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Functional Consequences of the Deletion Mutation ΔGlu160 in Human Cardiac Troponin T[1]

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To explore the functional consequences of a deletion mutation of troponin T (ΔGlu160) found in familial hypertrophic cardiomyopathy, the mutant human cardiac troponin T, and wild-type troponins T, I, and C were expressed in *Escherichia coli* and directly incorporated into isolated porcine cardiac myofibrils using our previously reported troponin exchange technique. The mutant troponin T showed a slightly reduced potency in replacing the endogenous troponin complex in myofibrils and did not affect the inhibitory action of troponin I but potentiated the neutralizing action of troponin C, suggesting that the deletion of a single amino acid, Glu-160, in the strong tropomyosin-binding region affects the tropomyosin binding affinity of the entire troponin T molecule and alters the interaction between troponin I and troponin C within ternary troponin complex in the thin filament. This mutation also increased the Ca^{2+} sensitivity of the myofibrillar ATPase activity, as in the case of other mutations in troponin T with clinical phenotypes of poor prognosis similar to that of ΔGlu160. These results provide strong evidence that the increased Ca^{2+} sensitivity of cardiac myofilament is a typical functional consequence of the troponin T mutation associated with a malignant form of hypertrophic cardiomyopathy.

Key words: ATPase, calcium sensitivity, heart, hypertrophy, troponin.

The contraction of vertebrate striated muscle is regulated by Ca^{2+} through two regulatory proteins, troponin (Tn) and tropomyosin (TM), both of which are located in the thin filament (*1*, *2*). Tn consists of three subunits that differ in structure and function; a Ca^{2+}-binding subunit, troponin C (TnC), an inhibitory subunit, troponin I (TnI), and a tropomyosin-binding subunit, troponin T (TnT). All three components are essential for an effective Ca^{2+} regulation of muscle contraction.

Hypertrophic cardiomyopathy (HCM) is an autosomal dominant disorder of heart muscle associated with a high incidence of sudden death in young adults (*3–5*). HCM is a genetically heterogenous disease resulting from mutations in genes encoding cardiac myofibrillar proteins, including TnT (*6*, *7*), TnI (*8*), β-myosin heavy chain (*9*), myosin essential and regulatory light chains (*10*), α-tropomyosin (*6*, *11*), and myosin-binding protein C (*12*, *13*).

At least 13 different mutations in the TnT gene have been identified to date (*14*), and several studies have been carried out to explore their functional consequences (*15–22*). We have previously reported the effects of five missense mutations (Ile79Asn, Arg92Gln, Glu244Asp, Arg278Cys, and Phe110Ile) and a splice donor site mutation that results in aberrant splice products encoding two truncated TnT molecules lacking carboxyl termini on the Ca^{2+}-activated myofibrillar ATPase activity (*23*) and force generation of skinned muscle fibers (*24–27*). The present study was undertaken to investigate the functional effects of a mutation in the TnT gene involving the deletion of three nucleotides encoding glutamic acid at position 160 (ΔGlu160), which occurs within the region constituting a primary TM-binding site (*28*, *29*). We prepared recombinant human cardiac Tn subunits (wild-type TnC, TnI, and TnT) and the HCM-causing ΔGlu160 TnT mutant, and these proteins were then incorporated into porcine cardiac myofibrils using our previously reported method for replacing endogenous Tn complex in myofibrils with exogenously added TnT (*30–32*). The ΔGlu160 mutant and wild-type TnTs were equally effective in replacing the endogenous Tn complex in myofibrils at saturating levels of added TnT. The mutation did not affect the inhibitory action of TnI but potentiated the neutralizing action of TnC. In addition, the ΔGlu160 TnT shifted the pCa-ATPase activity relationship to the left, as in the case of other mutations associated with poor prognosis (*i.e.*, Ile79Asn, Arg92Gln, and splice donor site mutation) (*23*, *24*, *27*), and also reduced the steepness of the pCa-ATPase activity relationship, as in the case of the mutation Arg278Cys and the splice donor site mutation in

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Abbreviations: Tn, troponin; TM, tropomyosin; HCM, hypertrophic cardiomyopathy; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; 2-ME, 2-mercaptoethanol; pCa, -log [Ca^{2+}].

the COOH-terminal, second TM-binding region (*23*, *26*, *27*). The results suggest that an increase in the Ca^{2+} sensitivity and a decrease in the cooperativity of the cardiac myofilament response to Ca^{2+} are common functional defects of the TnT mutations in the TM-binding regions and provide strong evidence that increased Ca^{2+} sensitivity is involved in the pathogenesis of HCM with poor prognosis caused by mutations in TnT.

