer image analysis

Computation of Fourier transforms and Fourier filtered images of the complexes of C0–C2 with F-actin was performed as previously described (Kensler et al., 2002, 2005a,b, Kensler and Harris, 2008). For computation of Fourier transforms, selected areas of the complexes at a pixel size corresponding to $\sim$ 0.42 nm were floated in 2048 $\times$ 2048 arrays. The images were straightened, rotated and rescaled as necessary to have exactly 24 axial repeats per 2048 pixels to insure that the helical layer lines fell on the sampled lines of the transform. Filtered images of the complexes were computed by Fourier inversion of the layer line data for the first six layer lines. Spacings in the filtered images were calculated relative to the average axial helical periodicity of F-actin in the micrographs which was assigned to be 36 nm.

3. Results

3.1 Appearance of the C0–C2 decorated F-actin complexes

Figures 1a and 1b show low magnification micrographs of negatively-stained aliquots of cardiac F-actin filaments (Fig. 1a) and the mixture of C0–C2 recombinant fragments with the F-actin filaments (fig. 1b). The cardiac F-actin filaments show the expected double helical arrangement of actin monomers with a helical repeat of 36 nm. Even at this low magnification, the mixture of C0–C2 with F-actin (Fig. 1b) can be seen to form long linear complexes with diameter greater than the F-actin filaments alone. The C0–C2 plus F-actin complexes show a periodic beaded appearance (Fig. 1b, tick marks), except in a few areas (asterisks) where the binding of C0–C2 to F-actin appears less complete. In these areas (asterisks) the thickness appears similar to that of the F-actin filaments in (Fig. 1a). In higher magnification images (Figs. 2a and 2b), both the greater diameter of the C0–C2 plus F-actin complexes (Fig. 2b) compared to the F-actin filaments (Fig. 2a), as well as the distinct periodic beaded appearance of the C0–C2 plus F-actin complexes (Fig. 2b) is apparent. As in the lower magnification micrographs, in a few regions in which the binding of C0–C2 to F-actin is less complete (asterisks, Fig. 2b), the similarity in thickness to that of the F-actin filaments (Fig. 2a) is apparent. Direct measurements of the diameter of the F-actin filaments and the C0–C2 plus F-actin complexes gave values of 9.73 ± 0.51 nm (n=350) and 19.29 ± 1.75 nm (n=350), respectively.

At the higher magnification, the helical axial repeat spacing of the F-actin filaments (Fig. 2a) and the axial repeat spacing along the C0–C2 plus F-actin complex (Fig. 2b) appear similar if not identical. The spacings between the tick marks in Fig. 2a of the F-actin filaments and in Fig. 2b of the C0–C2 plus F-actin complexes are identical, thus illustrating the similarity of the helical axial repeat of the F-actin filaments to the axial periodicity of the

C0–C2 plus F-actin complexes. This similarity in the periodicity of the F-actin filaments and the C0–C2 plus F-actin complex is consistent with the binding of the C0–C2 fragments to the actin monomers to give a complex which reflects the underlying F-actin helical symmetry, but has a diameter greater than expected for the F-actin.

The effect of phosphorylation of C0–C2 on its binding to F-actin was also examined. Although phosphorylation of C0–C2 did not abolish the binding to F-actin, the binding appeared less robust (Fig. 2c). F-actin filaments labeled with phosphorylated C0–C2, while clearly thicker than actin alone, usually appeared thinner in diameter than compared to labeling with unphosphorylated C0–C2. In many regions the binding of the phosphorylated C0–C2 partially obscured the actin helical structure. While it was still evident that the phosphorylated C0–C2 was binding with the periodicity of the underlying F-actin, the strong "beads on a string" appearance of the C0–C2 plus F-actin complexes was typically much less apparent. Although phosphorylated C0–C2 still produced some bundling of the F-actin filaments at higher concentrations (6:1 ratio of phosphorylated C0–C2 to F-actin), both the number and the size of the bundles was clearly reduced compared to the unphosphorylated C0–C2. Phosphorylation of the C0–C2 thus appears to reduce and modify its binding to F-actin and its ability to bundle actin.

3.2 Fourier transforms of the C0–C2 plus F-actin complexes

To further examine the periodicity of the binding of the C0–C2 fragments with F-actin, Fourier transforms of the complexes were computed. Regions of the complexes displaying a distinct periodicity in the micrographs were selected, boxed, straightened, and resized to have exactly 24 repeats in 2048 pixels. The images were floated in 2048×2048 pixel arrays for computation of Fourier transforms as previously described (Kensler, 2002, 2005a, 2005b). Figures 3a–h illustrate Fourier transforms obtained from the complexes. The computed transforms show a set of layer lines corresponding to the 36 nm periodicity for actin. Typically, the patterns extend to at least the 6th layer line. The transforms from different regions of individual C0–C2 plus F-actin complexes consistently show strong 1st and 6th layer lines. Weaker reflections on the 2nd, 3rd, 4th, 5th and 7th layer lines were also frequently present. This was confirmed in transforms (Fig. 3i) obtained by averaging the individual transforms from ten different C0–C2 plus F-actin complexes. In the averaged transforms the strong 1st and 6th layer lines and the presence of the weaker layer lines was much clearer.

