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Structural and kinetic effects of hypertrophic cardiomyopathy related mutations R146G/Q and R163W on the regulatory switching activity of rat cardiac troponin I

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ABSTRACT

Mutations in cardiac troponin I (cTnI) that cause hypertrophic cardiomyopathy (HCM) have been reported to change the contractility of cardiac myofilaments, but the underlying molecular mechanism remains elusive. In this study, Förster resonance energy transfer (FRET) was used to investigate the specific structural and kinetic effects that HCM related rat cTnI mutations R146G/Q and R163W exert on Ca^{2+} and myosin S1 dependent conformational transitions in rat cTn structure. Ca^{2+}-induced changes in interactions between cTnC and cTnI were individually monitored in reconstituted thin filaments using steady state and time resolved FRET, and kinetics were determined using stopped flow. R146G/Q and R163W all changed the FRET distances between cTnC and cTnI in unique and various ways. However, kinetic rates of conformational transitions induced by Ca^{2+}-dissociation were universally slowed when R146G/Q and R163W were present. Interestingly, the kinetic rates of changes in the inhibitory region of cTnI were always slower than that of the regulatory region, suggesting that the fly casting mechanism that normally underlies deactivation is preserved in spite of mutation. In situ rat myocardial fiber studies also revealed that FRET distance changes indicating mutation specific disruption of the $cTnI_{IR}$–actin interaction were consistent with increased passive tension.

Introduction

Hypertrophic cardiomyopathy (HCM) is characterized by ventricular hypertrophy, myofibrillar disarray, and a too often asymptomatic progression toward serious complications [1,2]. Unfortunately, it is also one of the most common genetic cardiovascular diseases, with a 1:500 incidence in adults [2]. HCM is linked to dominant missense mutations in almost all of the genes encoding sarcomeric proteins, including the trimeric cardiac troponin (cTn) complex [3–5]. The goal of characterizing the pathological link between cTn mutation and HCM is thus of immense scientific importance due to the critical role that cTn plays in the Ca^{2+} dependent regulation of myocardial contractility. Comprised of subunits cardiac troponin C (cTnC), I (cTnI), and T (cTnT), cTn acts as the Ca^{2+} triggered molecular switch that controls the activation state of the thin filament. Integral to the regulatory switching function of cTn is cTnI, which itself switches from strongly interacting with actin during relaxation to strongly interacting with Ca^{2+} bound cTnC during contraction. Mutations found in human cTnI and linked to the development of HCM were first reported in 1997 and included R145G/Q, R162W, K206Q, and G203S [6]. These mutations occur in ~5% of families with HCM [7,8]. Since 1997, more than 20 HCM-related mutations in cTnI have been reported [3,7,9–11].

From among the first five HCM related cTnI mutations reported in 1997 [6], R145Q, R162W, and especially R145G have received significant empirical attention. Investigations of these HCM related mutations have uncovered several mutation specific physiological effects on myocardium [3,7,9–11]. In the year 2000, it was demonstrated in situ by exchange of human cTnI(R145G) into porcine cardiac myofibrils that R145G reduces the extent to which human cTnI can inhibit actomyosin ATPase activity and increases the Ca^{2+} sensitivity of the myofibrillar ATPase activity profile [12]. Surprisingly, a mutation specific reduction in maximal ATPase activity was also observed, implying that R145G affects both cTnI–actin and cTnI–cTnC interactions. Similar observations were made in a later 2002 report on the effects of human cTnI(R145G) exchange into detergent skinned, porcine, left ventricular myocardial fibers, which showed decreased maximal force generation, increased passive tension, and an enhanced Ca^{2+} sensitivity and reduced steepness of force development [13]. In 2008, a very thorough investigation of the properties of skinned papillary fibers from transgenic mice bearing human cTnI(145G) largely corroborated the findings of both prior studies, but showed additionally that force per crossbridge (XB) was increased [14]. Similar observations have been made about R145Q and R162W [15]. Yet R145Q involves substitution of a positively charge arginine by glutamine which is more polar than

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glycine, whereas R162W involves a hydrophobic substitution at a completely different amino acid position. What mechanism could be responsible for common effects from three different mutations?