MATERIALS AND METHODS

Cloning and Mutagenesis of Human Cardiac Tn cDNAs—Human cardiac Tn (TnC, TnI, and TnT) cDNAs were amplified by reverse transcriptase-polymerase chain reaction of human heart mRNA purchased from Clontech (Palo Alto, CA). The polymerase chain reaction products were purified using agarose gels and subsequently treated with restriction enzymes. The digested cDNAs were further purified and subcloned into the pUC119 vector for screening by restriction analysis and DNA sequencing. The nucleotide sequence of the TnT cDNA cloned in this study was identical to that reported by Mesnard *et al.* (*33*), which lacks an acidic domain of ~10 amino acids that has been identified in the NH_2-terminal region of fetal isoforms in various animals. The obtained wild-type Tn cDNAs were then constructed into a pET-3d vector for expression. To obtain the deletion mutant ΔGlu160 TnT, mutagenesis was carried out by polymerase chain reaction according to the method described by Horton (*34*). Oligonucleotides employed for mutagenesis were: forward, 5′-CGA CGA GAG GAG GAG AAC AGG AGG AAG GCT-3′; reverse, 5′-CCT GTT CTC CTC CTC TCG TCG AGC CCT CTC-3′. The result of the mutation in the TnT cDNA was confirmed by DNA sequencing.

Expression and Purification of Recombinant Tns—The human cardiac wild-type Tn and mutant TnT were expressed in *Escherichia coli* strain BL21(DE3) and purified as described previously (*23*).

Preparation of Myofibrils—Cardiac myofibrils were prepared from porcine left ventricular muscle by the methods of Solaro *et al.* (*35*) and stored in a solution containing 50% (v/v) glycerol, 100 mM KCl, 20 mM MOPS/KOH (pH7.0), and 0.5% 2-ME at –20°C.

Tn Exchange in Myofibrils—Tn exchange in the myofibrils was carried out according to our previously reported method (*30–32*) with slight modifications. Myofibrillar suspensions containing 20 mM MOPS/KOH (pH 6.2), 270 mM KCl, 2 mM EGTA, 2 mM $MgCl_2$, 0.5% 2-ME, 500 μg/ml myofibrils, and variable amounts of recombinant human cardiac TnTs were gently shaken for 1 h at 25°C and then spun. The resulting sediments were washed with a solution containing 0.5% (w/v) Brij-58, 20 mM MOPS/KOH (pH 7.0), 265 mM KCl, 2 mM $MgCl_2$, then with a solution containing 60 mM KCl and 1 mM $NaHCO_3$, and finally resuspended in a small amount of the solution containing 60 mM KCl and 1 mM $NaHCO_3$. The TnT-treated myofibrils (0.175 mg/ml) were then reconstituted with variable amounts of recombinant human cardiac TnI and TnC in a solution containing 90 mM KCl, 5 mM $MgCl_2$, 20 mM MOPS/KOH (pH 7.0), 1 mM EGTA for 1 h at 4°C.

ATPase Activity Measurement—The reaction mixture (200 μl) for the ATPase assay consisted of 90 mM KCl, 5 mM $MgCl_2$, 20 mM MOPS/KOH (pH 7.0), 1 mM Ca^{2+}-EGTA, 4 mM Na_2ATP, and 35 μg of myofibrils. The reaction was started by adding ATP at 25°C and was terminated by adding 1.6 ml of a mixture of 50% (v/v) acetone, 2.5 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, and 1.25 N H_2SO_4. The contents were mixed carefully, then 160 μl of 1 M citric acid was added to tubes, and the yellow color was measured at OD_{355} (*36*). Ca^{2+} concentration in the reaction mixtures was calculated as described previously (*37*).

