Characterization of Troponin T Dilated Cardiomyopathy Mutations in the Fetal Troponin Isoform*

The major goal of this study was to elucidate how troponin T (TnT) dilated cardiomyopathy (DCM) mutations in fetal TnT and fetal troponin affect the functional properties of the fetal heart that lead to infantile cardiomyopathy. The DCM mutations R141W and ΔK210 were created in the TnT1 isoform, the primary isoform of cardiac TnT in the embryonic heart. In addition to a different TnT isoform, a different troponin I (TnI) isoform, slow skeletal TnI (ssTnI), is the dominant isoform in the embryonic heart. In skinned fiber studies, TnT1-wild-type (WT)-treated fibers reconstituted with cardiac TnT-troponin C (TnC) or ssTnI-TnC significantly increased Ca2⁺ sensitivity of force development when compared with TnT3-WT-treated fibers at both pH 7.0 and pH 6.5. Porcine cardiac fibers treated with TnT1 that contained the DCM mutations (R141W and ΔK210), when reconstituted with either cardiac TnT-TnC or ssTnI-TnC, significantly decreased Ca2⁺ sensitivity of force development compared with TnT1-WT at both pH values. The R141W mutation, which showed no significant change in the Ca2⁺ sensitivity of force development in the TnT3 isoform, caused a significant decrease in the TnT1 isoform. The ΔK210 mutation caused a greater decrease in Ca2⁺ sensitivity and maximal isometric force development compared with the R141W mutation in both the fetal and adult TnT isoforms. When complexed with cardiac TnT-TnC or ssTnI-TnC, both TnT1 DCM mutations strongly decreased maximal actomyosin ATPase activity as compared with TnT1-WT. Our results suggest that a decrease in maximal actomyosin ATPase activity in conjunction with decreased Ca2⁺ sensitivity of force development may cause a severe DCM phenotype in infants with the mutations.

Tropinin T (TnT) isoforms are encoded by distinct genes in different muscle types: fast skeletal, slow skeletal, and cardiac muscle (1). Multiple isoforms of TnT have been identified in skeletal and cardiac muscle. The expression of more than one cardiac TnT isoform was first identified in chicken. Subsequently, multiple cardiac TnT isoforms were found in different species including the rabbit, rat, mouse, bovine, and human heart, and the number of isoforms expressed varies among different species. In the human heart, alternative splicing of exons 4 and 5 generates up to four different isoforms (TnT1–TnT4) with varying electrophoretic mobility. The four isoforms differ by the presence or absence of exons 4 and 5. Cardiac TnT1 has both exons 4 and 5, in cardiac TnT2 exon 4 is missing, in cardiac TnT3 exon 5 is missing, and in cardiac TnT4 both exons 4 and 5 are missing (2). The multiple cardiac TnT isoforms are expressed in a developmentally regulated manner. Anderson et al. (3) showed that TnT3 is the dominant TnT isoform in the adult human heart, and TnT1 and TnT2 are the two isoforms present in the fetal heart, with TnT2 being present at low levels (4). The switch in the expression of these different isoforms is believed to be at least partially responsible for the different Ca2⁺ sensitivity seen between the neonatal and adult cardiac muscle (5).

Dilated cardiomyopathy (DCM) characterized by increased left ventricular cavity dimensions and systolic dysfunction is caused by mutations in sarcemeric proteins including cardiac TnT, troponin I (TnI), troponin C (TnC), actin, ß-myosin heavy chain, and α-tropomyosin (Tm) (6, 7). Kamisago et al. (8) reported the first TnT mutation as the cause of DCM in two unrelated families. This mutation leads to a deletion of the amino acid lysine at position 210 in exon 13. None of the individuals affected with DCM exhibited ventricular hypertrophy, a hallmark feature of hypertrophic cardiomyopathy. In affected family members, the TnT-ΔK210 mutation caused early-onset ventricular dilation. It also caused a high incidence of sudden cardiac death in both infants and adults. Two children (age, 1 month and 8 months, respectively) who died suddenly had a clinical diagnosis of infantile cardiomyopathy. Li et al. (9) reported another novel TnT mutation, R141W, in a large family of 72 members. Fourteen living members of the family clinically manifested DCM, predominantly in the second decade of their life. Five children (between the ages of 1 and 15 years) in this family died of DCM. Also, 17 members of this family died before genotypic analysis was carried out, presumably due to DCM. It is not known how many out of these 17 members were children. Previously, we investigated the functional properties of TnT DCM mutations in the adult isoform of TnT (TnT3) (10). Because a different TnT isoform is present in the embryonic heart (TnT1) and because both these DCM mutations cause infantile cardiomyopathy, we created mutations in the TnT1 isoform to elucidate the behavior of the mutations in the fetal TnT isoform.

In addition to a different TnT isoform, a different TnI isoform is expressed in the embryonic heart. Both slow skeletal TnI (ssTnI) and cardiac TnI (cTnI) are expressed in the heart during development (11). In humans, only ssTnI is present during the 12th week of gestation (12). In the human heart, ssTnI and cTnI mRNA and protein are both expressed in the neonate, and...
only cTnI is expressed in the adult heart. However, ssTnI expression persists in the human ventricle well into the first year of life (13, 14). Although ssTnI and cTnI serve the same role in the regulation of muscle contraction by inhibiting the actomyosin ATPase activity, their responses to Ca\(^{2+}\) and H\(^+\) ions are markedly different (15). In order to understand the behavior of DCM mutants in fetal troponin, we made the mutations in fetal TnT (TnT1) and utilized ssTnI instead of cTnI in our assays. Because the isoforms used in the assays represent the troponin isoforms in the fetal heart, these studies were designed to reveal how DCM mutations affect the functional properties of troponin in the fetal heart.