One feature of the transforms of the C0–C2 plus F-actin complexes not expected for the transform of actin was the presence of a meridional reflection on the 1st layer line. Low pass filtering of the transforms to include just this meridional reflection and the origin of the pattern suggests that this low frequency reflection arises from the 36 nm axial spacing of the very strong undulating "beads on a string" shape of the repeating densities formed by the binding of the C0–C2 to the F-actin. Projection of the 3-dimensional helical density of the complex onto the 2-dimensional plane of the micrograph also results in spots of density at the helical cross-over points every 36 nm axially. A weak meridional reflection on the 1st layer line also occurred occasionally in our transforms of actin-alone. Non-inclusion of this meridional reflection had only a very limited effect on the appearance of the filtered images of the C0–C2 plus F-actin complex. Thus this reflection is unlikely to suggest a perturbation or other major deviation in the structure of the C0–C2 plus F-actin complex from the underlying actin helical symmetry.

Although the transforms of the C0–C2 plus F-actin complexes resembled the appearance of transforms from the F-actin alone, the positions of the primary reflections along the 1st and 6th layer lines in the transforms from the complexes were consistently closer to the meridian than the reflections for F-actin alone. Measurements of the position of the primary maxima

on the 1st layer (relative to the meridian) in the averaged transforms gave values of 0.097 nm^{-1} for F-actin and 0.079 nm^{-1} for C0–C2 plus F-actin. On the 6th layer lines the values were 0.070 nm^{-1} for the F-actin transform and 0.054 nm^{-1} for the C0–C2 plus F-actin transform. This difference correlates with the greater diameter of the C0–C2 plus F-actin complexes as compared to F-actin filaments alone. The transforms from the C0–C2 plus F-actin complexes support the observations from the micrographs which suggest that the recombinant C0–C2 fragments bind to F-actin with a periodicity which reflects the underlying F-actin periodicity.

3.3 Fourier filtered images of the C0–C2 decorated F-actin complexes

Fourier filtered images of F-actin filaments (Figs. 4a and b) and the C0–C2 complexes with F-actin (Figs. 4c–h) were computed as previously described (Kensler, 2002, 2005a;b) by inverse Fourier transforming of the data along the first six layer lines in the transforms from the F-actin filaments and the C0–C2 plus F-actin complexes. The filtered images of the F-actin filaments (4a and b) showed the expected double helical arrangement of the actin molecules previously established and modeled at the atomic level (Holmes et. al., 1990; Lorenz et al., 1993, 1995; Oda et al., 2009). The Fourier filtered images of the C0–C2 plus F-actin complexes (Figs. 4c–h) showed a structure with a similar axial repeat to the helical repeat of the F-actin filaments, but with a distinctly greater diameter than the F-actin filaments. The bead-like structures seen in the electron micrographs of the C0–C2 complex are resolved as a series of sub-densities with a similar axial spacing to that of the underlying actin monomers, but extending to a greater radius than for the F-actin monomers alone (Figs. 4a and b). Using the average 36 nm axial repeat of F-actin as a ruler for measurements, the diameter of the F-actin filaments in the filtered images was $9.59 \pm 0.3 \text{ nm}$ ($n = 25$ filtered images) and for the C0–C2 plus F-actin complexes was $16.85 \pm 1.01 \text{ nm}$ ($n = 25$ filtered images). While the diameter of the F-actin filaments obtained from direct measurements of the micrographs ($9.73 \pm 0.51 \text{ nm}$) and measurements of the filtered images ($9.59 \pm 0.3 \text{ nm}$) are very similar, the average diameters of the C0–C2 plus F-actin complexes measured directly on the micrographs ($19.29 \pm 1.75 \text{ nm}$) and for the filtered images ($16.85 \pm 1.01 \text{ nm}$) varied significantly. Since the filtered images only show structural details that are well ordered with a periodicity corresponding to the layer lines, the difference in diameter suggests that while the portions of C0–C2 that bind directly to F-actin are well ordered, the outer portions of C0–C2 extending away from the actin are probably less well ordered. Consistent with this idea, in a number of the filtered images additional weaker density appeared to extend beyond the well-defined sub-densities. Thus, using the measured values for diameter, the C0–C2 plus F-actin complex extends to a radius of at least 3.5 – 5 nm beyond the F-actin monomers, with less ordered features extending further.

The sub-densities on either side of the midline of the C0–C2 plus F-actin complex typically appeared to be staggered axially (arrows, Fig. 4d) by a spacing consistent with the stagger of $\sim 2.75 \text{ nm}$ expected between the adjacent actin monomers along the two strands of the F-actin filament. The similarity in the number and axial spacing of the sub-densities in the 36 nm axial repeat to that of the F-actin monomers suggests that the C0–C2 fragments are binding to F-actin monomers with a 1:1 ratio.