Electrophoresis—SDS-PAGE was carried out at 12% acrylamide concentration according to the method of Laemmli (*38*). The gel was stained with Coomassie Brilliant Blue R-250. An optical densitometric scan was performed using Phoretix gel analysis software (Phoretix International).

RESULTS

Effect of Wild-type TnT and ΔGlu160 TnT Treatment on the ATPase Activity of Porcine Cardiac Myofibrils—Figure 1 shows the result of SDS-PAGE of the bacterially expressed and purified wild-type and ΔGlu160 mutant of human cardiac TnTs. These recombinant human cardiac TnTs had slightly faster electrophoretic mobility than native porcine

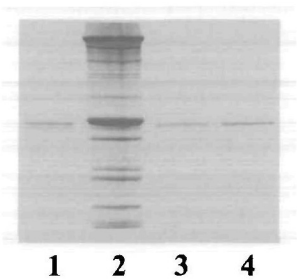

Fig. 1. **SDS-PAGE of purified recombinant human cardiac wild-type and ΔGlu160 mutant TnTs.** All samples were separated on 12% SDS-PAGE gel. Lane 1, porcine cardiac tissue–derived (native) TnT; lane 2, porcine cardiac myofibrils; lane 3, human cardiac wild-type TnT; lane 4, human cardiac ΔGlu160 TnT. The gel was stained with Coomassie Brilliant Blue R-250.

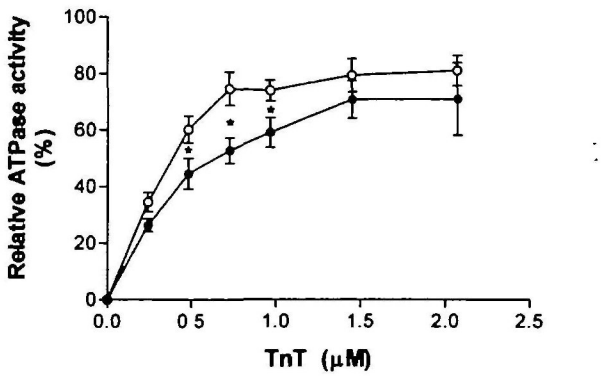

Fig. 2. **The ATPase activity of porcine cardiac myofibrils treated with recombinant human cardiac TnT.** Myofibrils were treated with various amounts of wild-type TnT (○) or ΔGlu160 TnT (●) for 1 h at 25°C, and the ATPase activity of these myofibrils were measured in the absence of Ca^{2+} (pCa > 8.5). The data were normalized to the ATPase activity in the presence of Ca^{2+} (pCa 4.5) at each concentration of TnT. The results are the mean ± SE of 5–10 experiments. *$p < 0.05$ *vs.* wild-type TnT-treated myofibrils (unpaired *t* test).

cardiac TnT on this 12% SDS-PAGE gel. We have previously shown that the treatment of myofibrils with an excess amount of purified TnT in acidic and high-ionic-strength solution causes an increase in the ATPase activity of myofibrils in the absence of Ca^{2+}, due to the replacement of endogenous Tn complex with exogenous TnT (*30–32*). To optimize the exchange of TnT into porcine cardiac myofibrils, the myofibrils were treated with variable amounts of recombinant TnT, and their ATPase activities were measured in the absence of Ca^{2+} (Fig. 2). The ATPase activity in the absence of Ca^{2+} increased with increase in the concentration of TnT. The ΔGlu160 mutant TnT showed slightly, but statistically significantly, lower potency than the wild type at low concentrations of TnT, suggesting that the TM-binding affinity is slightly impaired in this mutant. However, the difference disappeared at TnT concentrations higher than 1.45 μM. In the following experiments, myofibrils were treated with 1.45 μM TnT.

Determination of the Extent of TnT Exchanged into Myofibrils—To determine the extents of the exchange of Tn complex in the myofibrils treated with wild-type TnT and ΔGlu160 mutant TnT, SDS-PAGE analyses were carried out (Fig. 3). After treatment with wild-type and mutant TnT (lanes 4 and 5), the amount of endogenous TnI significantly decreased compared to that in the untreated myofibrils (lane 3). Previous studies demonstrated that the extent of the exchange of Tn complex is directly proportional to the decreased amount of endogenous TnI after the TnT treatment (*24*, *28*). Quantification of the TnI/TM ratio by densitometric scans showed that 63.6 ± 6.4% (mean ± SE, n = 3) and 61.1 ± 8.7% (mean ± SE, n = 3) of the endogenous TnI were removed by wild-type TnT treatment and ΔGlu160 mutant TnT treatment, respectively, indicating that the wild-type and ΔGlu160 mutant TnTs were equally effective in replacing the endogenous Tn complex, and that approximately 60% of these recombinant human cardiac TnT were incorporated into the porcine cardiac myofibrils.