**Materials and Methods**

**Cloning of Cardiac Muscle Troponin T WT, Isoform, and Mutants**

The cDNA for human adult cardiac troponin T (TnT3-WT) was previously cloned in our laboratory by reverse transcription-PCR using a template of total RNA from human myocardium and oligonucleotide primers specific for the 5′ and 3′ regions of the respective coding sequences (16). The TnT1 isoform, which is the primary TnT isoform in the embryonic heart, was constructed using TnT3-WT as the template. Two mutations, R141W and K210, were introduced in TnT1 by the method of sequential overlapping PCR using the following primers (17):

- TnT1-R141W
- TnT1-K210

The proteins were initially purified on an S-Sepharose column, and eluted with a linear gradient of 0–600 mM KCl. The fractions were 95% pure protein. The purity of the proteins was verified on SDS-PAGE after each purification step.

**Expression and Purification of TnT-WT, Isoform, and Mutants**

Recombinant TnT3-WT, TnT isoform (referred to as TnT1-WT), and TnT1-DCM mutants were expressed in BL21 bacterial cells. Standard methods were utilized for purification of various TnTs (18, 19). Briefly, bacterially expressed TnT3-WT was passed through an S-Sepharose column and eluted with a linear gradient of 0–600 mM KCl. The fractions containing TnT were loaded onto a fast flow Q-Sepharose column and eluted with a 0–500 mM salt gradient. The purity of the protein was >90%. The proteins were further passed through a DE-52 column that gave >95% pure protein. The purity of the proteins was verified on SDS-PAGE after each purification step.

**Purification of cTnI and ssTnI**

The cDNAs for human cTnI and ssTnI were previously cloned in our laboratory by reverse transcription-PCR using a template of total RNA from human myocardium and oligonucleotide primers specific for the 5′ and 3′ regions of the respective coding sequences (20). *Escherichia coli* BL21 bacterial cells were transformed with pET-3d constructs containing cTnI or ssTnI.

The proteins were initially purified on an S-Sepharose column, and the purity of cTnI and ssTnI was determined by SDS-PAGE. Fractions containing cTnI or ssTnI were purified using a TnT affinity column and eluted with a linear gradient consisting of 2 mM CaCl\(_2\) and 1 mM urea and 3 mM EDTA and 6 mM urea. The purity of the proteins was verified on SDS-PAGE.

**Measurement of the Ca\(^{2+}\) Dependence of Force Development**

This experiment was used to measure the steady-state isometric force and the Ca\(^{2+}\) sensitivity of force development at both pH 6.5 and pH 7.0. The experiment was performed after displacement of the endogenous TnT complex and replacement with either TnT3-WT, TnT1-WT, or TnT1-DCM mutants and reconstitution with the human cTnT3TnC or human ssTnT3TnC complex. This protocol is well established in our laboratory (2, 16, 20).

**Cardiack Skinned Muscle Preparation**—Porcine hearts obtained from the slaughterhouse were transported to the laboratory in an ice-cold oxygenated solution containing 140 mM NaCl, 4 mM CaCl\(_2\), 1.8 mM HEPES, pH 7.4. Cardiac muscle was isolated from the left ventricle and chemically skinned by incubation with 50% glycerol and 1% Triton X-100 in the pCa 8.0 relaxing solution. The composition of the relaxing solution was 10 mM CaCl\(_2\), 1 mM MgCl\(_2\), 7 mM EGTA, 5 mM MgATP, 20 mM imidazole, pH 7.0, 20 mM creatine phosphate, and 15 units/ml creatine phosphokinase, ionic strength = 150 mM, at 4 °C for 24 h. This partially skinned preparation was then stored at –20 °C in the same solution without Triton X-100 and dissected into small bundles (100–150 μm in diameter) before each experiment.

**TnT3TnC Complex Formation**—The cardiac TnT was mixed with either ssTnI or cTnI in a molar ratio of 1:1.25 and dialyzed in a solution containing 6 mM urea, 20 mM MOPS, pH 7.0, 0.5 mM CaCl\(_2\), 1 mM KCl, 1 mM EGTA, and 1 mM dithiothreitol. After overnight dialysis, the TnT3TnC complex was dialyzed in solution containing 20 mM MOPS, pH 7.0, 0.5 mM MgCl\(_2\), and 1 mM dithiothreitol and decreasing KCl concentrations (1, 0.7, 0.4, 0.2, and 0.1 mM). The excess TnI that precipitated from the complex was removed by centrifugation. The TnT3TnC complex was run on SDS-PAGE to verify stoichiometry before storage at –70 °C.