Close inspection of the filtered images suggests that the arrangement of the bound C0–C2 fragments is polar. In many cases, the arrangement of the sub-densities appears to have a distinctly arrowhead-like appearance (Figs. 4d–g) consistent with polar binding. The similarity between the filtered images from different regions of individual C0–C2 plus F-actin complexes suggests that the C0–C2 fragments are binding to the F-actin in an ordered manner that reflects the underlying F-actin helical periodicity and is consistent with specific binding.

4. Discussion

In the studies presented here, we used electron microscopy to examine the ability of the C0–C2 N-terminal fragment of cMyBP-C to bind to F-actin at physiological ionic strength and pH 7.4. The major finding of these studies is that C0–C2 binds to F-actin to form well-ordered periodic complexes. The results provide direct evidence for binding of the N-terminal region of cMyBP-C to F-actin.

These results are consistent with several earlier studies that provided evidence that native purified MyBP-C can bind to actin. Moos et al (1978) reported evidence from cosedimentation binding assays that skeletal muscle MyBP-C can bind to actin in the micromolar range, and further demonstrated (Moos, 1981) that fluorescently-labeled skeletal muscle MyBP-C labels the I-band of myofibrils at calcium levels comparable to those activating the myofibrillar ATPase. Hartzell (1985) reported that the addition of MyBP-C to actomyosin solutions produced an increase in the light-scattering of the actomyosin solution and a distinct precipitation of the actomyosin with time. Phosphorylated MyBP-C had a smaller effect on light-scattering than dephosphorylated MyBP-C. These results were interpreted as suggesting that MyBP-C stimulates actin-activated myosin ATPase activity by enhancing the formation of stable aggregates between actin and myosin filaments. Yamamoto (1986) similarly reported that the addition of skeletal muscle MyBP-C to regulated actin caused an increase in solution turbidity, especially in the presence of Ca^{+2} , and this was found to result from side-by-side association of actin filaments into bundles.

Although these early studies suggested that MyBP-C can bind to actin, the precise location of the actin binding site(s) is still subject to debate. It has been suggested that MyBP-C could interact with actin through a proline/alanine-rich region that lies in the N-terminus region of MyBP-C (Squire et al., 2003; Kulikovskaya et al., 2003) or through other N-terminal domains including the C1 domain and the regulatory motif of cMyBP-C (Razumova et al., 2006; Shaffer et al., 2009). Interestingly, Rybakova et al (Rybakova et al., 2010) recently failed to detect specific interactions of any N-terminal domains with actin but instead found evidence for a distinct actin binding site in the C-terminal half of cMyBP-C. However, because interactions of the cMyBP-C N-terminus with actin are likely mediated in part by electrostatic charge interactions (Razumova et al., 2006; Shaffer et al., 2009), the higher pH and increased phosphorylation state of baculovirus expressed recombinant N-terminal fragments used in their study may have precluded detection of N-terminal binding interactions. Other differences including slower centrifugation speeds and the use of some refolded N-terminal proteins from bacterial inclusion bodies may have also contributed to differences in their results. Nonetheless, the idea that cMyBP-C contains additional actin binding sites near its C-terminus is intriguing and warrants further investigation.

Results of Shaffer et al. (2009) using only soluble bacterially expressed fragments in cosedimentation binding assays showed that unphosphorylated C1–C2, C1-m, and C0–C2 fragments of cMyBP-C bind in a saturable manner to F-actin. Our results in the current study using electron microscopy confirm and extend these observations by providing a direct demonstration that the C0–C2 fragment can bind to individual F-actin filaments to form long ordered complexes. Furthermore, the electron microscope data appears consistent with the binding assay data indicating that C0–C2 binds to F-actin at a 1:1 ratio. The additional demonstration provided here that phosphorylation of C0–C2 appears to reduce but not abolish its binding to F-actin, is also consistent with the results in Shaffer et al. (2009). In that study it was reported that phosphorylated C1–C2 bound to actin with a similar affinity to that of the unphosphorylated C1–C2, but binding was significantly decreased as indicated by a decrease in B_{max} . The reduced ability of phosphorylated C1–C2 to crosslink actin

found in that study is consistent with our observation here that phosphorylation of C0–C2 reduces its ability to bundle F-actin filaments.

Our results are also consistent with the neutron scattering study of Whitten et al. (2008) showing that binding of C0–C2 to G-actin formed well-ordered decorated F-actin complexes. However, the study by Whitten et al. (2008) was done under conditions that started with G-actin at low ionic strength and at pH 8.0, leaving open the possibility that binding might be qualitatively different than starting with F-actin at physiological ionic strength and pH 7.4 as in our study. Despite this, the periodic beaded appearance of the binding of C0–C2 to F-actin revealed here by electron microscopy is similar to the general appearance of the derived model projection by Whitten et al. (2008) and to a preliminary supporting electron microscope study by Orlova et al. (2010) using conditions similar to ours. In addition, the diameter of 19.3 nm which we measured directly from our micrographs for the C0–C2 plus F-actin complex is consistent with the diameter of 18–20 nm suggested by the model of Whitten et al. (2008).

