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Hypertrophic Cardiomyopathy Linked Mutation D145E Drastically Alters Calcium Binding By the C-domain of Cardiac Troponin C†

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Abstract

The role of the C-domain sites of cardiac troponin C in the modulation of the calcium signal remains unclear. In this study we investigated the effects of hypertrophic cardiomyopathy linked mutations A8V, E134D and D145E in cardiac troponin C on the properties of the C-domain sites. The A8V mutation had essentially no effect on the calcium or magnesium binding properties of the C-domain sites, while E134D mutation moderately decreased calcium and magnesium binding affinities. On the other hand, D145E mutation affected cooperative interactions between sites III and IV, significantly reducing calcium binding affinity of both sites. Binding of the anchoring region of cardiac troponin I (corresponding to residues 34-71) to cardiac troponin C with D145E mutation was not able to recover normal calcium binding to the C-domain. Experiments utilizing the fluorescent hydrophobic probe bis-ANS suggest that D145E mutation dramatically reduced the extent of calcium-induced hydrophobic exposure by the C-domain. At high non-physiological calcium concentration, A8V, E134D and D145E mutations minimally affected the affinity of cardiac troponin C for the regulatory region of cardiac troponin I (corresponding to residues 128-180). In contrast, at lower physiological calcium concentration, D145E mutation led to ~8-fold decrease in the affinity of cardiac troponin C for the regulatory region of cardiac troponin I. Our results suggest that calcium binding properties of the C-domain sites might be important for the proper regulatory function of cardiac troponin C.

Familial hypertrophic cardiomyopathy (HCM)¹ is a genetic disorder of cardiac muscle, characterized by a hypertrophied left ventricle, with a wide range of clinical phenotypes and outcomes (for review, see (1–3)). HCM has been linked to hundreds of mutations of various sarcomeric proteins, including the thin filament proteins actin, tropomyosin, cardiac troponin I (cTnI) and cardiac troponin T (cTnT) (for review, see (4,5)). Until recently, cardiac troponin C (cTnC), which is highly conserved among vertebrate species, was believed to have fewer HCM linked mutations. However, new evidence indicates that prevalence of HCM linked mutations in cTnC might be similar to that of other thin filament proteins (6,7).

cTnC, comprised of the N- and C-terminal globular domains connected by a central α -helix (for review, see (8,9)), is a member of the EF-hand super-family of Ca^{2+} binding proteins. A canonical helix-loop-helix EF-hand Ca^{2+} binding motif consists of a 12 residue loop flanked

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¹Abbreviations: Hypertrophic cardiomyopathy, HCM; dilated cardiomyopathy, DCM; cTn, cardiac troponin; cTnC, cardiac troponin C; cTnI, cardiac troponin I; cTnT, cardiac troponin T; sTnC, skeletal troponin C; sTnI, skeletal troponin I; IANBD, N-((2-iodoacetoxy) ethyl)-N-methylamino-7-nitrobenz-2-oxa-1,3-diazole; cTnI₃₄₋₇₁, peptide corresponding to residues from 34 to 71 of human cTnI, cTnI₁₂₈₋₁₈₀, peptide corresponding to residues from 128 to 180 of human cTnI; EGTA, ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid; Quin-2, 2-[[2-bis(carboxymethyl)amino-5-methylphenoxy]methyl]-6-methoxy-8-bis(carboxymethyl)aminoquinoline; bis-ANS, 4,4'-Dianilino-1,1'-binaphthyl-5,5'-disulfonic acid dipotassium salt; DTT, dithiothreitol; MOPS, 3-(N-morpholino) propanesulfonic acid; Tween-20, polysorbate 20; K_d , dissociation constant.

by α -helices. The loop residues at positions 1 (+X), 3 (+Y), 5 (+Z), 7 (-Y), 9 (-X) and 12 (-Z) coordinate the Ca^{2+} ion through seven oxygen atoms (for review, see (10–12)). Each domain of cTnC contains a pair of EF-hands numbered I–IV, but EF-hand I is not capable of binding Ca^{2+} due to several loop residue substitutions (13). The α -helices are designated A–H, with an additional 14-residue N-helix at the N-terminus. Because of the defunct first EF-hand, the N-domain of cTnC does not undergo a large conformational “opening” upon Ca^{2+} binding (14).

Binding of Ca^{2+} to the second EF-hand of cTnC is believed to play a direct role in regulation of muscle contraction. The C-domain EF-hands, which possess dramatically higher Ca^{2+} affinity and slower exchange rates compared to the N-domain EF-hand (for review see (15)), are occupied by either Ca^{2+} or Mg^{2+} under resting physiological conditions, and thus thought to play a structural role of anchoring cTnC into the thin filament.

Despite the fact that the C-domain sites are considered structural rather than regulatory, a number of mutations associated with either dilated cardiomyopathy (DCM) or HCM are located in the C-domain of cTnC (for review, see (4)). Recently, several novel mutations of cTnC (A8V, E134D and D145E) were linked to HCM (6,7). Two of the mutations (A8V and D145E) led to higher force recovery and increased Ca^{2+} sensitivity of force development in skinned fibers, despite being located in separate domains of cTnC (6,7). Since E134D mutation did not affect either the extent of force recovery or the Ca^{2+} sensitivity of force generation, it was hypothesized to be a polymorphism (4).