Effects of ΔGlu160 Mutation in TnT on the Inhibitory or Neutralizing Action of Recombinant Human Cardiac TnI and TnC—The Ca^{2+}-insensitive ATPase activities of myofibrils treated with wild-type TnT and ΔGlu160 TnT were reduced by the addition of the recombinant human cardiac TnI in a similar dose-dependent manner and were completely inhibited at 1.15 μM (Fig. 4A). No significant differences in the ATPase activity were detected at any concentrations of TnI between the wild-type and ΔGlu160 TnT-treated myofibrils, indicating that the ΔGlu160 mutation does not affect the inhibitory action of TnI. In the presence of Ca^{2+}, the addition of the recombinant human cardiac TnC reactivated the suppressed ATPase activities of the TnI-reconstituted myofibrils containing wild-type TnT and

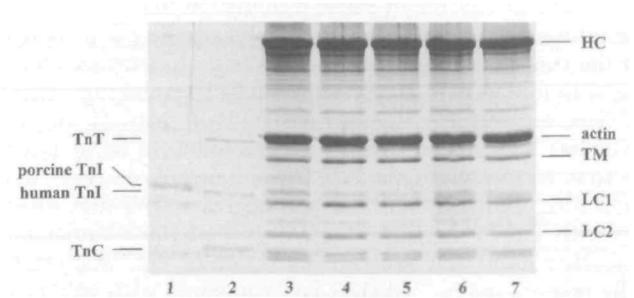

Fig. 3. **The gel electrophoresis pattern of TnT-treated and TnI•TnC-reconstituted myofibrils.** The myofibrils (500 μg/ml) were treated with 1.45 μM wild-type TnT or ΔGlu160 TnT and reconstituted with recombinant human cardiac TnI (1.15 μM) and TnC (0.61 μM). The samples were separated on 12% SDS-PAGE. Lane 1, native porcine cardiac TnI; lane 2, recombinant human cardiac TnT, TnI, TnC-mixture; lane 3, untreated myofibrils; lane 4, wild-type TnT-treated myofibrils; lane 5, ΔGlu160 TnT-treated myofibrils; lane 6, reconstituted myofibrils after treatment with wild-type TnT; lane 7, reconstituted myofibrils after treatment with ΔGlu160 TnT. The data are representative of three independent experiments. HC, myosin heavy chain; TM, tropomyosin; LC1, myosin light chain 1; LC2, myosin light chain 2.