Whitten et al. (2008) proposed that the C0 and C1 domains bind directly to the actin monomers while the m-domain and C2 domains extend out to a radius of ~9 – 10 nm. Although the filtered images in the current study do not provide information about which domains of C0–C2 are involved in the binding to F-actin, the binding assays in Shaffer et al. (2009) were performed under similar conditions of ionic strength and pH as those used here and thus suggest that the C1 and m-domains mediate binding. Our observations that binding is reduced when the m-domain is phosphorylated also support this view. The difference in conclusions between the Whitten et al. (2008) study and Shaffer et al. (2009) concerning the binding of the m-domain could also be related to differences in pH between the studies since Shaffer et al. (2009) found that the binding of the m-domain is weakened at pH 8.0, which was the pH used in the Whitten et al. (2008) study. To confirm this, a more detailed analysis by 3D-image reconstruction will be necessary to establish whether the binding to F-actin which we observe here is the same as the binding observed by Whitten et al. (2008), and to confirm which domains of C0–C2 are involved in the binding to the F-actin.

An important question is whether the binding of the C0–C2 fragment to actin is specific. Our conditions using F-actin at an ionic strength of 200 mM and pH 7.4 are close to the conditions expected for the intact myocardial sarcomere, and identical to the conditions under which we have previously shown bundling of F-actin by C0–C2 (Shaffer et al., 2009). The higher ionic strength used in our studies was important to reduce the possibility that the observed binding of the C0–C2 to F-actin was non-specific and due to the well known “stickiness” of actin for other proteins. Also, under the identical conditions of both ionic strength and pH used here, binding of C0–C2 to F-actin was saturable at approximately a 1:1 molar ratio, suggesting binding interactions are mediated by a limited number of sites on each actin monomer. In contrast, binding of a two Ig domain cMyBP-C fragment (C3–C4) was relatively low and not saturable and therefore likely to be nonspecific. Thus the binding of the C0–C2 fragment to actin is unlikely to be due simply to a nonspecific affinity of the Ig domains for actin since not all of the N-terminal fragments bind with equal affinity.

Because of the location of cMyBP-C on the thick filament as an accessory protein, much of the attention regarding cMyBP-C has focused on its known phosphorylation-dependent binding to the S2 region of myosin. It will thus be of interest to determine whether the same N-terminal regions of cMyBP-C that decorate F-actin can also bind to S2 and decorate thick filaments in a periodic manner. If so, several studies (Weisberg and Winegrad, 1996,1998; Calaghan et al., 2000) have suggested that the regulatory role of cMyBP-C may be mediated through the binding of the C1–C2 region to the S2 region of myosin when C1–C2 is unphosphorylated. This binding could act as a tether of the myosin head to the filament

backbone thus constraining the crossbridge kinetics. Upon phosphorylation, the interaction of cMyBP-C with the S2 region of myosin is abolished (Gruen and Gautel, 1999) thus freeing the tether of the crossbridge and allowing for faster crossbridge kinetics. Consistent with this idea, Colson et al. (2007) concluded from X-ray diffraction studies that ablation of cMyBP-C results in a radial displacement of myosin crossbridges away from the thick filament backbone.

The significance of our direct demonstration by electron microscopy that the N-terminal fragment C0–C2 of cMyBP-C can bind to F-actin is that it, together with the results of the studies by other investigators supporting the binding of cMyBP-C to actin (Moos et al., 1978; Moos, 1981; Hartzell, 1985; Yamamoto, 1986; Kulikovskaya et al., 2003; Razumova et al., 2006; Whitten et al., 2008; Shaffer et al., 2009), raises the possibility that cMyBP-C may regulate myocardial contractile function through direct interactions with F-actin in the thin filament, in addition to its ability to bind to the S2 region of myosin. Although the precise mechanism by which cMyBP-C may influence myocardial contraction through interactions with actin is still far from clear, binding of cMyBP-C to the thin filament could potentially create a drag that slows sarcomere shortening, influence thin filament activation or relaxation dynamics, modulate cross-bridge kinetics, or affect sarcomere lattice spacing.

The idea that the mechanism of regulation of myocardial contraction by cMyBP-C may involve interactions with both myosin S2 and with F-actin is not without precedent. Myosin essential and regulatory light chains may also interact with actin to influence cross-bridge cycling kinetics (Farman et al., 2009 and for reviews, see Timson, 2003 and Morano, 1999). In addition, the thick filament-associated protein twitchin in molluscan smooth muscle is involved in the maintenance of the “catch” contractile process in these muscles. Funabara et al. (2003) have noted that, although this protein is much larger than cMyBP-C, there are similarities in the DXD1 region of twitchin and the N-terminal portion of cMyBP-C. Current models of the function of twitchin (Funabara et al., 2007) involve a phosphorylation-dependent binding to both myosin and actin. Although the molluscan catch state and regulation of myocardial contraction by cMyBP-C are likely to be different, the possibility that the regulatory role of cMyBP-C may also involve binding to both myosin S2 on the thick filament and to F-actin in the thin filament is intriguing and requires further study.