Considering that mutations in the C-domain sites of cTnC have been linked to both HCM and DCM, the importance of the C-domain in the modulation of muscle contraction might have been under-appreciated. The objective of this study was to determine the effect of recently discovered cTnC mutations linked to HCM (A8V, E134D and D145E) on the $\text{Ca}^{2+}/\text{Mg}^{2+}$ binding properties of the C-domain sites, and on interactions of cTnC with the regulatory region of cTnI.

EXPERIMENTAL PROCEDURES

Materials

Phenyl-Sepharose CL-4B, CaCl_2 and EGTA were purchased from Sigma-Aldrich (St. Louis, MO). Quin-2 was purchased from Calbiochem (La Jolla, CA). IANBD was purchased from Invitrogen (Carlsbad, CA). The human cardiac TnI peptides: residues 34–71, herein designated as TnI_{34–71}, and residues 128–180, herein designated as TnI_{128–180}, were synthesized and purified by GenScript USA, Inc (Piscataway, NJ).

Protein Mutagenesis and Purification

The pET3a plasmid encoding human cTnC was a generous gift from Dr. Lawrence B. Smillie (University of Alberta, Canada). The cTnC construct used in this work contained C35S, T53C and C84S substitutions, to enable fluorescent labeling of cTnC on Cys⁵³. Fluorescently labeled cTnC was used to determine the effect of the mutations on the affinity of cTnC for the regulatory fragment of cTnI (cTnI_{128–180}). The HCM cTnC mutants were generated as previously described, and confirmed by DNA sequencing (16,17). Expression and purification of cTnC and its mutants was carried out as previously described (16,17).

Labeling of cTnC and its Mutants

cTnC and its mutants were labeled with the environmentally sensitive thiol-reactive fluorescent probe IANBD on Cys⁵³ as previously described (16,17).

Determination of Ca²⁺ Binding Sensitivities

All steady-state fluorescence measurements were performed using a Perkin-Elmer LS55 fluorescence spectrometer at 15°C. Tyr fluorescence was excited at 275 nm and monitored at 303 nm as microliter amounts of CaCl₂ were added to 2 ml of each unlabeled TnC protein (0.5 μM) in titration buffer (200 mM MOPS (to prevent pH changes upon addition of Ca²⁺), 150 mM KCl, 2 mM EGTA, 1 mM DTT, 3 mM MgCl₂, pH 7.0) at 15°C with constant stirring. The [Ca²⁺]_{free} was calculated using the computer program EGCA02 developed by Robertson and Potter (18). The Ca²⁺ sensitivities of conformational changes were reported as a dissociation constant K_d, representing a mean of at least three titrations ± SE. The data were fit with a logistic sigmoid function (mathematically equivalent to the Hill equation), as previously described (19).

Determination of Mg²⁺ Binding Sensitivities

Mg²⁺ sensitivities were calculated from a decrease in the apparent Ca²⁺ affinities caused by 3 mM Mg²⁺, assuming competitive binding of Ca²⁺ and Mg²⁺, as described (20).

Determination of Ca²⁺ Dissociation Kinetics

All kinetic measurements were performed utilizing an Applied Photophysics Ltd. (Leatherhead, UK) model SX.18 MV stopped-flow instrument with a dead time of ~1.4 ms at 15°C. The rates of conformational changes induced by EGTA removal of Ca²⁺ from unlabeled cTnC or its mutants were measured following intrinsic Tyr fluorescence. Tyr was excited at 275 nm. The Tyr emission was monitored through a UG1 interference filter from Oriel (Stratford, CT). Ca²⁺ dissociation rates were also measured using the fluorescent Ca²⁺ chelator Quin-2. Quin-2 fluorescence was excited at 330 nm with its emission monitored through a 510 nm BrightLine Basic™ filter from Semrock (Rochester, NY). The changes in Quin-2 fluorescence were converted to moles of Ca²⁺ dissociating from unlabeled cTnC or its mutants by mixing increasing concentrations of Ca²⁺ with Quin-2, as previously described (21). The data were fit using a program (by P. J. King, Applied Photophysics Ltd) that utilizes the nonlinear Levenberg-Marquardt algorithm. Each k_{off} represents an average of at least three separate experiments, each averaging at least five traces fit with a single exponential equation.

Determination of cTnI₁₂₈₋₁₈₀ Peptide Affinities

IANBD fluorescence was monitored with excitation at 480 nm and emission at 525 nm. Microliter amounts of cTnI₁₂₈₋₁₈₀ were added to 2 ml of each labeled cTnC protein (0.15 μM) in 10 mM MOPS, 150 mM KCl, 3 mM MgCl₂, 1 mM CaCl₂ or 2 μM CaCl₂, 0.02 % Tween-20, 1 mM DTT, pH 7.0 at 15°C. Each peptide affinity represents a mean of at least three titrations ± SE fit to the root of a quadratic equation for binary complex formation as previously described (17,22).