The finding that R145G/Q and R162W all affect both cTnI–actin and cTnI–cTnC interactions suggests that their mutation specific effects can be traced to their impact on the functional regions of the C-domain of cTnI (C-cTnI). It is now known that the three functional regions of C-cTnI, namely the inhibitory region ($cTnI_{IR}$), regulatory region ($cTnI_{RR}$), and mobile domain ($cTnI_{MD}$), each play a unique role in making regulatory switching possible. Each cTn regulatory switch controls one regulatory unit (RU), which in addition to one trimeric cTn consists also of one dimeric coiled-coil Tm and a seven monomer stretch of F-actin [16]. In relaxed myocardium, $cTnI_{IR}$ and $cTnI_{MD}$ bind to F-actin [17–19] and act together with tropomyosin (Tm) [20,21] to sterically block the formation of "strong," or force generating, actomyosin XBs within the RU [22–24]. After sarcomeric [Ca^{2+}] rises and Ca^{2+} binds to the N-domain of cTnC (N-cTnC), N-cTnC "opens" and exposes a previously buried hydrophobic pocket which binds strongly to $cTnI_{RR}$ [25,26], sensitizing cTnC to Ca^{2+} [27,28]. The $cTnI_{RR}$–cTnC interaction "drags" $cTnI_{IR}$ and $cTnI_{MD}$ off of actin, thus "releasing" the regulatory inhibition of strong XB formation within the RU in what is known as the "drag and release" mechanism [29–31]. This in turn causes $cTnI_{IR}$ to switch into interacting with cTnC [29], whereas $cTnI_{MD}$ becomes highly dynamic but maintains transient contacts with the thin filament [19]. Upon dissociation of Ca^{2+} from cTnC, a fly casting mechanism [32] is triggered wherein $cTnI_{MD}$ rapidly nucleates into a binding interaction with actin, pulling $cTnI_{RR}$ out of interaction with cTnC and further pulling $cTnI_{IR}$ back down onto F-actin for inhibitory interaction [33]. Finally, should a strong XB form in the absence of Ca^{2+}, the $cTnI_{IR}$ and $cTnI_{MD}$ interactions with actin are disrupted [33–35], leaving the thin filament in a fourth "pre relax" state of regulation [36].

We have shown previously through fluorescence anisotropy measurements that each functional region of C-cTnI exhibits distinct regional protein dynamics and kinetics that play a critical role in facilitating regulatory switching [33]. R145G/Q occurs in $cTnI_{IR}$, and R162W in $cTnI_{RR}$, and all three mutations involve residue substitutions that modify amino acid side chain chemistry. For example, loss of charge resulting from R145G/Q may impact the interactions of $cTnI_{IR}$ with either actin or cTnC, whereas increased regional hydrophobicity from R162W may change the way $cTnI_{RR}$ interacts with the open hydrophobic pocket of Ca^{2+} bound N-cTnC. Furthermore, in affecting the $cTnI_{IR}$, R145G/Q may also affect $cTnI_{RR}$ which lies downstream. By the same logic, R162W may indirectly affect $cTnI_{MD}$ (or $cTnI_{IR}$ due to the fly casting mechanism) by affecting $cTnI_{RR}$. Finally, any conformational change in $cTnI_{RR}$ or impact on its conformational kinetics may be expected to affect the Ca^{2+} sensitivity of N-cTnC. It is therefore reasonable to hypothesize that changes in surface charge and hydrophobicity inherent in mutation result in mutation specific changes to regional conformation and kinetic behavior, which in turn affects regional functions essential to regulatory switching. We hypothesized further that different HCM related mutations result in similar pathology due to common mechanisms of slowing the kinetics of relaxation and changing the nature of protein–protein interactions involving predominately the $cTnI_{RR}$ and $cTnI_{IR}$.