Acknowledgments

We wish to thank our colleagues at the University of Puerto Rico School of Medicine and the University of California, Davis for their helpful comments and suggestions. Appreciation is also expressed to Vanessa Cartagener for technical help. This work was supported by Supported by NIH 5SC1HL096017 (RWK) and NIH HL080367 (SPH).

References

1. Alyonycheva T, Cohen-Gould L, Siewert C, Fischman DA, Mikawa T. Skeletal muscle-specific myosin binding protein-H is expressed in Purkinje fibers of the cardiac conduction system. *Circ. Res* 1997;80:665–672. [PubMed: 9130447]
2. Ababou A, Rostkova E, Mistry S, Le Masurier C, Gautel M, Pfuhl M. Myosin binding protein C positioned to play a key role in regulation of muscle contraction: Structure and interactions of Domain C1. *J. Mol. Biol* 2008;384:615–630. [PubMed: 18926831]
3. Calaghan SC, Trinick J, Knight PJ, White E. A role for C-protein in the regulation of contraction and intracellular Ca²⁺ in intact rat ventricular myocytes. *J. Physiol* 2000;528(Pt 1):151–156. [PubMed: 11018113]
4. Colson BA, Bekyarova T, Fitzsimons DP, Irving TC, Moss RL. Radial displacement of myosin cross-bridges in mouse myocardium due to ablation of myosin binding protein-C. *J. Mol. Biol* 2007;367:36–41. [PubMed: 17254601]

5. Einheber S, Fischman D. Isolation and characterization of a cDNA clone encoding avian skeletal C-protein: an intracellular member of the immunoglobulin superfamily. *Proc. Natl. Acad. Sci* 1990;87:2157–2161. [PubMed: 2315308]
6. Farman GP, Miller MS, Reedy MC, Soto-Adames FN, Vigoreaux JO, Maughan DW, Irving TC. Phosphorylation and the N-terminal extension of the regulatory light chain help orient and align the myosin heads in *Drosophila* flight muscle. *J. Struct. Biol* 2009;168:240–249. [PubMed: 19635572]
7. Freiburg A, Gautel M. A molecular map of the interactions between titin and myosin-binding protein C. Implications for sarcomeric assembly in familial hypertrophic cardiomyopathy. *Eur. J. Biochem* 1996;235:317–323. [PubMed: 8631348]
8. Funabara D, Watabe S, Mooers SU, Narayan S, Dudas C, Hartshone DJ, Siegman MJ, Butler TM. Twitchin from molluscan catch muscle: primary structure and relationship between site-specific phosphorylation and mechanical function. *J. Biol. Chem* 2003;278:29308–29316. [PubMed: 12756258]
9. Funabara D, Hamamoto C, Inoue K, Ueda M, Osawa R, Kanoh S, Hartshone DJ, Suzuki S, Watabe S. Unphosphorylated twitchin forms a complex with actin and myosin that may contribute to tension maintenance in catch. *J. Exp. Biol* 2007;210:4399–4410. [PubMed: 18055628]
10. Furst DO, Vinkemeyer U, Weber K. Mammalian skeletal muscle C-protein: purification from bovine muscle, binding to titin and the characterization of a full length cDNA. *J. Cell Sci* 1992;102:769–778. [PubMed: 1429890]
11. Garvey JL, Kranias EG, Solaro RJ. Phosphorylation of C-protein, troponin I, and phospholamban in isolated rabbit hearts. *Biochem. J* 1988;249:709–714. [PubMed: 2895634]
12. Gautel M, Zuffardi O, Freiburg A, Labeit S. Phosphorylation switches specific for the cardiac isoform of myosin binding protein-C: a modulator of cardiac contraction? *The EMBO Journal* 1995;14:1952–1960. [PubMed: 7744002]
13. Gruen M, Gautel M. Mutations in beta (β)-Myosin S2 that cause familial hypertrophic cardiomyopathy (FHC) abolish the interaction with the regulatory domain of myosin-binding protein-C. *J. Mol. Biol* 1999;286:933–949. [PubMed: 10024460]
14. Harris SP, Bartley CR, Hacker TA, McDonald KS, Douglas PS, Greaser ML, Powers PA, Moss RL. Hypertrophic cardiomyopathy in cardiac myosin binding protein-C knockout mice. *Circ. Res* 2002;90:594–601. [PubMed: 11909824]
15. Harris SP, Rostkova E, Gautel M, Moss RL. Binding of myosin binding protein-C to myosin subfragment S2 affects contractility independent of a tether mechanism. *Circ. Res* 2004;95:930–936. [PubMed: 15472117]
16. Hartzell HC. Effects of phosphorylated and unphosphorylated C-protein on cardiac actomyosin ATPase. *J. Mol. Biol* 1985;186:185–195. [PubMed: 2934553]
17. Hartzell HC, Glass DB. Phosphorylation of purified cardiac muscle C-protein by purified cAMP-dependent and endogenous Ca^{2+} -calmodulin-dependent protein kinases. *J. Biol. Chem* 1984;259:15587–15596. [PubMed: 6549009]
18. Hartzell HC, Titus L. Effects of cholinergic and adrenergic agonists on phosphorylation of a 165,000-dalton myofibrillar protein in intact cardiac muscle. *J. Biol. Chem* 1982;257:2111–2120. [PubMed: 6276407]
19. Hofmann PA, Hartzell HC, Moss RL. Alterations in Ca^{2+} sensitive tension due to partial extraction of C-Protein from rat skinned cardiac myocytes and rabbit skeletal muscle fibers. *J. Gen. Physiol* 1991;97:1141–1163. [PubMed: 1678777]
20. Holmes KC, Popp D, Gebhard W, Kabsch W. Atomic model of the actin filament. *Nature* 1990;347(6288):44–49. [PubMed: 2395461]
21. Jeacocke S, England P. Phosphorylation of a myofibrillar protein of Mr 150,000 in perfused rat heart, and the tentative identification of this as C-protein. *FEBS Letters* 1980;122:129–132. [PubMed: 6260526]
22. Jefferies CM, Whitten AE, Harris SP, Trehwella J. Small-angle X-ray scattering reveals the N-terminal domain organization of cardiac myosin binding protein C. *J. Mol. Biol* 2008;377:1186–1199. [PubMed: 18313073]