Statistical Analysis

Statistical significance was determined by an unpaired two-sample t-test using the statistical analysis software Minitab (State College, PA). The two means were considered to be significantly different when the P value was < 0.05. All data is shown as a mean value ± SE.

RESULTS

Location of the HCM Linked Mutations in Different Domains of cTnC

Figure 1 shows that the A8V mutation is located in the N-helix of the N-domain of cTnC, while E134D and D145E mutations are located in the C-domain of cTnC. The E134D mutation is located between Ca²⁺ binding sites III and IV, while the D145E mutation is located in the +Z chelating loop position of site IV.

Effect of HCM Linked cTnC Mutations on the Ca^{2+} and Mg^{2+} Binding Properties of the C-domain Sites

Tyr fluorescence was utilized to determine the effect of HCM linked cTnC mutations on the Ca^{2+} sensitivity of the C-domain sites in the absence and presence of 3 mM Mg^{2+} . The Ca^{2+} dependent changes in intrinsic Tyr fluorescence are due to Ca^{2+} binding to sites III and IV of cTnC (23,24). The C35S, T53C and C84S substitutions present in all the proteins had no effect on the Ca^{2+} affinity (in the absence or presence of Mg^{2+}) or the rate of Ca^{2+} dissociation from the C-domain sites of cTnC (data not shown). The Ca^{2+} induced increases in Tyr fluorescence, occurring when Ca^{2+} binds to the C-domain site of cTnC, cTnC^{A8V}, cTnC^{E134D} and cTnC^{D145E} in the absence or presence of 3 mM Mg^{2+} are shown in Figure 2. In the absence of Mg^{2+} , cTnC exhibited a half-maximal Ca^{2+} dependent increase in its Tyr fluorescence at 224 ± 2 nM. In the presence of 3 mM Mg^{2+} , cTnC exhibited a half-maximal Ca^{2+} dependent increase in its Tyr fluorescence at 534 ± 13 nM. Thus, 3 mM Mg^{2+} produced ~2.4-fold decrease in the Ca^{2+} sensitivity of the C-domain sites of cTnC. Assuming competitive Mg^{2+} binding, the $K_{d(\text{Mg})}$ of the C-domain sites of cTnC was calculated to be 2.17 mM. The $K_{d(\text{Ca})}$ of the C-domain sites of cTnC^{A8V} was measured at 196 ± 1 nM and 550 ± 5 nM in the absence and presence of 3 mM Mg^{2+} , respectively (Figure 2A). Assuming competitive Mg^{2+} binding, the $K_{d(\text{Mg})}$ of the C-domain sites of cTnC^{A8V} was calculated to be 1.66 mM. For cTnC^{E134D}, the half-maximal Ca^{2+} dependent increase in Tyr fluorescence occurred at 301 ± 2 nM in the absence of Mg^{2+} and at 605 ± 33 nM in the presence of 3 mM Mg^{2+} (Figure 2B). Assuming competitive Mg^{2+} binding, the $K_{d(\text{Mg})}$ of the C-domain sites of cTnC^{E134D} was calculated to be 2.97 mM. These results indicate that E134D mutation produced ~1.3- and 1.4-fold decreases in the Ca^{2+} and Mg^{2+} affinities, respectively, of the C-domain sites. The cTnC^{D145E} underwent a biphasic increase in its Tyr fluorescence in both absence and presence of 3 mM Mg^{2+} (Figure 2C). The half-maximal increase of the first phase occurred at 314 ± 31 nM ($K_{d(\text{Ca})1}$) in the absence of Mg^{2+} and at 3801 ± 1093 nM in the presence of 3 mM Mg^{2+} . The half-maximal increase of the second phase occurred at 513 ± 36 μM ($K_{d(\text{Ca})2}$) in the absence of Mg and at 464 ± 53 μM in the presence of 3 mM Mg^{2+} . The $K_{d(\text{Ca})2}$ values were not significantly different from each other in the absence and presence of Mg^{2+} . Since D145E mutation is located in the +Z position of site IV, while site III was unchanged, $K_{d(\text{Ca})1}$ was tentatively assigned to site III and $K_{d(\text{Ca})2}$ was tentatively assigned to site IV. These results suggest that D145E mutation produced a dramatic 2,290-fold decrease in the Ca^{2+} affinity of site IV, and abolished Mg^{2+} binding to that site. Furthermore, D145E mutation produced ~1.4-fold decrease in the Ca^{2+} affinity of site III. Assuming competitive Mg^{2+} binding, the $K_{d(\text{Mg})}$ of site III of cTnC^{E134D} was calculated to be 0.27 mM.

