Biochemical and Biophysical Research Communications **261,** 79–82 (1999)
Article ID bbrc.1999.1000, available online at http://www.idealibrary.com on IDEAL®

# Functional Consequences of a Carboxyl Terminal Missense Mutation Arg278Cys in Human Cardiac Troponin T

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Received June 10, 1999

**A carboxyl terminal missense mutant Arg278Cys of human cardiac troponin T that causes familial hypertrophic cardiomyopathy was expressed in *Escherichia coli,* purified, and exchanged into rabbit cardiac skinned muscle fibers using a troponin exchange technique. Compared to the fibers exchanged with human cardiac wild-type troponin T, the fibers exchanged with the mutant Arg278Cys developed less maximum force with a decreased cooperativity and a slightly increased $Ca^{2+}$ sensitivity, resulting in a significant elevation of sub-half-maximal force. Since intact cardiac muscle is thought to never be activated beyond the half-maximum level, the results suggest that an enhanced myofilament response to $Ca^{2+}$ may be responsible for the pathogenesis of hypertrophic cardiomyopathy associated with this mutation. The results also provide the first evidence that the carboxyl terminal region of cardiac troponin T plays an important role probably through its interaction with tropomyosin in allowing troponin complex to inhibit the muscle contraction at low $Ca^{2+}$, in agreement with the hypothesis deduced from the previous studies on fast skeletal troponin T.** 

Troponin is a $Ca^{2+}$ regulatory protein complex determining the contraction and relaxation states in vertebrate striated muscle. This protein complex consists of three different components; a $Ca^{2+}$-binding component, troponin C (TnC), an inhibitory component, troponin I (TnI), and a tropomyosin (TM)-binding component, troponin T (TnT). The contractile interaction of myosin and actin-TM is inhibited by TnI, and this inhibition by TnI is removed by the activating or neutralizing action of TnC. However, the neutralizing action of TnC is mostly insensitive to $Ca^{2+}$ in the absence of TnT, and all the three troponin components are required for the $Ca^{2+}$ regulation of contraction (1).

The present study was undertaken to examine the functional consequences of a missense mutation Arg278Cys in human cardiac TnT that had been shown to cause familial hypertrophic cardiomyopathy (HCM) (2). It has previously been shown that a carboxyl terminal small region of fast skeletal TnT has an important role in the TM-binding and thus in the $Ca^{2+}$ regulation of contraction (3–5). The HCM-causing Arg278Cys mutation occurs in a carboxyl terminal region of cardiac TnT that is highly homologous to this carboxyl terminal region of fast skeletal TnT. Investigation of the $Ca^{2+}$ activation of force development in the skinned rabbit cardiac muscle fibers exchanged with Arg278Cys mutant human cardiac TnT showed that this mutation decreased the maximum force and increased the submaximum force at low $Ca^{2+}$, which leads to a reduction in the overall cooperativity of $Ca^{2+}$ regulation. The results suggest that the carboxyl terminal region of TnT plays an important role in the $Ca^{2+}$ regulation of contraction in cardiac muscle as well as in fast skeletal muscle and that an impairment of the normal function of carboxyl terminal region is involved in the pathogenesis of HCM caused by the missense mutation Arg278Cys.

## MATERIALS AND METHODS

*Cloning and mutagenesis of human cardiac TnT cDNA.* Human cardiac TnT cDNA was amplified by RT-PCR of human heart mRNA purchased from Clontech (Palo Alto, CA). The PCR products were subcloned into the pUC119 vector for screening by restriction analysis and DNA sequencing. The obtained wild-type TnT cDNA was then constructed in pET3-d vector for expression and mutagenesis. Mutagenesis was carried out by PCR according to the method described by Horton (6); oligonucleotides employed for the mutation Arg278Cys were 5′-CTCCAAGACCTGCGGGAAGG-3′ (changed base was underlined). The complete nucleotide sequence of the mutant TnT cDNA were confirmed by DNA sequencing.

