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ALTERED INTERACTIONS BETWEEN CARDIAC MYOSIN BINDING PROTEIN-C AND α-CARDIAC ACTIN VARIANTS ASSOCIATED WITH CARDIOMYOPATHIES

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Abstract

The two genes most commonly associated with mutations linked to hypertrophic or dilated cardiomyopathies are β-myosin and cardiac myosin binding protein-C (cMyBP-C). Both of these proteins interact with cardiac actin (ACTC). Currently there are 16 ACTC variants that have been found in patients with HCM or DCM. While some of these ACTC variants exhibit protein instability or polymerization-deficiencies that might contribute to the development of disease, other changes could cause changes in protein-protein interactions between sarcomere proteins and ACTC. To test the hypothesis that changes in ACTC disrupt interactions with cMyBP-C, we examined the interactions between seven ACTC variants and the N-terminal C0C2 fragment of cMyBP-C. We found there was a significant decrease in binding affinity (increase in Kd values) for the A331P and Y166C variants of ACTC. These results suggest that a change in the ability of cMyBP-C to bind actin filaments containing these ACTC protein variants might contribute to the development of disease. These results also provide clues regarding the binding site of the C0C2 fragment of cMyBP-C on F-actin.

Keywords

cardiac actin; myosin binding protein-C; heart disease; cardiomyopathy

Introduction

Cardiovascular disease is the leading cause of death in the developing world [1, 2]. Mutations or defects in muscle sarcomere proteins are involved in the development of either

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hypertrophic cardiomyopathy (HCM, increased left ventricular wall thickness without chamber expansion) or dilated cardiomyopathy (DCM, thin ventricular walls with enlarged chamber volume)[3]. However, the precise molecular mechanisms leading to disease states as a result of sarcomere protein changes are incompletely understood and are likely to be multi-factorial. At least part of the heterogeneity arises because over 450 different mutations in sarcomeric and myofilament-related genes have been associated with HCM so far in the human population [4]. Of these, the second-most frequently mutated gene is cMyBP-C, accounting for ~33% of all known mutations [5]. By contrast, mutations in the ACTC gene encoding cardiac actin are extremely rare, with only 16 variants identified so far [6-14].

cMyBP-C is encoded by a single polypeptide chain expressed in the C-zone of sarcomeres that consists of eight immunoglobulin I-like domains (IgI) and three fibronectin 3 domains (Fn3) along with a 100 amino acid MyBP-C specific motif (the m-motif) that links IgI domains C1 and C2. cMyBP-C interacts with both myosin and actin in the sarcomeres of muscle through domains near the N-terminus of cMyBP-C including the m-motif. Shaffer et al. showed the N-terminal C0C2 fragment of cMyBP-C interacts with actin in a phosphorylation-regulated manner [15]. Binding of these domains and other N-terminal domains to actin occurs at a position where cMyBP-C could have a role in regulating actin-myosin interactions overall by influencing tropomyosin binding or myosin subfragment-1 interactions with actin thin filaments [16, 17].

We hypothesized that mutations in ACTC found in patients suffering from HCM or DCM disrupt interactions between the N-terminal domains of cMyBP-and ACTC. Therefore, we tested the binding affinities of seven ACTC variants using the C0C2 actin-binding fragment of cMyBP-C. We found that two ACTC variants (A331P and Y166C) resulted in a significantly increased K_d for binding of cMyBP-C. Our findings suggest that changes in interactions between cMyBP-C and ACTC in heart muscle could contribute to disease for patients affected with these mutations. These results also contribute to wider discussion about the location of C0C2 fragment binding on actin filaments.