**Measurement of the Ca\(^{2+}\) Dependence of Force**—Porcine muscle fiber bundles (a bundle of three to five fibers isolated from a batch of glycinated fibers) were mounted on a force transducer and treated with the pCa 8.0 relaxing solution containing 1% Triton X-100 for 1 h. The sarcomere length and diameter of the fibers selected for each experiment were ~1.2–1.5 mm and 120–150 μm. The composition of pCa 8.0 solution was 10 mM CaCl\(_2\), 1 mM MgCl\(_2\), 7 mM EGTA, 5 mM MgATP, 20 mM imidazole, pH 7.0, 20 mM creatine phosphate, and 15 units/ml creatine phosphokinase, ionic strength = 150 mM. This permeabilizes the muscle membrane and allows us to gain access to the myofilament proteins. Subsequently, the fibers were transferred to pCa 8.0 solution containing 250 mM KCl, 20 mM Tris and 100 μM F-actin. The fibers were then washed with the same solution without the protein (10 min at room temperature) and tested for the Ca\(^{2+}\)-regulated force that developed due to the absence of the endogenous porcine cTnI and TnC. The Ca\(^{2+}\) regulation of steady-state force was restored with a preformed human cTnT3TnC complex. The reconstitution with the TnT3TnC complex (30 μM) was performed in the pCa 8.0 solution for ~1.5 h at room temperature for the force to reach a stable level. Control fibers were run in parallel and treated with the same solutions without the proteins. The final Ca\(^{2+}\) sensitivity of force development was determined after human cTnT3TnC reconstitution as described before by exposing the fiber to increasing Ca\(^{2+}\) concentration from pCa 8.0 to pCa 4.0. The data were analyzed using the Hill equation in Sigma Plot. The Hill equation is written as follows: % relative force = 100 × [(Ca\(^{2+}\)\(^{-1+m}\) / (Ca\(^{2+}\)\(^{-1+m}\) + pCa\(_{0.5}\)\(^{1+m}\))), where pCa\(_{0.5}\) determines Ca\(^{2+}\) concentration of the solution in which 50% of force is produced, and nH is the Hill coefficient.

**Reconstituted Actomyosin ATPase Assay**

The native proteins used are rabbit skeletal actin, porcine cardiac myosin, and porcine cardiac tropomyosin. Rabbit skeletal F-actin was prepared as described by Strzelecka-Golaszewka et al. (21). Porcine cardiac myosin was purified as described by Murakami et al. (22). Porcine cardiac TnT was prepared from pig ventricles according to Potter (23). Recombinant TnT, TnC, and TnC were used to make functional troponin complexes. The ATPase assays were performed with rabbit skeletal muscle F-actin (3.5 μM) containing porcine cardiac Tm (1 μM) and pre-formed Tn complexes (1 μM) as described previously (2, 16). The concentration of porcine cardiac myosin was 0.6 μM. All ATPase assays were performed in the presence (0.5 mM CaCl\(_2\)) or absence (1 mM EGTA) of Ca\(^{2+}\). The ATPase reactions (in 10 mM MOPS, 50 mM KCl, 1.8 mM MgCl\(_2\), pH 7.0, 1 mM dithiothreitol, and 1 mM dithiothreitol and 1 mM dithiothreitol) were initiated with 2.5 mM ATP. After a 20-min incubation at 30 °C, the reaction was terminated with 5% trichloroacetic acid. The inorganic phosphate that was released was measured according to the method of Fiske and SubbaRow (24).

**Data Analysis**

Statistical analysis of the differences between mean values was performed by Student’s t-test. Values are presented as mean ± S.D.

**Results**

**Alterations in the Ca\(^{2+}\) Sensitivity of Force Development Caused by TnT1 DCM Mutations at pH 7.0**—In order to study...
the functional differences between TnT3 and TnT1, the endogenous porcine troponin complex was exchanged with either TnT3-WT or TnT1-WT and reconstituted with human cTnI/H18528 TnC, and the Ca²⁺ sensitivity of contraction was measured at pH 7.0 and analyzed using the Hill equation. Before troponin exchange, the Ca²⁺ dependence of force development was measured, and there was no statistically significant difference in either pCa₅₀ or Hill coefficient between the different fibers used (data not shown). As shown in Fig. 1A, porcine fibers treated with TnT3-WT increased Ca²⁺ sensitivity of force development compared with TnT3-WT-treated fibers. This shift of +0.12 pCa unit is consistent with previous results obtained from our laboratory (2) for the same proteins. Also, the Hill coefficient (an indicator of the cooperativity) significantly increased in TnT1 isoform-treated fibers. When porcine cardiac fibers were exchanged with TnT1-R141W and TnT1-K210 (Fig. 1B), both of the DCM mutations decreased the Ca²⁺ sensitivity of force development compared with TnT1-WT. The Ca²⁺ unregulated force produced by fibers treated with TnT1 isoforms and mutants is shown in Table I. Although the ΔK210 mutant had a lower Ca²⁺ unregulated force than its respective TnT1-WT, this difference was not statistically significant. Previous studies from our laboratory (10) showed that in the TnT3 isoform, only the TnT3-ΔK210 decreased Ca²⁺ sensitivity of force development (and not TnT3-R141W) when compared with the TnT3-WT. However, in the TnT1 isoform, both mutations decreased the Ca²⁺ sensitivity of force development, and the magnitude of the shift in Ca²⁺ sensitivity was increased compared with what was observed in the TnT3 isoform. There was no significant difference in the Hill coefficient between TnT1-WT and TnT1 DCM mutant-treated fibers.