Effect of HCM Linked TnC Mutations on the Rates of Ca^{2+} Dissociation from the C-domain Sites

Fluorescence stopped-flow measurements, utilizing intrinsic Tyr fluorescence, were conducted to determine the effect of HCM linked cTnC mutations on the kinetics of Ca^{2+} dissociation from the C-domain sites. Figure 3A shows that excess EGTA removed Ca^{2+} from the C-domain sites of cTnC, cTnC^{A8V}, cTnC^{E134D} and cTnC^{D145E} at 1.02 ± 0.01 , 0.91 ± 0.01 , 1.64 ± 0.02 and $3.09 \pm 0.05/\text{s}$, respectively. To verify that Tyr signal changes were accurately reporting the true Ca^{2+} dissociation rates and not slower or faster structural change, Ca^{2+} dissociation rates were also measured using the fluorescent Ca^{2+} chelator Quin-2. Figure 3B shows the time course of the increases in Quin-2 fluorescence as Ca^{2+} dissociated from the C-domain sites of cTnC and HCM linked cTnC mutants. Similar Ca^{2+} dissociation rates were measured using Quin-2 fluorescence for cTnC, cTnC^{A8V}, cTnC^{E134D} and cTnC^{D145E} at 1.25 ± 0.02 , 1.16 ± 0.01 , 1.79 ± 0.01 , and $3.03 \pm 0.08/\text{s}$, respectively, as were measured by EGTA induced Tyr changes. Therefore, E134D and D145E mutations led to ~1.6- and 3.0-fold faster rate of Ca^{2+} dissociation from the C-domain sites, respectively. The stoichiometry of Ca^{2+} dissociation from the C-domain sites of cTnC, cTnC^{A8V} and cTnC^{E134D} was 1.91 ± 0.07 , 1.83

± 0.07 , and 1.94 ± 0.08 mol of Ca^{2+} /mol of protein, respectively. In contrast, the stoichiometry of Ca^{2+} dissociation from the C-domain sites of cTnC^{D145E} was 0.87 ± 0.03 mol of Ca^{2+} /mol of protein. Thus, at $15 \mu\text{M}$ Ca^{2+} , the C-domain of cTnC^{D145E} bound $\sim$ one-half of mol Ca^{2+} /mol of protein bound by the C-domains of cTnC, cTnC^{A8V} and cTnC^{E134D}.

The rates of Ca^{2+} dissociation from the C-domain sites of cTnC and HCM linked cTnC mutants were also measured in the presence of anchoring fragment of cTnI that binds to the C-domain of cTnC (residues 34-71). Figure 3C shows the time course of the increases in Quin-2 fluorescence as Ca^{2+} dissociated from the C-domain sites of cTnC, cTnC^{A8V}, cTnC^{E134D} and cTnC^{D145E} in the presence of cTnI₃₄₋₇₁ peptide. The rates of Ca^{2+} dissociation from the C-domain sites of cTnC, cTnC^{A8V}, cTnC^{E134D} and cTnC^{D145E} in the presence of cTnI₃₄₋₇₁ were measured at 0.0357 ± 0.0005 , 0.0356 ± 0.0005 , 0.0369 ± 0.0006 , and 0.0489 ± 0.0013 /s, respectively. The stoichiometry of Ca^{2+} dissociation from the C-domain sites of cTnC, cTnC^{A8V} and cTnC^{E134D} in the presence of cTnI₃₄₋₇₁ was 1.37 ± 0.07 , 1.25 ± 0.07 and 1.20 ± 0.07 mol of Ca^{2+} /mol of protein. The stoichiometry for the C-domain sites of cTnC, cTnC^{A8V} and cTnC^{E134D} in the presence of cTnI₃₄₋₇₁ was less than expected 2 mol of Ca^{2+} /mol of protein, likely due to the fact that Quin-2 was unable to remove all the Ca^{2+} from the C-domain sites of cTnC proteins in the presence of cTnI₃₄₋₇₁. Similarly, Quin-2 was unable to remove all the Ca^{2+} from the C-domain of cTnC in the cTn complex (21), likely due to the fact that Ca^{2+} affinity of the C-domain sites of cTnC in the cTn complex is $\sim$ 24-fold higher than that of isolated cTnC (25). The stoichiometry of Ca^{2+} dissociation from the C-domain sites of cTnC^{D145E} in the presence of cTnI₃₄₋₇₁ was 0.69 ± 0.03 mol of Ca^{2+} /mol of protein, or $\sim$ one-half of that for cTnC. Thus, cTnI₃₄₋₇₁ was not able to recover normal Ca^{2+} binding to the C-domain of cTnC^{D145E}.