**Experimental Procedures**

**Protein expression and purification**

To obtain recombinant ACTC proteins, S/9 cells at cell density of ~1 x 10^6 cell/ml were infected at an optimal MOI (multiplicity of infection) of 1 with recombinant baculovirus for an optimal post infection time of 72 hr. Infected cells were harvested by centrifugation for 10 min at 3000 rpm using the J25.2 rotor (Beckman Coulter, Mississauga, ON). The pellet was lysed with a high Tris-buffer (1 M Tris–HCl, pH 7.5, 0.6 M KCl, 0.5 mM MgCl2, 0.5 mM ATP, 4% Triton X-100, 1 mg/mL Tween 20, 1 mM DTT and a protease inhibitor cocktail (benzamidine, leupeptin, aprotinin, antipain, TLCK, TPCK, E-64, each at 1.25 µg/mL) by vortexing as described [18]. The cell lysate was then spun for 30 min at 30 000 rpm using TLA 110 rotor (Beckman Coulter, Mississauga, ON). The cell supernatant was filtered through glass wool (Costar, Corning Inc., NY) in a 60 ml syringe (Becton, Dickinson and company (BD), Franklin, NJ) equilibrated with G-buffer (10 mM Tris-HCl, pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0.2 mM βME, and a protease inhibitor cocktail (antipain, aprotinin, BAEE, benzamidine, E-64, leupeptin, pepstatin, PMSF, TLCK, and
TPCK at 0.25 μg/ml, and PMSF at 0.125 mM) to remove lipids. The filtered lysate was purified by DNase-I affinity chromatography as described [19]. Purified recombinant ACTC mutant protein was dialyzed overnight in G-buffer followed by being concentrated using Amicon 10 000 MWCO concentrator (Millipore, Etobicoke, ON) the next day. Purified ACTC proteins were stored at 4 °C on ice and used within 3 days of purification.

**Cosedimentation assay**

The cosedimentation assay used recombinant ACTC and cMyBP-C C0C2 domains as described previously [15]. The cMyBP-C C0C2 was expressed and purified as previously described and proteins were stored in cosedimentation buffer until use (in mmol/liter: 20 mM imidazole, pH 7.4, 180 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT) [15]. C0C2 concentration was determined by using the Beckman Coulter DU800 spectrophotometer (Beckman, Mississauga, ON) with the absorbance at 280 nm and 310 nm with the extinction coefficient of (ε₂₈₀) 0.854 cm²/mg.

Before using the ACTC mutant protein, the activity was monitored by using the pyrene polymerization assay as previously described [19]. 10 μM actin polymerization reactions including 2.5% pyrene labeled actin were used to ensure the ACTC mutant protein was active. Upon confirming the protein was active, ACTC proteins were polymerized with the addition of polymerization salts (25 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM EGTA, 2 mM MgCl₂, 0.1 mM ATP) at room temperature for at least 2 hours.

When the recombinant ACTC mutant protein was polymerized, the actin (5 μM final concentration with 1 mM DTT and 1 mM ATP) was combined with the recombinant cMyBP-C C0C2 proteins (1-30 μM final concentrations) to achieve a total volume of 50 μl in ultra clear centrifuge tubes (Beckman, 5×20 mm). Then the ACTC mutant protein and C0C2 reaction mixtures were incubated for 30 min at room temperature. The samples were then separated using ultra tabletop centrifuge (Beckman) at 95 000 rpm with the TLA 100 rotor for 32 min at 4°C. The supernatants were removed and pellets were washed with 50 μl of cosedimentation buffer. The pellets were then resuspended in 100 μl of a 1:1 mixture of cosedimentation buffer to urea/thiourea sample buffer [20]. The pellet fraction samples were separated by gel electrophoresis on 10% SDS-PAGE. In addition to running the experimental samples, standard samples were run on each gel as well. The standards consisted of known molar ratios of C0C2 to actin (0.1, 0.2, 0.35, 0.50, 0.65, 0.80, and 1.0 mol/mol). The standard samples, C0C2 and alpha skeletal chicken F-actin were diluted in a 1:1 mixture of cosedimentation buffer to urea/thiourea sample buffer. This assay was repeated at least four times for each ACTC mutant purification (exception for WT and R312H on one day with n=2). The gels were stained overnight in Coomassie brilliant blue dye (R-250) and then destained the following day. To improve visualization of C0C2 and actin separation, gels were dried using a BioRad gel dryer system and then scanned.