In the next series of experiments, porcine cardiac fibers were treated with either TnT3-WT, TnT1-WT, or TnT1 DCM mutants and reconstituted with ssTnI/H18528 TnC instead of cTnI/H18528 TnC. Results depicted in Fig. 1C show that the pCa₅₀ of TnT3-WT-treated fibers reconstituted with cTnI/H18528 TnC is 5.55 ± 0.02. However, when cTnI/H18528 TnC was used to reconstitute TnT3-WT-treated fibers, the pCa₅₀ observed was 5.39 ± 0.02 (Fig. 1A). The increase in Ca²⁺ sensitivity (+0.16 pCa unit) observed upon ssTnI/H18528 TnC reconstitution is due to the well-known effect of ssTnI to lower the threshold for Ca²⁺ activation of force development (25–27). The pCa-force relationship in fibers exchanged with TnT1-WT and reconstituted with ssTnI/H18528 TnC is also depicted in Fig. 1C. The Ca²⁺ sensitivity of force development significantly increased in TnT1-WT-treated fibers compared with TnT3-WT-treated fibers reconstituted with ssTnI/H18528 TnC. Previous results show that (Fig. 1A) TnT1 isoform is more sensitive to Ca²⁺ than TnT3 upon reconstitution with cTnI/H18528 TnC. Our results show that both the TnT1 isoform and ssTnI contribute to the additive increase in Ca²⁺ sensitivity of

**FIG. 1.** Normalized pCa-force relationship in skinned cardiac muscle fibers at pH 7.0. Each skinned muscle preparation was treated with 0.8 mg/ml TnT to displace the endogenous troponin complex at pH 7.0. Data in each experiment are the average of many experiments and expressed as mean ± S.D. A, fibers treated with TnT3-WT versus TnT1-WT and reconstituted with cTnI/H18528 TnC. B, fibers treated with TnT1-WT versus TnT1-R141W and ΔK210 and reconstituted with cTnI/H18528 TnC. C, fibers treated with TnT3-WT versus TnT1-WT and reconstituted with ssTnI/H18528 TnC. D, fibers treated with TnT1-WT versus TnT1-R141W and ΔK210 and reconstituted with ssTnI/H18528 TnC.

**TABLE I.** Summary of normalized pCa₅₀ and Hill coefficient for skinned cardiac muscle fibers treated with TnT isoforms and mutants. Results are expressed as mean ± S.D. of at least 5 experiments.

| Fiber Type       | pCa₅₀ (± S.D.) | Hill Coefficient (± S.D.) |
|------------------|---------------|--------------------------|
| TnT3-WT          | 5.55 ± 0.02   | 2.05 ± 0.02              |
| TnT3-R141W       | 5.39 ± 0.02   | 2.06 ± 0.02              |
| TnT3-ΔK210       | 5.23 ± 0.02   | 2.05 ± 0.02              |
| TnT1-WT          | 5.64 ± 0.02   | 2.31 ± 0.02              |
| TnT1-R141W       | 5.55 ± 0.02   | 2.31 ± 0.02              |
| TnT1-ΔK210       | 5.46 ± 0.02   | 2.20 ± 0.02              |

**DCM Mutations in the Fetal Troponin Isoform**
TABLE I
Summary of pCa-force relationship curves in fibers reconstituted with either cardiac TnI-TnC or ssTnI-TnC complex at pH 7.0

The pCa50 maximal force, and nH values are the average of many independent fiber experiments, and the errors are the S.D. values. The DCM mutants in TnT3 and TnT1 are compared against their respective wild types. The data for TnT3-R141W and TnT3-ΔK210 were previously published by our group. The Ca2+ unregulated force was calculated by the following equation: (FpCa8/FpCa4) × 100. FpCa8 and FpCa4 are the force at pCa 8.0 and pCa 4.0, respectively.

| TnT  | TnI    | pCa50 | nH | % Ca2+ unregulated force | Maximum force | n |
|------|--------|-------|----|--------------------------|---------------|---|
| TnT3-WT | cTnI | 5.39 ± 0.02 | 1.82 ± 0.11 | 93.4 ± 4.2 | 65.4 ± 2.8 | 7 |
| TnT3-R141W | ssTnI | 5.55 ± 0.03 | 1.74 ± 0.04 | 92.2 ± 5.4 | 71.8 ± 10.1 | 4 |
| TnT3-ΔK210 | ssTnI | 5.27 ± 0.02 | 1.74 ± 0.11 | 89.6 ± 6.2 | 46.8 ± 1.1b | 4 |
| TnT1-WT | cTnI | 5.51 ± 0.03 | 2.05 ± 0.15 | 91.8 ± 4.7 | 72.6 ± 3.5 | 5 |
| TnT1-R141W | ssTnI | 5.30 ± 0.03 | 1.94 ± 0.13 | 93.8 ± 3.1 | 71.2 ± 1.2 | 4 |
| TnT1-ΔK210 | ssTnI | 5.22 ± 0.02 | 1.9 ± 0.21 | 87.9 ± 5.3 | 54.6 ± 5.5 | 4 |
| TnT3-WT | ssTnI | 5.55 ± 0.02 | 2.22 ± 0.17 | 94.6 ± 3.2 | 84.9 ± 2.5 | 4 |
| TnT1-WT | ssTnI | 5.64 ± 0.03 | 1.84 ± 0.11 | 93.6 ± 4.9 | 96.2 ± 2.5b | 4 |
| TnT1-R141W | ssTnI | 5.46 ± 0.03 | 2.09 ± 0.15 | 93.1 ± 3.5 | 86.1 ± 1.9 | 3 |
| TnT1-ΔK210 | ssTnI | 5.47 ± 0.02 | 2.08 ± 0.09 | 89.8 ± 4.3 | 70.3 ± 2.6 | 3 |

* Values are compared against TnT3-WT-cTnI.

b p < 0.01.