23. Jia W, Shaffer JF, Harris SP, Leary JA. Identification of novel protein kinase A phosphorylation sites in the M-domain of human and murine cardiac myosin binding protein-C using mass spectrometry analysis. *J. Proteome Res* 2010;9:1843–1853. [PubMed: 20151718]
24. Kasahara H, Itoh M, Sugiyama T, Kido N, Hayashi H, Saito H, Tsukita S, Kato N. Autoimmune myocarditis induced in mice by cardiac C-protein. Cloning of complementary DNA encoding murine cardiac C-protein and partial characterization of the antigenic peptides. *J. Clin. Invest* 1994;94:1026–1036. [PubMed: 8083344]
25. Kensler RW. Mammalian cardiac muscle thick filaments: their periodicity and interactions with actin. *Biophys. J* 2002;82:1497–1508. [PubMed: 11867464]
26. Kensler RW. The mammalian cardiac muscle thick filament: crossbridge arrangement. *J. Struct. Biol* 2005a;149:303–312. [PubMed: 15721584]
27. Kensler RW. The mammalian cardiac muscle thick filament: backbone contributions to meridional reflections. *J. Struct. Biol* 2005b;149:313–324. [PubMed: 15721585]
28. Kensler RW, Shaffer JF, Harris SP. Binding of the N-terminal fragment C0–C2 of cardiac MyBP-C to cardiac F-actin. *Biophys. J* 2010;98:554a–554a. (abstract).
29. Kensler RW, Harris SP. The Structure of Isolated Myosin Thick Filaments from cMyBP-C Knockout Mice. *Biophys. J* 2008;94:1707–1718. [PubMed: 17993479]
30. Korte FS, McDonald KS, Harris SP, Moss RL. Loaded shortening, power output, and rate of force redevelopment are increased with knockout of cardiac myosin binding protein-C. *Circ. Res* 2003;93:752–758. [PubMed: 14500336]
31. Kulikovskaya I, McClellan G, Flavigny J, Carrier L, Winegrad S. Effect of MyBP-C binding to actin on contractility in heart muscle. *J. Gen. Physiol* 2003;122:761–774. [PubMed: 14638934]
32. Kunst G, Kress M, Gruen M, Utenweiler D, Gautel M, Fink RHA. Myosin binding protein C, a phosphorylation-dependent force regulator in muscle that controls the attachment of myosin heads by its interaction with myosin S2. *Circ. Res* 2000;86:51–58. [PubMed: 10625305]
33. Lorenz M, Popp D, Holmes KC. Refinement of the F-actin model against X-ray fiber diffraction data by the use of a directed mutation algorithm. *J. Mol. Biol* 1993;234:826–836. [PubMed: 8254675]
34. Lorenz M, Poole kJ, Popp D, Rosenbaum G, Holmes KC. An atomic model of the unregulated thin filament obtained by X-ray diffraction on oriented actin-tropomyosin gels. *J. Mol. Biol* 1995;246:108–119. [PubMed: 7853391]
35. Marian AJ, Roberts R. The molecular genetic basis for hypertrophic cardiomyopathy. *J. Mol. Cell Cardiol* 2001;33:655–670. [PubMed: 11273720]
36. McClellan G, Kulikovskaya I, Winegrad S. Changes in cardiac contractility related to calcium-mediated changes in phosphorylation of myosin-binding protein C. *Biophys. J* 2001;81:1083–1092. [PubMed: 11463649]
37. Moos C. Fluorescence microscope study of the binding of added C-protein to skeletal muscle myofibrils. *J. Cell Biol* 1981;90:25–31. [PubMed: 6788782]
38. Moos C, Mason CM, Besterman JM, Feng IN, Dubin JH. The binding of skeletal muscle C-protein to F-actin, and its relation to the interaction of actin with myosin subfragment-1. *J. Mol. Biol* 1978;124:571–586. [PubMed: 152359]
39. Morano I. Tuning the human heart molecular motors by myosin light chains. *J. Mol. Biol* 1999;77:544–555.
40. Oda T, Iwasa M, Aihara T, Maeda Y, Narita A. The nature of the globular-to fibrous-actin transition. *Nature* 2009;457:441–445. [PubMed: 19158791]
41. Okagaki T, Weber FE, Fischman DA, Vaughan KT, Mikawa T, Reinach FC. The major myosin-binding domain of skeletal muscle MyBP-C (C-protein) resides in the COOH-terminal immunoglobulin C2 motif. *J. Cell Biol* 1993;123:619–626. [PubMed: 8227129]
42. Orlova A, Galkin VE, Jeffries CM, Trewheella J, Engelman EH. Binding of N-terminus fragments of Cardiac Myosin-binding C-protein to Actin. *Biophys. J* 2010;98:157a–157a. (Abstract).
43. Palmer BM, Georgakopoulos D, Janssen PM, Wang Y, Alpert NR, Belardi DF, Harris SP, Moss RL, Burgon PG, Seidman CE, Seidman JG, Maughan DW, Kass DA. Role of Cardiac Myosin Binding Protein C in Sustaining Left Ventricular Systolic Stiffening. *Circ. Res* 2004a;94:1249–1255. [PubMed: 15059932]