To test our hypotheses, it would be necessary to monitor changes in the structural behavior of the functional regions of C-cTnI. Hence in this study we designed experiments to use Förster resonance energy transfer (FRET) as a spectroscopic ruler to detect mutation specific changes in distance between C-cTnI functional regions and cTnC. Recombinant single-cysteine mutant rat proteins cTnC(S89C) and either cTnI(S151C) or cTnI(S167C) were fluorescently labeled and reconstituted into thin filaments for in vitro steady state, time resolved, and stopped flow FRET measurements. This scheme is convenient because Cys-151 is positioned at the interface between $cTnI_{RR}$ and $cTnI_{IR}$, whereas Cys-167 is located at the interface between $cTnI_{RR}$ and $cTnI_{MD}$ [33]. Furthermore, it may be seen from X-ray crystal structures 1YTZ, 1YV0 [37], 1J1D and 1J1E [38] and our prior FRET studies [39,40] that Cys-151 and Cys-167 of cTnI both experience significant Ca^{2+} dependent changes in proximity to Cys-89 of cTnC, which is located in the central linker of cTnC between its N and C domains. Thus changes in distance between Cys-89 of cTnC and Cys-151 of cTnI should reflect conformational changes involving especially the N-terminal end of $cTnI_{RR}$ and to some extent $cTnI_{IR}$, whereas Cys-167 should indicate conformational changes involving especially the C-terminal end of $cTnI_{RR}$ and to some extent $cTnI_{MD}$. To test for mutation specific effects, R146G, R146Q, or R163W (murine analogs of human HCM related mutations) were also introduced into the rat cTnI(S151C) or cTnI(S167C) construct. The major finding of our study is that R146G/Q and R163W affected C-cTnI functional region conformations uniquely, but this produced a common kinetic outcome in which $cTnI_{RR}$ and especially $cTnI_{IR}$ kinetics were slowed while leaving the fly casting mechanism itself intact. Additionally, R146Q and R163W eliminated S1-ADP dependent conformational changes usually seen in the absence of Ca^{2+}, strongly indicating a mutation-specific disruption of the $cTnI_{IR}$–actin interaction that resembles the fourth pre-relax state of thin filament regulation. These observations directly explained the pathophysiological outcomes of these mutations which were verified in situ in detergent skinned myocardial fibers from rat.

Materials and methods

Protein sample preparation and characterization

To implement FRET in this study, a series of recombinant single-cysteine mutants were generated from wild type rat protein clones using approaches similar to those previously reported [41]. The mutants generated included: cTnI(S151C), cTnI(S167C), cTnC(S89C), cTnI(S151C/R146G), cTnI(S151C/R146Q), cTnI(S151C/R163W), cTnI(S167C/R146G), cTnI(S167C/R146Q) and cTnI(S167C/R163W). Note that in these mutants, endogenous cysteine residues Cys-35 and Cys-84 in cTnC have been substituted with serines [25] and Cys-81 and Cys-98 in cTnI have been substituted with serine and isoleucine, respectively [42]. Furthermore, the substitutions R146G/Q and R163W were used in our rat proteins to mimic the HCM related mutations R145G/Q and R162W found in human cTnI. All cTnI proteins were purified and modified with 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (AEDANS) as FRET donor according to previously described procedures [25,43]. cTnC(S89C) was purified and labeled with *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide (DDPM) as FRET acceptor by following a previously described procedure [29] to produce $cTnC(S89C_{DDPM})$. The labeling ratio was determined spectroscopically using ε_{325nm} = 6000 cm^{-1} M^{-1} for AEDANS and ε_{442nm} = 2930 - cm^{-1} M^{-1} for DDPM. Labeling ratios for all protein modification were >95%. Recombinant wild-type rat cTnT was purified as previously reported [25]. cTm and S1 from the chymotryptic digestion of myosin were obtained from bovine cardiac tissue [44,45]. Actin was purified from rabbit skeletal muscle [46]. Single-cysteine cTnC and cTnI mutants were reconstituted into thin filament samples using a cTn:Tm:actin molar ratio of 1 μM:1:7.5 and checked by Native Gel as previously reported [33,39]. The biochemical activity of the labeled cTnI and cTnC mutants, used before in our laboratory, was also verified by Ca^{2+}-dependent regulation of acto-S1 ATPase activity [39] (data not shown). Multiple experiments were performed on the samples prepared within 2 weeks, and no protein degradation was observed during electrophoretic analysis.