** p < 0.05; ***, p < 0.001.

Values are compared against TnT3-WT-ssTnI.

Values are compared against TnT1-WT-cTnI.

Values are compared against TnT1-WT-ssTnI.

Values are compared against TnT1-ΔK210-ssTnI.

values are the average of many independent fiber experiments, and the errors are the S.D. values. The DCM mutations in the fetal TnT isoform indicates that the R141W mutation behaves similar to the wild type in both the fetal and adult isoform. On the other hand, the ΔK210 mutation decreased Ca2+ activated maximal force in both the fetal and adult TnT isoforms (Table I).

Fig. 2 illustrates the Ca2+ activated maximal force achieved in fibers treated with TnT3-WT, TnT1-WT, and TnT1 DCM mutants and reconstituted with ssTnI-TnC. The maximal force recovered for fibers treated with TnT3-WT was ~85%. When the same TnT3-WT-treated fibers were reconstituted with cTnI-TnC, the maximal force recovered was 65% (Fig. 2A). Therefore, our results indicate that ssTnI is able to markedly increase the amount of maximal force recovered, in addition to increasing the Ca2+ sensitivity of force development. The maximal isometric force obtained for TnT1-WT-treated fibers reconstituted with ssTnI-TnC was 96%. This is a significant increase compared with 73% force recovered when TnT1-WT-treated fibers were reconstituted with cTnI-TnC (Fig. 2A; Table I). Our results indicate that the maximal recovered force is the highest when ssTnI is in combination with TnT1-WT rather than TnT3-WT. TnT1-R141W-treated fibers did not show significant changes in maximal force compared with TnT1-WT in the presence of ssTnI-TnC. However, in the ΔK210 mutation-
In order to understand how the DCM mutations would affect ATPase inhibition in the fetal heart at the level of troponin, we utilized ssTnI-TnC to make troponin complexes containing TnT3-WT, TnT1-WT, and TnT1 DCM mutants. In the absence of Ca\textsuperscript{2+}, when increasing concentrations of Tn complexes are added, the basal activity starts to fall. At 2 \mu M, TnT3-WT:ssTnI-TnC complex was able to inhibit 60.5% of the basal ATPase activity. When TnT3-WT was complexed with cTnI-TnC at the same concentration (2 \mu M), 86.4% inhibition was achieved (Fig. 3A). TnT1-WT:ssTnI-TnC was able to inhibit only 49.5% of the basal ATPase activity. When complexed with cTnI-TnC, TnT1-WT was able to inhibit 75% of the basal ATPase activity (Fig. 3A). TnT1 DCM mutants complexed with ssTnI-TnC inhibited ATPase activity to a similar extent as TnT1-WT (35% for both proteins investigated). Our results show that complexes containing ssTnI-TnC are not able to inhibit as well as cTnI-TnC at all concentrations, and this difference was found to be statistically significant by Student’s t test (p < 0.001).

Fig. 4A illustrates ATPase activation in the presence of Ca\textsuperscript{2+} (-Ca\textsuperscript{2+}) at 1 \mu M Tn (the concentration at which the maximal inhibition was achieved), and the results are indicated as a bar chart. The results show that ATPase activity in the absence of Tn was considered to be the basal activity (100% activity). Tn complex containing TnT3-WT and cTnI-TnC achieved ~490% activity. The ATPase activity of TnT1-WT was 157 ± 6%. With respect

treated fibers, we observed a significant decrease in the maximal force recovered (96% for TnT1-WT versus 70% for TnT1-\Delta K210) when reconstituted with ssTnI-TnC.

Reconstituted Actomyosin ATPase Assays on TnT1 DCM Mutations—It is well known that TnI by itself inhibits ATPase activity and that the addition of TnC relieves this inhibition. When TnT is added, there is an activation of actomyosin ATPase activity that is Ca\textsuperscript{2+}-dependent (28). Fig. 3A illustrates the ATPase inhibition in the absence of Ca\textsuperscript{2+} (-Ca\textsuperscript{2+}) in Tn complexes containing cTnI-TnC. The actomyosin ATPase activity in the absence of human cardiac troponin complex was considered to be the basal activity. In the absence of Ca\textsuperscript{2+}, ATPase was maximally inhibited at 2 \mu M Tn. Tn complexes containing TnT3-WT were able to inhibit 86.4% of the basal activity. TnT1-WT complexes containing TnT3-WT were able to inhibit 75% of the basal activity. Tn complexes containing TnT3-WT and TnT1-WT versus TnT1-\Delta K210 and \Delta K210 and complexed with ssTnI-TnC. The assay conditions were as follows: 3.5 \mu M actin, 1 \mu M Tn, 0 and 1 \mu M Tn, and 0.6 \mu M myosin were dissolved in 10 mM MOPS, 50 mM KCl, 4 mM MgCl\textsubscript{2}, pH 7.0, and 0.5 mM CaCl\textsubscript{2}. Each data point represents the average of four to five experiments, each performed in triplicate, and is expressed as mean ± S.D. 100% corresponds to the activity in the absence of Tn.