44. Palmer BM, McConnell BK, Li GH, Seidman CE, Seidman JG, Irving TC, Alpert NR, Maughan DW. Reduced cross-bridge dependent stiffness of skinned myocardium from mice lacking cardiac myosin binding protein-C. *Mol. Cell Biochem* 2004b;263:73–80. [PubMed: 15524168]
45. Palmer BM, Noguchi T, Wang Y, Heim JR, Alpert NR, Burgon PG, Seidman CE, Seidman JG, Maughan DW, Lewinter MM. Effect of cardiac myosin binding protein-C on mechanoenergetics in mouse myocardium. *Circ. Res* 2004c;94:1615–1622. [PubMed: 15155526]
46. Pardee JD, Spudich JA. Purification of muscle actin. *Methods Enzymol* 1982;85:164–181. [PubMed: 7121269]
47. Razumova MV, Shaffer JF, Tu AY, Flint GV, Regnier M, Harris SP. Effects of the N-terminal Domains of Myosin Binding Protein-C in an in Vitro Motility Assay: Evidence for long-lived cross-bridges. *J. Biol. Chem* 2006;281:35846–35854. [PubMed: 17012744]
48. Razumova MV, Bezold KL, Tu AY, Regnier M, Harris SP. Contribution of the myosin binding protein C motif to functional effects in permeabilized rat trabeculae. *J. Gen. Physiol* 2008;132:575–585. [PubMed: 18955596]
49. Rybakova IN, Greaser ML, Moss RL. Myosin binding protein C interactions with actin: characterization and mapping of the binding site. *J. Biol. Chem.* 2010 10.1074/jbc.M110.170605e.
50. Schlender K, Bean LJ. Phosphorylation of chick cardiac C-protein by calcium/calmodulin-dependent protein kinase II. *J. Biol. Chem* 1991;266:2811–2817. [PubMed: 1671569]
51. Schlender KK, Hegazy MG, Thysseril TJ. Dephosphorylation of cardiac myofibril C-protein by protein phosphatase 1 and protein phosphatase 2A. *Biochim. Biophys. Acta* 1987;928:312–319. [PubMed: 3032283]
52. Shaffer JF, Kensler RW, Harris SP. The myosin-binding protein C motif binds to F-actin in a phosphorylation-sensitive manner. *J. Biol. Chem* 2009;284:12318–12327. [PubMed: 19269976]
53. Squire JM, Luther PK, Knupp C. Structural evidence for the interaction of C-protein (MyBP-C) with actin and sequence identification of a possible actin-binding domain. *J. Mol. Biol* 2003;331:713–724. [PubMed: 12899839]
54. Stelzer JE, Fitzsimons DP, Moss RL. Ablation of myosin-binding protein C accelerates force development in mouse myocardium. *Biophys. J* 2006;90:4119–4127. [PubMed: 16513777]
55. Timson DJ. Fine tuning the myosin motor: The role of the essential light chain in striated muscle myosin. *Biochimie* 2003;85:639–645. [PubMed: 14505818]
56. Tong CW, Stelzer JE, Greaser ML, Powers PA, Moss RL. Acceleration of crossbridge kinetics by protein kinase A phosphorylation of cardiac myosin binding protein C modulates cardiac function. *Circ. Res* 2008;103:974–982. [PubMed: 18802026]
57. Vaughan KT, Weber FE, Einheber S, Fischman DA. Molecular cloning of chicken myosin-binding protein H (MyBP) H (86 KDa protein) reveals extensive homology with the MyBP-C (C-protein) with conserved immunoglobulin C2 and fibronectin type III motifs. *J. Biol. Chem* 1993;268:3670–3676. [PubMed: 7679114]
58. Weisberg A, Winegrad S. Alteration of myosin cross-bridges by phosphorylation of myosin-binding protein C in cardiac muscle. *Proc. Natl. Acad. Sci* 1996;93:8999–9003. [PubMed: 8799143]
59. Weisberg A, Winegrad S. Relation between crossbridge structure and actomyosin ATPase activity in rat heart. *Circ. Res* 1998;83:60–72. [PubMed: 9670919]
60. Whitten AE, Jeffries CM, Harris SP, Trewella J. Cardiac myosin-binding protein C decorates F-actin: Implications for cardiac function. *PNAS* 2008;105:18360–18365. [PubMed: 19011110]
61. Yamamoto K. The binding of skeletal muscle C-protein to regulated actin. *FEBS Lett* 1986;208:123–127. [PubMed: 3770206]
62. Yasuda M, Koshida S, Sato N, Obinata T. Complete primary structure of chicken cardiac C-protein (MyBP-C) and its expression in developing striated muscles. *J. Mol. Cell Cardiol* 1995;27:2275–2286. [PubMed: 8576942]