**Data Analysis**

The band intensities of C0C2 to actin in each pellet were measured by using ImageJ (National Institute of Health, MD) and were converted to a molar ratio (mol of cMyBP-C C0C2 to mol of actin) with a standard curve run on each gel (Figure 1). The molar ratio of...
C0C2 to actin was plotted against the total concentration of C0C2 added and fitted against a one ligand binding model using Sigmaplot 11 (San Jose, CA) to determine the binding dissociation ($K_d$) and stoichiometry ($B_{max}$) (Figure 1). To assess statistical differences of each ACTC mutant compared to WT ACTC for the $K_d$ and $B_{max}$ values, a series of statistical tests was used: one-way ANOVA followed by post-hoc analysis to determine which ACTC mutant is significantly different than WT using the Statistical Product and Service Solutions software package (SPSS, Chicago, IL).

**Results and Conclusions**

Interactions between ACTC mutants and the N-terminus of c-MyBPC were analyzed by cosedimentation assays. ACTC variant proteins were polymerized and incubated with varying amounts of recombinant C0C2 protein. The reactions were sedimented and the pellet fractions analyzed by densitometry of SDS-PAGE to determine the binding affinity ($K_d$) and stoichiometry ($B_{max}$) of C0C2 binding (Figure 1).

$B_{max}$ and $K_d$ values for each ACTC variant were determined on different days post-purification and showed equal variances and no significant mean differences ($\alpha = 0.05$) between days. The exception was the $K_d$ for the R312H ACTC mutant, which had a significant difference between means between days (data not shown), perhaps reflecting the instability of that ACTC mutant observed previously [21].

The $B_{max}$ mean values of C0C2 binding for each ACTC mutant were not significantly different (Table 1). The $B_{max}$ values for the C0C2 protein revealed that all ACTC mutant proteins bound at a molar ratio of about 1:1, similar to that previously determined for cMyBP-C C0C2 binding to bovine cardiac F-actin (~ 0.92 ± 0.11 mol/mol actin) [15]. These data suggest that there is no C0C2 binding stoichiometry differences between recombinant and tissue purified ACTC proteins.

The $K_d$ mean values of C0C2 binding for each ACTC mutant were likewise similar to WT ACTC, with the exceptions of the $K_d$ mean values of the A331P and Y166C ($\alpha = 0.05$) and E361G ACTC mutants ($\alpha = 0.15$) (Table 1). The binding curves for the A331P, Y166C, and E361G to WT ACTC proteins are shown in Figure 2.

The E361G ACTC protein produced greater distribution of the data about the mean. However, the E361G ACTC protein appears to be more similar to WT ACTC in its intrinsic properties [21], suggesting that the change in $K_d$ is due to a protein-protein interaction change. Although the statistical significance is low, it is interesting to note that the E361G mutant ACT1 protein produced in yeast had a reduced binding to $\alpha$-actinin [22], suggesting that the C0C2 fragment of MyBP-C may share part of the same binding region on ACTC.

Increases in $K_d$ were observed for Y166C and A331P ACTC proteins indicating that both mutants decreased binding affinity for C0C2. Both of these ACTC variants exhibited some polymerization deficiency [18, 21], suggesting that the changes in $K_d$ may be due to an increased binding energy requirement of the actin subunits to remain filamentous. Ala-331 is located within a myosin binding loop [23] and a tropomyosin binding site in the absence of $\text{Ca}^{2+}$ [24]. Tyr-166 is located in a hydrophobic cleft of the barbed end of actin molecules.
involved in along-strand interactions with the D-loop of neighboring actin protomers in thin filaments [25]. Recent structural work locates the binding site of the N-terminal C0-C1 fragment of cMyBP-C on subdomain 1 of actin [17, 26, 27], overlapping the tropomyosin binding site. Both Ala-331 and Tyr-166 are included in the binding site for the C0C1 portion of the C0C2 fragment used in our experiments. Our data showing reduced binding of cMyBP-C with the A331P mutant supports the hypothesis that binding of myosin, tropomyosin, and cMyBP-C converge on this region of actin.