Effect of HCM Linked Mutations on the Interactions of cTnC with bis-ANS

Non-covalent binding of bis-ANS to the hydrophobic segments of proteins is accompanied by an increase in its fluorescence, and has been widely used to follow conformational changes. Addition of Ca^{2+} to a bis-ANS solution in the presence of cTnC (in the absence or presence of Mg^{2+}) causes a biphasic increase in fluorescence (26). The first phase of the increase in bis-ANS fluorescence is related to the Ca^{2+} induced binding of bis-ANS to the C-domain sites of cTnC (26). The second phase is likely associated with non-specific Ca^{2+} binding to the additional weak binding sites for $\text{Ca}^{2+}/\text{Mg}^{2+}$ present in the C-domain (27). Figure 4 shows that Ca^{2+} binding to unlabeled cTnC, cTnC^{A8V} and cTnC^{E134D} induced a biphasic increase in the fluorescence of bis-ANS in the absence (Figure 4A) or the presence of 3 mM Mg^{2+} (Figure 4B). In the absence of Mg^{2+} , the half-maximal increase of the first phase occurred at 277 ± 3 , 308 ± 10 , and 408 ± 3 nM for cTnC, cTnC^{A8V} and cTnC^{E134D}, respectively. In the presence of 3 mM Mg^{2+} , the half-maximal increase of the first phase occurred at 666 ± 12 , 760 ± 14 , and 1087 ± 50 nM for cTnC, cTnC^{A8V} and cTnC^{E134D}, respectively. The $K_{d(\text{Ca})}$ values determined from the first phase of the bis-ANS titration are in reasonable agreement with those measured using intrinsic Tyr fluorescence. These results indicate that the C-domains of cTnC, cTnC^{A8V} and cTnC^{E134D} gradually expose hydrophobic surface to the solvent upon binding Ca^{2+} , leading to the first phase of the increase in bis-ANS fluorescence. It is worth noting that both A8V and E134D mutations reduced the magnitude of the first phase of bis-ANS fluorescence, suggesting a reduction in hydrophobic exposure. The Ca^{2+} binding to the C-domain of cTnC^{D145E} did not lead to an increase in bis-ANS fluorescence at the pCa range where site III was binding Ca^{2+} in either absence or presence of 3 mM Mg^{2+} . These results suggest that the C-domain of cTnC^{D145E} either does not undergo a conformational “opening” upon the Ca^{2+} binding to site III, or the exposed hydrophobic pocket is substantially altered to prevent bis-ANS binding.

Effect of HCM Linked cTnC Mutations on the cTnI₁₂₈₋₁₈₀ Binding Properties of Ca²⁺ Saturated cTnC

The HCM linked TnC mutations could have affected the strength of regulatory cTnC-cTnI interactions, considering that the N-domain of cTnC interacts with the switch region of cTnI (corresponding to residues 150-159 (28)) in a Ca²⁺ dependent manner, while the C-domain of cTnC interacts with the inhibitory region of cTnI (corresponding to residues 128-147) (29). A change in fluorescence of Ca²⁺ saturated cTnC labeled with environmentally sensitive probe IANBD on Cys⁵³ was utilized to determine whether the HCM linked mutations affected the affinity of cTnC for cTnI peptide corresponding to residues 128-180 of human cTnI (cTnI₁₂₈₋₁₈₀). The cTnI₁₂₈₋₁₈₀ peptide is homologous to the skeletal TnI₉₆₋₁₄₈ peptide, shown to be a good model system to study the Ca²⁺-dependent interactions between skeletal TnI (sTnI) and skeletal TnC (sTnC) (22,30), and includes both the inhibitory and the switch region. Figure 5A demonstrates that at 1 mM Ca²⁺, the affinity of cTnC for cTnI₁₂₈₋₁₈₀ was at $\sim 73 \pm 3$ nM. At 1 mM Ca²⁺, the affinities of cTnC^{A8V}, cTnC^{E134D} and cTnC^{D145E} for cTnI₁₂₈₋₁₈₀ were $\sim 56 \pm 2$ nM, 62 ± 2 nM and 75 ± 2 nM, respectively. Thus, at 1 mM Ca²⁺, HCM linked mutations A8V, E134D and D145E had minimal effect on the affinity of cTnC for cTnI₁₂₈₋₁₈₀.

During cardiac muscle contraction, intracellular Ca²⁺ concentration elevates to a level of 1–10 μ M (for review, see (31)). Thus, the effect of HCM linked mutations on the affinity of cTnC for cTnI₁₂₈₋₁₈₀ peptide was also determined at 2 μ M Ca²⁺. Figure 5B demonstrates that at 2 μ M Ca²⁺, the affinity of cTnC for cTnI₁₂₈₋₁₈₀ was at 447 ± 10 nM. At 2 μ M Ca²⁺, the affinities of cTnC^{A8V}, cTnC^{E134D} and cTnC^{D145E} for cTnI₁₂₈₋₁₈₀ were at 286 ± 5 nM, 336 ± 6 nM and 3561 ± 157 nM, respectively. Thus, at 2 μ M Ca²⁺, HCM linked mutations A8V and E134D led to modest ~ 1.6 and ~ 1.3 -fold, respectively, increases in the affinity of cTnC for cTnI₁₂₈₋₁₈₀, while D145E mutation led to a dramatic ~ 8.0 -fold decrease in the affinity of cTnC for cTnI₁₂₈₋₁₈₀.