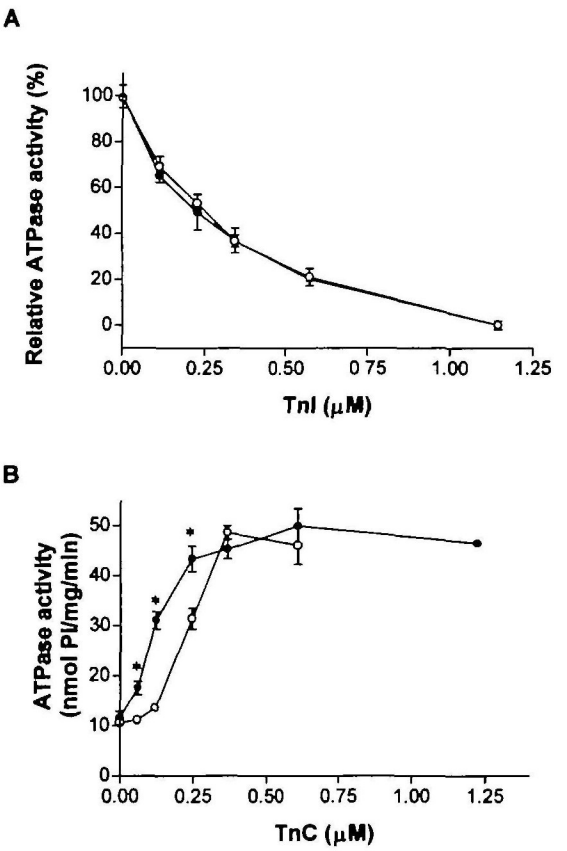

Fig. 4. **The effects of ΔGlu160 mutation in recombinant human cardiac TnT on the inhibitory action of human cardiac TnI and on the neutralizing action of human cardiac TnC.** A: The inhibitory action of TnI on the ATPase activity of TnT-treated myofibrils. The myofibrils (500 μg/ml) were treated with 1.45 μM wild-type TnT (○) or ΔGlu160 TnT (●) for 1 h at 25°C with shaking, then reconstituted with various amounts of human cardiac TnI for 1 h at 4°C. The ATPase activity of the TnI-reconstituted myofibrils was measured in the absence of Ca^{2+} (pCa > 8.5). The results are expressed as a percentage of the ATPase activity of TnT-treated myofibrils without adding TnI and are the mean ± SE of five experiments. B: The neutralizing action of TnC on the ATPase activity of TnT-treated and TnI-reconstituted myofibrils. The myofibrils treated with wild-type TnT (○) or ΔGlu160 TnT (●) were reconstituted with 1.15 μM TnI for 1 h at 4°C. The myofibrils was further reconstituted with various amounts of human cardiac TnC for 1 h at 4°C, then the ATPase activity was measured in the presence of Ca^{2+} (pCa 4.5). The results are the mean ± SE of five experiments. *$p < 0.05$ *vs.* wild-type TnT-treated myofibrils (unpaired t test).

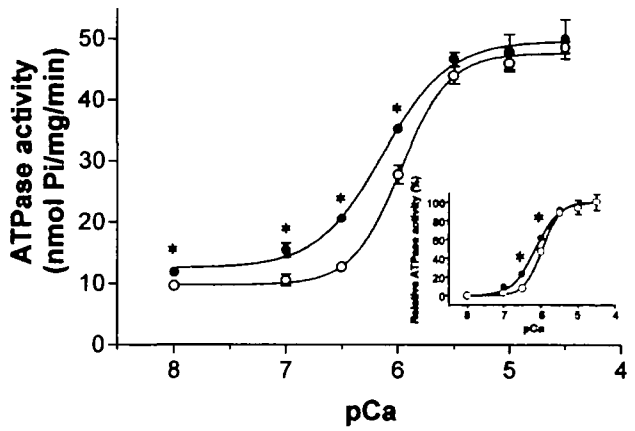

Fig. 5. **The Ca^{2+}-activated ATPase activity of myofibrils reconstituted with human cardiac TnI and TnC after treatment with recombinant TnT.** Myofibrils were treated with 1.45 μM wild-type TnT (○) or ΔGlu160 TnT (●) and then reconstituted with 1.15 μM TnI and 0.61 μM TnC, then the Ca^{2+}-activated ATPase activity of these myofibrils was measured. Inset. The Ca^{2+}-activated ATPase activity normalized to the maximum ATPase activity. The results are mean ± SE of three or six experiments. $^*p < 0.05$ *vs.* wild-type TnT-exchanged myofibrils (unpaired *t* test).

ΔGlu160 TnT in a different dose-dependent manner (Fig. 4B). The ATPase activity of the TnI-reconstituted myofibrils containing ΔGlu160 TnT was reactivated at significantly lower concentrations of TnC than that of the myofibrils containing wild-type TnT, indicating that the ΔGlu160 mutation potentiates the neutralizing action of TnC.