Testing the hypothesis that interactions between cMyBP-C and actin might be disrupted by mutations of ACTC related to cardiomyopathy development revealed that some ACTC mutations did affect cMyBP-C C0C2 binding. The scale of the changes in $K_d$ we observe between ACTC variants and the C0C2 fragment are on the same order of changes seen with other ACTC proteins and myosin, where altered $K_M$ values for actin-activated myosin ATPase activity can infer changes in binding affinity. For example, with the M305L and Y166C ACTC mutations, changes in myosin ATPase activity were within error compared to WT actin [28]. With the E99K ACTC variant, the $K_M$ of the myosin ATPase activity increased about 4-fold, while direct measurement of $K_d$ in the absence of nucleotide showed the $K_d$ of E99K ACTC binding by myosin was 61% that of WT ACTC protein [29].

The changes in $K_d$ observed would translate to potential functional changes in heart muscle if the effective concentration of cMyBP-C in sarcomeres is in the range of the measured $K_d$ values. Given the organized structural arrangement of sarcomeres, the effective concentrations of actin and cMyBP-C could be quite high because the two proteins do not freely diffuse, but are held in close proximity.

The location of the ACTC mutations affecting cMyBP-C binding relates to the debate regarding where cMyBP-C binds actin or myosin and how cMyBP-C might regulate force generation in sarcomeres (reviewed in [30]). One emerging picture is plasticity of binding by cMyBP-C. Models suggest that phosphorylation of the N-terminus of cMyBPC may regulate alternate binding between actin and myosin [31]. The impact of different ACTC mutations on cMyBP-C binding suggests potential variability in the binding of actin itself leading to heart disease, adding another layer of complexity to the mechanism of muscle regulation by cMyBP-C.

The number of mutations in cMyBP-C related to cardiomyopathy development in patients indicates that cMyBP-C plays a significant role in normal muscle function. The enigmatic binding and regulatory properties of cMyBP-C on key sarcomere proteins require further study to address the mechanisms of this protein in muscle regulation. Future work will examine the role of phosphorylation and the interplay between cMyBPC, myosin, and tropomyosin in the context of ACTC mutations to further define the interactions and binding sites of this important regulator of cardiac function.

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ABB Highlights

- binding between 7 cardiac actin variants and c-MyBP-C C0C2 fragment were determined
- all actin variants had about a 1:1 molar binding stoichiometry with C0C2
- A331P and Y166C actin showed significantly reduced binding affinity
- changed cardiac actin and cMyBP-C binding might contribute to disease
Figure 1. SDS-PAGE analysis of C0C2 binding reactions to WT-ACTC protein
5 μM of WT-ACTC filament (final concentration) was mixed with C0C2 (1-30 μM) and separated by centrifugation. The pellet fractions containing C0C2 bound to actin (left) were quantified by SDS-PAGE using a standard curve ran on each gel with known molar ratio amounts of actin to C0C2 (right).
Figure 2. Binding curves for ACTC variants and C0C2
The binding curve for C0C2 to WT-ACTC protein is shown in A with error bars (n=3) and reproduced in the other panels without error bars for comparison. C0C2 binding data for A331P (B, n=7), Y166C (C, n=4), and E361G (D, n=11) ACTC mutant proteins. In all cases, the error bars show the standard error about the mean.
Table 1

Summary data of the dissociation constants (Kd) and molar binding ratios (B$_{\text{max}}$) for binding of recombinant C0C2 to variant ACTC filaments.

| ACTC Variant | n  | B$_{\text{max}}$ (mol/mol actin) | Kd (μM)  |
|--------------|----|---------------------------------|----------|
|WT            | 6  | 0.76±0.09                       | 5.02±1.10|
|E99K          | 6  | 1.08±0.04                       | 6.73±1.82|
|Y166C         | 4  | 0.73±0.09                       | 11.86±3.27*|
|A230V         | 4  | 0.81±0.15                       | 7.24±0.86|
|M305L         | 4  | 0.68±0.05                       | 3.57±0.74|
|R312H         | 6  | 0.93±0.16                       | 4.51±0.67|
|A331P         | 7  | 0.91±0.12                       | 13.39±1.73*|
|E361G         | 11 | 0.99±0.09                       | 9.17±1.60**|

Data presented are averages ± standard error about the mean.

* p<0.05

** p<0.15 (one-way ANOVA followed by a Dunnett's t-test)