The ATPase activity of TnT1-WT was 157 ± 6%. With respect...
to maximal ATPase activity, the fetal isoform of TnT, TnT1, behaves very similar to TnT3, which is the adult TnT isoform. The ATPase activity of TnT1-R141W was 140% and the ATPase activity of TnT1-K210 was 108%. The maximal ATPase activity of both DCM mutants in the TnT1 isoform was significantly decreased compared with the TnT1-WT. This result is similar to what was observed when the mutations were present in the adult cardiac TnT isoform (10).

Fig. 4B depicts ATPase activity in the presence of Ca^2+ for TnT3-WT, TnT1-WT, and TnT1 DCM mutants complexed with ssTnI at 1 μM Tn. The ATPase activity of TnT3-WT was ~157 ± 3%, whereas TnT1-WT gave 172 ± 5% activity. The increase in ATPase activity for TnT1-WT found to be statistically significant (p < 0.01). The maximal ATPase activity of Tn complex containing TnT1-R141W was 143 ± 4%, and the maximal ATPase activity of Tn complex containing TnT1-K210 was 132 ± 1% (p < 0.001). The maximal activity for the TnT1 DCM mutants was significantly lower compared with TnT1-WT when complexed with ssTnI. Our results suggest that ssTnI modulates both ATPase activation and inhibition.

Alterations in Ca^2+ Sensitivity of Force Development and Maximal Force Caused due to TnT1 DCM Mutations at pH 6.5—Fig. 5A illustrates the effect of acidic pH on TnT1-WT and TnT1 DCM mutants in the presence of cTnI. As can be seen, in TnT1-WT-treated fibers, acidic pH caused a decrease of 0.65 pCa unit (pCa50 of 4.86 ± 0.04 at pH 6.5 compared with pCa50 of 5.51 ± 0.03 at pH 7.0). It is well known that the pCa-force relationship shifts to the right under acidic pH conditions. Also at pH 6.5, a significant decrease in the Hill coefficient was observed (nH = 1.44 at pH 6.5) in TnT1-WT-treated fibers. When TnT1-R141W and TnT1-K210 mutations were used in the fiber studies, after reconstitution with cTnI, both the TnT1 DCM mutations caused a statistically significant decrease in Ca^2+ sensitivity of force development when compared with TnT1-WT-treated fibers at pH 6.5. The relative magnitude of decrease in Ca^2+ sensitivity of force development increased as the pH value was altered from 7.0 to 6.5 for both mutations (0.8 pCa unit for TnT1-R141W and 0.7 pCa unit for TnT1-K210). The steady-state isometric force achieved with maximal calcium activation was lowered at pH 6.5 for TnT1-WT as well as for TnT1 DCM mutants (Fig. 6A). TnT1-WT-treated fibers recovered 57% of maximal force at pH 6.5 compared with 72% of maximal force at pH 7.0. The TnT1-R141W-treated fibers recovered 48.6% of the initial force, and the TnT1-K210-treated fibers restored only 30.3% of the initial force. Comparison of Ca^2+ activated maximal force at pH 6.5 in the fetal versus adult TnT isoform indicates that the
R141W mutation does not alter the maximal force recovered in both the fetal and adult TnT isoform (Table II). However, the ΔK210 mutation significantly decreased the maximal force in both the fetal and adult TnT isoforms (Table II).

We also investigated the pCa-force relationship under acid pH conditions in porcine fibers treated with TnT3-WT, TnT1-WT, and TnT1 DCM mutants and reconstituted with ssTnI/TnC. At pH 6.5, a pCa50 value of 5.22 ± 0.04 was obtained for TnT3-WT-treated fibers (Table II). At pH 7.0, TnT3-WT-treated fibers reconstituted with ssTnI/TnC caused a significant increase in Ca2+ sensitivity of force development compared with TnT1-WT. The R141W mutation significantly decreased the Ca2+ sensitivity of force development compared with TnT1-WT and increased even further in fibers treated with TnT1-WT and reconstituted with ssTnI/TnC, similar to what was reported by

**DISCUSSION**

Mutations in cardiac TnT have been shown to cause both hypertrophic cardiomyopathy and DCM. The functional properties of TnT mutations causing hypertrophic cardiomyopathy and DCM have been previously characterized by several laboratories (10, 16, 29–33). In all these studies, the mutations were created in the adult cardiac TnT isoform (TnT3). However, clinical data show that familial DCM caused due to TnT mutations affects infants as well as adults (8, 9). Multiple cardiac TnT isoforms are expressed in the mammalian heart in a developmentally regulated manner. It is well established that TnT1 is the primary TnT isoform found in the fetal and neonatal heart (3, 34). During perinatal development, expression of the TnT1 isoform decreases, and TnT3 expression increases and becomes the dominant TnT isoform. In order to elucidate the functional properties of DCM mutants at the level of fetal TnT, we created the TnT DCM mutations R141W and ΔK210 in the primary fetal isoform of TnT (TnT1) and performed skinned fiber studies and reconstituted actomyosin ATPase assays. In addition to a different TnT isoform, a different TnI isoform is present in the fetal heart. The dominant TnI isoform in the fetal and neonatal heart is ssTnI. In order to understand how the TnT DCM mutations alter the functional properties of the troponin in the fetal heart, we utilized ssTnI instead of cTnI in our assays.