DISCUSSION

While the N-domain site of cTnC responds to Ca²⁺ to regulate muscle contraction, the C-domain sites are thought to be permanently occupied by Ca²⁺ and/or Mg²⁺, thus playing a structural role of anchoring cTnC into the thin filament. However, a number of recently discovered cTnC mutations linked to DCM and HCM are located in the C-domain (for review, see (4)), indicating that properties of this domain might play an important role in the modulation of contraction. The main objective of this study was to examine the effect of HCM linked mutations A8V, E134D and D145E of cTnC on the Ca²⁺/Mg²⁺ binding properties of the C-domain sites. We also wanted to examine whether HCM linked mutations affected affinity of cTnC for the regulatory region of cTnI. First, we investigated whether A8V, E134D and D145E mutations affected the Ca²⁺ binding affinity and rate of Ca²⁺ dissociation from the C-domain of isolated cTnC by following the intrinsic Tyr fluorescence. The A8V mutation had very little effect on the Ca²⁺/Mg²⁺ binding affinities and the rate of Ca²⁺ dissociation from the C-terminal of cTnC. Likely, the main mechanism by which the A8V mutation leads to HCM is by altering the Ca²⁺ binding properties of the N-domain (6). In contrast, the E134D and D145E mutations significantly affected the Ca²⁺ and Mg²⁺ binding properties of the C-terminal sites. The E134D mutation moderately decreased both Ca²⁺ and Mg²⁺ binding affinities. The decrease in Ca²⁺ affinity was caused by the faster rate of Ca²⁺ dissociation from the C-domain sites of cTnC^{E134D}. Since the effects of E134D mutation on the properties of the C-domain were rather modest, further studies are needed to examine if this mutation causes altered regulation under certain physiological conditions, or whether it is simply a rare polymorphism.

Our results suggest that the D145E mutation, located in the +Z position of the fourth Ca²⁺ EF-hand, dramatically decreased Ca²⁺ affinity of site IV, and abolished Mg²⁺ binding to that site. Substitution of Asp residue with a larger Glu residue in the + Z position of site IV might lead

to a smaller Ca^{2+} binding cavity resulting in lower affinity. The effect of D145E mutation is consistent with the results obtained for the synthetic helix-loop-helix peptides, where substitution of Asp residue with Glu in the +Z position caused the peptide to lose the Ca^{2+} and Mg^{2+} binding capacities (32).

Binding of the anchoring region of cTnI, cTnI₃₄₋₇₁ was not able to restore normal Ca^{2+} binding to the C-domain of cTnC^{D145E}, as evidenced by the experiments utilizing Quin-2. In addition to drastically affecting Ca^{2+} binding to site IV, the D145E mutation significantly decreased Ca^{2+} affinity of site III, due to a faster rate of Ca^{2+} dissociation from that site. The lower Ca^{2+} affinity of site III is likely due to the loss of cooperativity between the C-domain sites. Our results are consistent with those observed with closely related EF-hand Ca^{2+} binding protein calmodulin, where D133E mutation in the +Z position of the fourth Ca^{2+} binding loop drastically reduced Ca^{2+} affinity of site IV and significantly reduced that of site III (33). A previous study demonstrated that disruption of Ca^{2+} binding to site III or IV of sTnC (by the substitution of Asp in the +X position of the Ca^{2+} binding loop with Ala) results in the increased Ca^{2+} sensitivity of force development (34). Perhaps cTnC^{D145E} increases the Ca^{2+} sensitivity of force development via a similar mechanism. However, at this time we do not have clear-cut data indicating that D145E mutation specifically inactivated site IV. Structural studies are needed to unequivocally determine whether D145E mutation specifically inactivates site IV.

The dramatic effect of D145E mutation on the properties of the C-domain sites was further revealed by the characterization of the interactions between the cTnC and fluorescent hydrophobic probe bis-ANS. Binding of Ca^{2+} to sites III and IV of cTnC, cTnC^{A8V} and cTnC^{E134D} (in the absence or presence of Mg^{2+}) leads to bis-ANS binding to the exposed hydrophobic pocket. It is worth noting that both A8V and E134D mutations reduced the magnitude of the increase in bis-ANS fluorescence associated with Ca^{2+} binding to sites III and IV. These results suggest that A8V and E134D mutations led to the reduction of hydrophobic surface exposed by the binding of Ca^{2+} to the C-domain of cTnC. The most dramatic result was observed for cTnC^{D145E}, where the D145E mutation prevented bis-ANS binding to the C-domain sites of cTnC at the pCa range where site III was binding Ca^{2+} . A possible interpretation is that binding of Ca^{2+} to the C-domain of cTnC^{D145E} either does not lead to the “opening” of the C-terminal hydrophobic pocket, or the exposed hydrophobic pocket differs substantially from that of cTnC. Presence of both Ca^{2+} and cTnI (residues 147-163) are needed to induce the “opening” of the N-domain of cTnC (35). On the other hand, binding of Ca^{2+} to the single functional site IV of F1 TnC (TnC isoform responsible for stretch activation in insect muscles) was sufficient to induce modest but clear “opening” of the C-domain (36). Structural studies are needed to unequivocally determine whether the C-domain of cTnC^{D145E} “opens” upon binding of Ca^{2+} and/or the anchoring region of cTnI.