pCa-ATPase Activity Relationships of the Myofibrils Exchanged with Wild-type TnT and ΔGlu160 Mutant TnT—The pCa-ATPase activity relationships were determined in myofibrils that had been treated with wild-type TnT and ΔGlu160 TnT and then reconstituted with recombinant human cardiac TnI and TnC (Fig. 5); SDS-PAGE analysis directly demonstrated that recombinant human cardiac TnI, which had slightly higher mobility than native porcine cardiac TnI, was incorporated into the TnT-treated myofibrils (Fig. 2, lanes 6 and 7). The ΔGlu160 TnT-exchanged myofibrils showed significantly higher sub-maximum ATPase activity than the wild-type TnT-exchanged myofibrils. The maximum ATPase activity, pCa at half-maximum activation (pCa_{50}, an index of Ca^{2+} sensitivity), and Hill coefficient (n_H, an index of cooperativity) in the pCa-ATPase activity relationships are summarized in Table I. As shown in Fig. 5 and Table I, the ΔGlu160 TnT caused a leftward shift in the position, and a reduction in the steepness, of the pCa-ATPase activity relationship, as demonstrated by a significant increase in the pCa_{50} and a significant decrease in the n_H. No significant difference was observed in the maximum ATPase activity. These results indicate that the ΔGlu160 mutation in TnT has a significant effect of increasing the Ca^{2+} sensitivity and decreasing the cooperativity in the cardiac contractile response to Ca^{2+}.

DISCUSSION

It has previously been shown that a highly α-helical region consisting of residues 72–152 in fast skeletal TnT has an important role in the strong binding of TnT to TM (*28*, *29*).

TABLE I. **Comparison of the effects of wild-type and mutant TnT on myofibrillar ATPase activity.** Myofibrils were treated with wild-type or ΔGlu160 TnT. SDS-PAGE analyses were carried out before reconstitution with TnI and TnC. The gels stained with Coomassie Brilliant Blue R-250 were analyzed by optical densitometric scanning using Phoretix gel analysis software to determine the amount of native TnI removed from myofibrils by the treatment with TnT. The Ca^{2+}-activated ATPase activity was measured after reconstitution with TnI and TnC. pCa values at half-maximum ATPase activity (pCa_{50}) and Hill coefficient (n_H) were calculated from the data shown in Fig. 5. The data are the mean ± SE of three or six experiments.

TnT	Extent of native TnI removed by treatment with TnT (%)	Ca^{2+}-activated myofibrillar ATPase activity		
		pCa_{50}	n_H	Maximum activity (nmol P_i/mg/min)
Wild-type	63.6 ± 6.4 (*n*=3)	5.98 ± 0.02 (*n*=6)	2.23 ± 0.20 (*n*=6)	48.7 ± 0.97 (*n*=6)
ΔGlu160	61.1 ± 8.7 (*n*=3)	6.12 ± 0.06* (*n*=3)	1.47 ± 0.20* (*n*=3)	51.3 ± 1.89 (*n*=3)

$^*p < 0.05$ *vs.* wild-type TnT (unpaired *t* test).

The Glu-160 of human cardiac TnT is in the region containing residues 101–181, which is highly homologous to the strong TM-binding region of fast skeletal TnT. Our present finding that the ΔGlu160 TnT has a reduced potency of desensitizing the ATPase activity to Ca^{2+} during the TnT treatment (Fig. 2) suggests that this mutant has lower ability to replace the endogenous Tn complex in myofibrils compared with wild-type TnT under these *in vitro* conditions. This may imply that the residue Glu-160 plays an important role in the strong TM-binding region, and its deletion causes a decrease in the affinity of the entire TnT molecule for TM. Deletion of the residue Glu-160 in the α-helical TM-binding region (residues 101–181) of cardiac TnT would cause a change in the rotational orientation of the residues by ~100° (360°/3.5) along the α-helical axis and lead to an altered interaction of this α-helical region with TM.

TnT interacts with TnI and TnC in the Tn complex located in the thin filament, and thus the HCM-causing mutations in TnT might cause a change in the intermolecular interactions within the Tn complex, leading to a change in the contractile interaction of myosin cross-bridges with the thin filament. In the present study, the inhibitory effect of TnI on the Ca^{2+}-insensitive ATPase activity of the ΔGlu160 TnT-treated myofibrils was found to be identical to that of the wild-type TnT-treated myofibrils (Fig. 4A), indicating that ΔGlu160 TnT and wild-type TnT may interact with TnI in the thin filament in a similar manner. On the other hand, the neutralizing action of TnC was markedly potentiated by ΔGlu160 TnT compared with wild-type TnT (Fig. 4B). This finding suggests that the apparent affinity of TnC for TnI is increased by the ΔGlu160 mutation in TnT. The potentiation of the neutralizing action of TnC might explain the Ca^{2+} sensitizing effect of ΔGlu160 TnT (Fig. 5), because Ca^{2+} binding to a smaller number of TnC molecules would be sufficient to activate the thin filament containing this TnT mutant. Using the fully reconstituted cardiac thin filament and skeletal muscle myosin subfragment 1, Tobacman *et al.* (*39*) recently reported that the ΔGlu160 mutation increased the Ca^{2+}-binding affinity of the regulatory site of TnC in the thin filament and also increased the Ca^{2+} sensitivity of the subfragment 1 ATPase