Studies on the Ca2+ sensitivity of force development demonstrated that TnT1-WT-treated fibers reconstituted with cTnI/TnC caused a significant increase in Ca2+ sensitivity and Hill coefficient of force development compared with TnT3-WT-treated fibers (Fig. 1A). This result is consistent with previous observations that TnT1 is more sensitive to Ca2+ than TnT3 and suggests that the hypervariable region in the NH2 terminus of cardiac TnT contributes to the Ca2+ sensitivity of force development in the cardiac muscle (2, 5, 35). The R141W mutation showed a slight but not statistically significant decrease (Table I) in the Ca2+ sensitivity of force development on the TnT3 isoform (10). However, in fetal troponin T (TnT1), the R141W mutation significantly decreased the Ca2+ sensitivity of force development compared with TnT1-WT. The R141W mutation is located in a region of TnT that mainly interacts with Tm. Previous studies by Lu et al. (36) using a quartz crystal microbalance showed that the R141W mutation increased the affinity of cardiac TnT for tropomyosin. The authors postulated that the change in affinity of TnT for tropomyosin might amplify the inhibition of cTnI on the thin filament, necessitating more Ca2+ binding to TnC to neutralize the TnI inhibition. The decrease in Ca2+ sensitivity observed here suggests that the highly acidic NH2-terminal hypervariable region may modulate the interaction of TnT-R141W with Tm. Patients harboring the TnT-ΔK210 mutation show a high incidence of sudden cardiac death in infants (20% of affected individuals) (8). In fiber studies, TnT1-ΔK210 showed a significant decrease in Ca2+ sensitivity of force development as well as maximal force.
Gomes et al. (37). This increase in Ca$^{2+}$ sensitivity is due to the
contribution of both TnT1 and ssTnI. Both TnT1-R141W- and
TnT1-ΔK210-treated fibers reconstituted with ssTnI-TnC de-
creased the Ca$^{2+}$ sensitivity compared with TnT1-WT-treated
fibers. It is not known whether the TnT1 NH$_2$-terminal hyper-
variable region alters the structure of TnT in the Ca$^{2+}$-inde-
pendent Tm binding region. A stronger interaction between
cardiac TnC and ssTnI compared with cTnI would lower the threshold for Ca$^{2+}$
activated force development. It is possible that
the interaction of ssTnI with TnC is altered in the pres-
ence of the TnT DCM mutations, leading to Ca$^{2+}$ desen-
sitization of the thin filament. Also, the Ca$^{2+}$ activated maximal
force increased significantly in fibers treated with TnT1-WT
and reconstituted with ssTnI-TnC compared with TnT3-WT-
treated fibers reconstituted with cTnI-cTnC. This could trans-
late in vivo to enhanced cardiac contractility. However, TnT1-
ΔK210-treated fibers showed a significant decrease in maximal
force, and the TnT1-R141W-treated fibers compared with
TnT1-WT-treated fibers reconstituted with ssTnI-TnC showed
no change in maximal force.

We also examined the ability of TnT1-WT and TnT1 DCM
mutants to inhibit and activate actomyosin ATPase activity.
In the absence of Ca$^{2+}$, we found that Tn complexes containing
TnT1-WT were unable to inhibit ATPase activity as well as
TnT3-WT. This result is consistent with what was observed by
Gomes et al. (2). Both the TnT1-R141W and ΔK210 mutations
were able to inhibit similar to TnT1-WT, indicating that the
DCM mutations in the fetal TnT are not disrupting the inhibi-
tory function of TnI. It is not clear whether the NH$_2$ terminus
exerts a direct effect via interactions with Tm or acts indirectly
through TnI and TnC. When Tn complexes containing
ssTnI-TnC were utilized in ATPase inhibitory assays, we
observed that Tn complexes containing ssTnI-TnC were not able
to inhibit actomyosin ATPase activity as well as cTnI-TnC at all
concentrations. The ability of ssTnI to significantly impair
ATPase inhibitory activity suggests that ssTnI possibly hin-
ders the release of myosin head binding to the actin thin
filament, and this would translate in vivo to incomplete
diastolic relaxation. Interestingly, impaired diastolic relaxation
was observed in transgenic mice overexpressing ssTnI. Again,
in the presence of ssTnI-TnC, both mutations were able to
inhibit as well as TnT1-WT.