The interaction of cTnC with the regulatory region of cTnI plays a crucial role in the regulation of muscle contraction (for review, see (37,38)). Thus, perturbations in affinity of cTnC for cTnI can potentially lead to adverse physiological consequences, such as development of cardiomyopathies. For instance, HCM linked cTnI mutation, R144G, led to ~ 6-fold reduction in the affinity of the Ca^{2+} -saturated C-domain of cTnC for the inhibitory region of cTnI (residues 128-147)(39). Our results show that A8V, E134D or D145E mutations did not lead to substantial alterations in affinity of cTnC for the regulatory region of cTnI (which includes inhibitory and switch regions), cTnI₁₂₈₋₁₈₀ at 1 mM Ca^{2+} . At this high non-physiological Ca^{2+} concentration, the C-domain of cTnC^{D145E} should be almost completely saturated with Ca^{2+} , apparently enabling cTnC^{D145E} to bind cTnI₁₂₈₋₁₈₀ with an affinity similar to that of cTnC. Consistent with these results, co-sedimentation analysis did not detect any changes in the ability of the cTnC complex reconstituted with cTnC^{A8V}, cTnC^{E134D} or cTnC^{D145E} to bind to the thin filament at 0.5 mM Ca^{2+} (6). In contrast, at lower physiological 2 μM Ca^{2+} , D145E mutation led to ~8-fold decrease in the affinity of cTnC for cTnI₁₂₈₋₁₈₀. At this low

Ca^{2+} concentration, the C-domain of cTnC^{D145E} should be only partially saturated with Ca^{2+} , while the C-domain of cTnC should be almost completely saturated with Ca^{2+} . Apparently, reduction in the level of Ca^{2+} saturation of the C-domain at low Ca^{2+} resulted in the decreased affinity of cTnC^{D145E} for cTnI₁₂₈₋₁₈₀, compared to that of cTnC. These results suggest that Ca^{2+} binding properties of the C-domain sites of cTnC play an important role in the interactions of cTnC with the regulatory region of cTnI.

In conclusion, our data demonstrate that the A8V mutation had minimal effect on the $\text{Ca}^{2+}/\text{Mg}^{2+}$ affinities of the C-domain sites. On the other hand, both E134D and D145E mutations altered the Ca^{2+} and Mg^{2+} binding affinities of the C-domain sites. While E134D substitution moderately decreased the Ca^{2+} and Mg^{2+} affinities, the D145E substitution drastically altered Ca^{2+} binding by the C-domain of cTnC. The cTnI₃₄₋₇₁ peptide was not able to recover normal Ca^{2+} binding to the C-domain of cTnC^{D145E}. Experiments utilizing the hydrophobic fluorescent probe bis-ANS suggest that D145E mutation led to a dramatic reduction in the Ca^{2+} -induced hydrophobic surface exposure by the C-domain. At high non-physiological Ca^{2+} concentration, A8V, E134D and D145E mutations had minimal effect on the affinity of cTnC for the regulatory region of cTnI. In contrast, at low physiological Ca^{2+} concentration, D145E mutation led to ~8-fold decrease in the affinity of cTnC for the regulatory region of cTnI. While more studies are needed to fully understand the role of the C-domain of cTnC in the modulation of the Ca^{2+} signal, our results suggest that Ca^{2+} binding properties of the C-domain sites are important for the proper regulatory function of cTnC.

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Figure 1. Location of HCM linked mutations in the N- and C-domains of cTnC

The figure shows ribbon representation of the cTnC in the Ca^{2+} bound state (Protein Data Bank entry 1AJ4 (14)). The A8V mutation (shown in red) is located in the N-helix of the N-domain, the E134D mutation (shown in green) is located between Ca^{2+} binding sites III and IV, and the D145E mutation (shown in blue) is located in the +Z position of Ca^{2+} binding site IV. This figure was generated using PyMOL (<http://www.pymol.org/>).

Figure 2. Effect of HCM linked cTnC mutations on the Ca^{2+} and Mg^{2+} binding properties of the C-domain sites

Panel A shows the Ca^{2+} dependent increases in Tyr fluorescence for cTnC (■) and cTnC^{A8V} (▼) in the absence of Mg^{2+} ; and for cTnC (□) and cTnC^{A8V} (▽) in the presence of 3 mM Mg^{2+} . Panel B shows the Ca^{2+} dependent increases in Tyr fluorescence for cTnC (■) and cTnC^{E134D} (▲) in the absence of Mg^{2+} ; and for cTnC (□) and cTnC^{E134D} (△) in the presence of 3 mM Mg^{2+} . Panel C shows the Ca^{2+} dependent increases in Tyr fluorescence for cTnC (■) and cTnC^{D145E} (●) in the absence of Mg^{2+} ; and for cTnC (□) and cTnC^{D145E} (○) in the presence of 3 mM Mg^{2+} . Microliter amounts of Ca^{2+} were added to 2 ml of each protein (0.5 μM) in 200 mM MOPS, 150 mM KCl, 2 mM EGTA, 1 mM DTT with or without 3 mM MgCl_2 , pH 7.0 at 15°C. Tyr fluorescence was excited at 275 nm and monitored at 303 nm at 15 °C. The data sets were normalized individually for each mutant. Each data point represents the mean $\pm$ SE of at least three titrations fit with a single logistic sigmoid function for cTnC, cTnC^{A8V} and cTnC^{E134D}, and with a double logistic sigmoid function for cTnC^{D145E}.