Figure 1.

Low magnification electron micrograph images of negatively stained F-actin alone (a) and the C0–C2 plus F-actin complexes (b). Both micrographs are at the same magnification and illustrate the increase in diameter of the C0–C2 plus F-actin complex (b) compared to the actin alone (a). Figure (b) also illustrates that the C0–C2 filamentous complexes have a bead-like periodic appearance (indicated by the tick marks). The axial spacing of the periodicity appears similar to that of the actin. The asterisks mark regions where the binding of the C0–C2 fragments to the F-actin is less complete and the thickness can be seen to be similar to actin alone. The bar in both micrographs is 50 nm.

Figure 2.

Higher magnification images of (a) actin alone, (b) unphosphorylated C0–C2 plus F-actin, and (c) phosphorylated C0–C2 plus F-actin (c). All micrographs are at the same magnification. At this magnification, the larger diameter and bead-like periodic appearance of the unphosphorylated C0–C2 plus F-actin complexes (b) compared to the actin alone (a) filaments is apparent. The tick marks in both images (a) and (b) are at equal spacings, and it is apparent that the axial periodicity of both the actin alone and C0–C2 plus F-actin complexes are identical or very similar. As in Figure 1, the asterisks in image (b) indicate regions where binding of the C0–C2 to the F-actin filament is less complete and it is apparent that the thickness is similar to that of F-actin alone. Image (c) shows an electron micrograph of the binding of phosphorylated C0–C2 to F-actin. Although binding of the phosphorylated C0–C2 to F-actin is apparent in the partial obscuring of the F-actin structure in many regions, it is clear that the binding is weaker and less ordered than for the unphosphorylated C0–C2 (b). The strong “beads on a string” appearance seen in labeling with unphosphorylated C0–C2 (b) is greatly reduced in the phosphorylated C0–C2 sample (c). The bars indicate 50 nm.

Figure 3.

(a-h) A gallery of the Fourier transforms (FFTs) calculated from images of selected regions of the C0-C2 filamentous complexes. Fig (i) shows an average of the FFTs from 10 different images of the C0-C2 complexes. The FFTs show strong 1st and 6th layer lines. Weaker peaks also appear to be present on the 2nd, 3rd, 4th, 5th, and 7th layer lines. This can be seen best in the averaged transform (i). The transforms clearly indicate the periodic structure of the binding of the C0-C2 fragments to the F-actin.

Figure 4.

A gallery of Fourier transform filtrations calculated from the data on the first 6 layer lines for both F-actin alone (a and b) and C0–C2 plus actin (c–h). The filtered images of the C0–C2 plus F-actin complexes show an increased diameter but similar axial periodicity to that seen for the actin alone. The greater diameter of the C0–C2 plus F-actin complexes can be seen to be due to a series of densities extending out to a greater radius than for actin alone. The arrows in (d) indicate the axial stagger of the densities on either side of the midline. The actin plus C0–C2 filtered images also appear to show a distinct almost arrow-head like appearance in many cases (d–g).