No difference in maximal ATPase activation was observed
between TnT3-WT and TnT1-WT complexed with cTnI-TnC
(Fig. 4A). This result is consistent with what was observed by
Gomes et al. (2). However, both DCM mutations in the TnT1
isofrom caused a significant decrease in maximal ATPase ac-
tivity (Fig. 4A). The importance of the NH$_2$-terminal domain
in the activation of actomyosin ATPase has been shown by Malnic
et al. (38). Deletion of the NH$_2$-terminal domain in TnT (resi-
dues 1–156 of chicken skeletal TnT) resulted in a significant
reduction in the maximal ATPase activation (39). We also
investigated maximal ATPase activity to determine whether
ssTnI plays a major role in modulating the ATPase activity in
the presence of Ca$^{2+}$. The maximal ATPase activity of
TnT3-WT complexed with ssTnI-TnC was similar to the activity
obtained with cTnI-TnC complex. However, the maximal
ATPase activity significantly increased for TnT1-WT and DCM
mutants when complexed with ssTnI-TnC instead of cTnI-TnC.
However, compared with TnT1-WT, the maximal activity for
the TnT1 DCM mutants was significantly lower. We also meas-
ured the Ca$^{2+}$ sensitivity of force development and maximal
force recovered at acid pH conditions (pH 6.5) for the TnT DCM
mutations in the fetal isoform (TnT1) of TnT and fetal Tn
(TnT1-ssTnI). Acidosis frequently occurs during birth, during
which the intracellular pH varies from 6.2 to 7.4. When the pH
was changed from 7 to 6.5, TnT3-WT-treated fibers showed a
decrease in pCa$_{50}$ of 0.58 pCa unit. A rightward shift in pCa$_{50}$
was observed in TnT1-WT-treated fibers, but there was no
significant difference in the Ca$^{2+}$ sensitivity of force develop-
ment between TnT3-WT and TnT1-WT at pH 6.5 (Table II). At
pH 6.5, in the TnT1 isoform, both the mutations significantly
decreased the Ca$^{2+}$ sensitivity of force development similar to
TnT1-WT, suggesting that the mutations are not resisting
changes in pH. This result is expected because the R141W
mutation is several residues away from the cluster of positively
charged residues (residues 91–94 in TnT3) that are responsible
for the determination of pH-dependent Ca$^{2+}$ regulation of car-
diac muscle contraction (40).

It has been demonstrated by many laboratories that the
change in pCa$_{50}$ and the force depression caused by acidic pH in
neonatal heart are less than what is observed in the adult heart
(26, 41, 42). The resistance to acidic pH is of great physiological
significance because this could be one of the protection mech-
анизms for embryonic or neonatal heart against ischemia. We
investigated the pCa-force relationship under acidic conditions
(pH 6.5) in porcine fibers treated with TnT3-WT, TnT1-WT,
and TnT1 DCM mutants and reconstituted with ssTnI-TnC.
The shift in pCa$_{50}$ obtained with ssTnI-TnC reconstitution at
pH 6.5 was significantly smaller than what was observed after
cTnI-TnC reconstitution for all four proteins. This is due to the
effect of ssTnI, which can resist changes in pH better than
cTnI. It has been shown that the amino acid histidine at posi-
tion 131 is primarily responsible for the ability of ssTnI to
resist acidic pH (43). However, both the TnT1 DCM mutations
causa a statistically significant decrease in Ca$^{2+}$ sensitivity of
force development when compared with TnT1-WT-treated fib-
ers at pH 6.5 (Fig. 5B). Table 2 summarizes the maximal force
recovered at pH 6.5 for all groups of fibers reconstituted with
ssTnI-TnC. The depression in maximal force normally induced
by acid pH was significantly decreased upon ssTnI-TnC recon-
stitution in all fiber groups. A similar result was also observed
by Wolska et al. (44) in transgenic mice overexpressing ssTnI
in the heart. This suggests that the composition of the TnI isoform
plays a major role in the determination of myocardial force
development under acidic pH conditions. However, TnT1-
R141W-treated fibers showed no change in maximal force com-
pared with TnT1-WT-treated fibers reconstituted with
ssTnI-TnC, whereas the TnT1-ΔK210 treated fibers showed a
significant decrease in maximal force.

In summary, this study provides insight into the functional
significance of DCM mutations in the fetal cardiac TnT isoform
and fetal cardiac Tn. Our results demonstrate that both DCM
mutations R141W and ΔK210 decrease the Ca$^{2+}$ sensitivity of
force development in both the TnT1 isoform and fetal Tn. Both
mutations have in common a decrease in the Ca$^{2+}$ activated
ATPase activity, with the decrease being more pronounced
in ΔK210 than in R141W in adult TnT (10), fetal TnT, and
fetal Tn.

The in vitro results presented here suggest that both the
R141W and ΔK210 mutations would cause a severe DCM phe-
notype in the fetal and neonatal stage for the following rea-
sons: the ΔK210 mutation consistently decreased Ca$^{2+}$ sensitivity of
force development, maximal force recovered, and maximal
ATPase activity, irrespective of whether the mutation was
present in the adult TnT, fetal TnT, or fetal Tn. The R141W
mutation decreased Ca$^{2+}$ sensitivity of force development in
the fetal TnT and fetal Tn. In the adult isoform of TnT, the
R141W mutation caused no change in Ca$^{2+}$ sensitivity of force
development compared with TnT3-WT. The TnT DCM muta-
tions in the fetal Tn isoform showed significantly greater
decreases in the Ca$^{2+}$ sensitivity of force development (compared
with wild-type TnT1) relative to the decreases observed when these mutations were present in the adult TnT3 isoform. This greater decrease in change in Ca\textsuperscript{2+} sensitivity in combination with a decrease in maximal actomyosin ATPase activity in fetal TnT1 may contribute to a more severe phenotype in children with these mutations, consistent with clinical data (8). In addition to TnT and TnI, actin and tropomyosin and myosin heavy chain also have different isoforms during cardiac development. How they affect the contribution of TnT and TnI to Ca\textsuperscript{2+} sensitivity and ATPase activity remains to be established.

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Characterization of Troponin T Dilated Cardiomyopathy Mutations in the Fetal Troponin Isoform

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