Figure 3. Effect of HCM linked TnC mutations on the Rates of Ca^{2+} dissociation from the C-domain sites

Panel A shows the time course of the decrease in Tyr fluorescence as Ca^{2+} was removed by EGTA from the C-domain sites of cTnC, cTnC^{A8V}, cTnC^{E134D} and cTnC^{D145E} at 15 °C. Each protein (5 μM) with 500 μM Ca^{2+} in the stopped-flow buffer (10 mM MOPS, 150 mM KCl, 3 mM MgCl_2 and 1 mM DTT, pH 7.0) was rapidly mixed with an equal volume of EGTA (10 mM) in the stopped-flow buffer. The traces have been normalized and displaced vertically for clarity. Panel B shows the time course of the increase in Quin-2 fluorescence as Ca^{2+} was removed by Quin-2 from the C-domain sites of cTnC, cTnC^{A8V}, cTnC^{E134D} and cTnC^{D145E} at 15 °C. Each protein (6 μM) with 15 μM Ca^{2+} in the stopped-flow buffer was rapidly mixed with an equal volume of Quin-2 (150 μM) in the stopped-flow buffer. The traces are not normalized but have been displaced vertically for clarity. Panel C shows the time course of the increase in Quin-2 fluorescence as Ca^{2+} was removed by Quin-2 from the C-domain sites of cTnC, cTnC^{A8V}, cTnC^{E134D} and cTnC^{D145E} at 15 °C in the presence of cTnI₃₄₋₇₁. Each protein (6 μM) in the presence of cTnI₃₄₋₇₁ (18 μM) with 15 μM Ca^{2+} in the stopped-flow buffer was rapidly mixed with an equal volume of Quin-2 (150 μM) in the stopped-flow buffer. The traces are not normalized but have been displaced vertically for clarity.

Figure 4. Effect of HCM linked mutations on the interactions of cTnC with bis-ANS

Panel A shows the Ca^{2+} dependent increases in bis-ANS fluorescence in the presence of cTnC (■), cTnC^{A8V} (▼), cTnC^{E134D} (▲) and cTnC^{D145E} (●) in the absence of Mg^{2+} . Microliter amounts of Ca^{2+} were added to 2 ml of each protein (2 μ M) with bis-ANS (2 μ M) in 200 mM MOPS, 150 mM KCl, 2 mM EGTA, 1 mM DTT, pH 7.0 at 15°C. Panel B shows the Ca^{2+} dependent increases in bis-ANS fluorescence for cTnC (□), cTnC^{A8V} (▽), cTnC^{E134D} (△) and cTnC^{D145E} (○) in the presence of 3 mM Mg^{2+} . The experimental conditions were identical to those in Panel A, except the buffer contained 3 mM $MgCl_2$. Bis-ANS fluorescence was excited at 400 nm and monitored at 495 nm at 15°C. F_0 is the fluorescence value (F) of bis-ANS before addition of Ca^{2+} . Each data point represents the mean $\pm$ SE of at least three

titrations fit with a double logistic sigmoid function for cTnC, cTnC^{A8V} and cTnC^{E134D}, and with a single logistic sigmoid for cTnC^{D145E}.

Figure 5. Effect of HCM linked mutations on cTnI₁₂₈₋₁₈₀ binding affinity of cTnC

Panel A shows the effect of the HCM linked mutations on the cTnI₁₂₈₋₁₈₀ binding properties of cTnC in the presence of 1 mM Ca²⁺. The cTnI₁₂₈₋₁₈₀ dependent changes in IANBD fluorescence are shown as a function of [cTnI₁₂₈₋₁₈₀] for the cTnC (□), cTnC^{A8V} (▽), cTnC^{E134D} (△) and cTnC^{D145E} (○). Panel B shows the effect of the HCM linked mutations on the cTnI₁₂₈₋₁₈₀ binding properties of the Ca²⁺ saturated cTnC in the presence of 2 μM Ca²⁺. The cTnI₁₂₈₋₁₈₀ dependent changes in IANBD fluorescence are shown as a function of [cTnI₁₂₈₋₁₈₀] for the cTnC (■), cTnC^{A8V} (▼), cTnC^{E134D} (▲) and cTnC^{D145E} (●). 100% IANBD fluorescence corresponds to the Ca²⁺-bound state, whereas 0% corresponds to the Ca²⁺-cTnI₁₂₈₋₁₈₀ bound state for each individual cTnC protein. In the case of cTnC^{D145E} in the presence of 2 μM Ca²⁺, the IANBD fluorescence increased upon addition of cTnI₁₂₈₋₁₈₀, so the plot of the data was inverted for the sake of comparison.