activity, consistent with our present results obtained under more physiological conditions by directly exchanging the mutant TnT into isolated cardiac myofibrils. The mechanism by which ΔGlu160 TnT reduces the cooperativity in the Ca^{2+}-sensitive myofibrillar ATPase activity (Fig. 5 and Table I) is presently unknown, but a possible reduction in the structural integrity of the Tn complex and TM in the thin filament might be responsible for this phenomenon.

In our previous study, the missense mutation Ile79Asn in TnT was demonstrated to cause an increase in the Ca^{2+} sensitivity of the rabbit cardiac myofibrillar ATPase activity (*23*). However, this TnT mutation slightly impaired the inhibitory action of TnI and did not affect the neutralizing action of TnC, in contrast to the mutation ΔGlu160. The Ile-79 residue of cardiac TnT is not located in the strong TM-binding α-helical region. This strongly indicates that the mutation Ile79Asn causes an increase in the Ca^{2+} sensitivity *via* molecular mechanism different from that involved in the mutation ΔGlu160 in the strong TM-binding region.

Sweeney *et al.* have recently reported the function of ΔGlu160 TnT using a quail skeletal myotube expression system, in which the mutant TnT is incorporated into the sarcomeres of myotubes *in vivo* (*15*). The results of the present study are quite different from their results, which showed that the mutant TnT greatly reduces the maximum Ca^{2+}-activated force by ~40% and also causes a decrease in the Ca^{2+} sensitivity in absolute force-pCa relationships as well as an apparent, slight increase in the Ca^{2+} sensitivity in relative force-pCa relationships. The reason for the discrepancy in the results, especially concerning the maximum level of Ca^{2+}-activation is not known, but it might derive from the difference in the experimental design, including the differences in the muscle lineage and its developmental maturity as well as in the animal species.

As discussed above, the mutation ΔGlu160 is considered to occur in the strong TM-binding region of TnT molecule. The HCM-causing missense mutation in TnT, Phe110Ile, also occurs in this strong TM-binding region. However, the functional effects of these two mutations are very different: Phe110Ile does not alter the Ca^{2+} sensitivity but enhances the maximal ATPase activity or maximal force generating capability (*23*, *25*). This suggests that these two mutations in the strong TM-binding region of TnT have very different effects on the protein-protein interactions in the regulatory complex in the thin filament. On the other hand, the Arg278Cys missense mutation and splice donor site mutation in the COOH-terminal, weak TM-binding region of TnT have been shown to have functional effects similar to those of the ΔGlu160 mutation (*i.e.*, an increase in the Ca^{2+} sensitivity and a decrease in the cooperativity) (*26*, *27*), suggesting that these mutations in the TM-binding regions may affect the thin filament Ca^{2+} regulation through a common molecular mechanism.

We have previously reported that the four missense mutations in TnT, Ile79Asn, Arg92Gln, Glu244Asp, and Arg278Cys, and the splice donor site mutation producing two truncated TnT molecules lacking COOH termini have a Ca^{2+}-sensitizing effect on the contraction of cardiac muscle, whereas the missense mutation Phe110Ile, which is the only TnT mutation shown to be associated with a favorable prognosis (*40*), has no Ca^{2+} sensitizing effect (*23–26*). A clinical study shows that the ΔGlu160 mutation is associated with a poor prognosis characterized by a high risk of sudden death as in the case of the missense mutations Ile79Asn and Arg92Gln and the splice donor site mutation (*6*). Thus, the present study provides strong evidence to support the hypothesis that increased Ca^{2+} sensitivity is responsible for the pathogenesis of HCM with a poor prognosis caused by the mutations in the TnT gene.

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