*Purification of proteins.* Expression and purification of the recombinant human cardiac TnTs were performed as described previously (7); the final yield was about 1.5 mg of pure protein/litter of



0006-291X/99 $30.00

**TABLE 1**

Maximum Force, $Ca^{2+}$ Sensitivity, and Cooperativity in Recombinant Human Cardiac TnT-Exchanged Fibers

| TnT | $pCa_{50}$ | $n_H$ | Maximum force[a] |
|---|---|---|---|
| Wild-type | 5.76 ± 0.02 | 3.14 ± 0.10 | 61.7 ± 1.2 (100) |
| Arg278Cys | 5.83 ± 0.01* | 2.69 ± 0.01* | 52.8 ± 1.8 (86)* |

*Note.* Values are means ± SE of measurements on three fibers.

[a] Maximum force was expressed as a percent of the maximum force developed in the same fiber before TnT exchange; values in parentheses express % of the maximum force developed in the wild-type TnT-exchanged fibers.

* $P < 0.05$ vs wild-type TnT-exchanged fibers (unpaired *t*-test).

culture. Rabbit native cardiac troponin and its components, TnI and TnC, were prepared from the left ventricular myocardiums of young male albino rabbits (~3 mo old) according to the method of Tsukui and Ebashi (8).

*Preparation of skinned fibers and force measurements.* Skinned fibers were prepared from the left ventricular trabeculae of young male albino rabbits (~3 mo old), and force measurements were performed as described previously (7). Relaxing solution consisted of (in mM) 50 MOPS/KOH (pH 7.0), 100 KCl, 6 $MgCl_2$, 5 ATP, 4 EGTA, 0.5 DTT, and 10 creatine phosphate, as well as 35 U/ml creatine kinase. Activating solutions with desired free $Ca^{2+}$ concentrations were prepared by adding appropriate amounts of $CaCl_2$, calculated as described previously (9), to the relaxing solution. All force measurements were done at 25°C.

To determine the pCa ($-\log[Ca^{2+}]$) value at half-maximum force generation ($pCa_{50}$) and the Hill coefficient ($n_H$), the force was normalized to the maximum force generated in the same fiber and the relationship between relative force and pCa in each fiber was fitted to the following form of Hill equation by means of the Marquardt nonlinear least-squares method (GraphPad Prism 2.01, GraphPad Software, Inc):

$$\text{relative force } (\%) = 100/\{1 + 10^{(pCa - pCa_{50}) \cdot n_H}\}.$$

Average values of $pCa_{50}$ and $n_H$ were then calculated and are summarized in Table 1.

*TnT exchange in skinned fibers.* TnT exchange in the skinned fibers was performed by the method described previously (7).

*SDS–PAGE.* SDS–PAGE was carried out at 12% acrylamide concentration according to the method of Laemmli (10). The fiber samples were lysed in Laemmli's sample buffer by heating for 4 min at 95°C after having been frozen (−80°C) and thawed several times. The gel was stained with silver using a staining kit (Pharmacia Biotech) or with Coomassie Brilliant Blue R-250. An optical densitometric scan was performed with Phoretix gel analysis software (Phoretix International Ltd) calibrated by a photographic step tablet (21 steps · density range 0.05–3.03, Eastman Kodak Company).

## RESULTS

Figure 1A shows SDS–PAGE of the bacterially expressed and purified human cardiac wild-type and Arg278Cys mutant TnTs as well as the purified rabbit cardiac TnT. The recombinant human cardiac TnTs had the same electrophoretic mobility as the major isoform of rabbit adult cardiac TnT, consistent with their molecular weights calculated from amino acid sequence. These recombinant human cardiac TnTs were attempted to be partially exchanged into rabbit skinned cardiac muscle fibers by using a previously developed troponin exchange technique (7, 11, 12). The skinned fibers were first treated with an excess amount of purified human cardiac wild-type or Arg278Cys mutant TnT under acidic and high-ionic-strength conditions. This procedure resulted in replacement of endogenous TnT · I · C complexes in the skinned fibers with exogenously added TnT, as evidenced by a significant decrease in the amount of endogenous TnI (Fig. 2B, lanes 2 and 3) and a significant increase in the force in the absence of $Ca^{2+}$ (data not shown) after treatment. Previous studies have demonstrated that the extent of TnT exchanged into the fibers is directly proportional to the decreased amount of endogenous TnI (7, 13). A

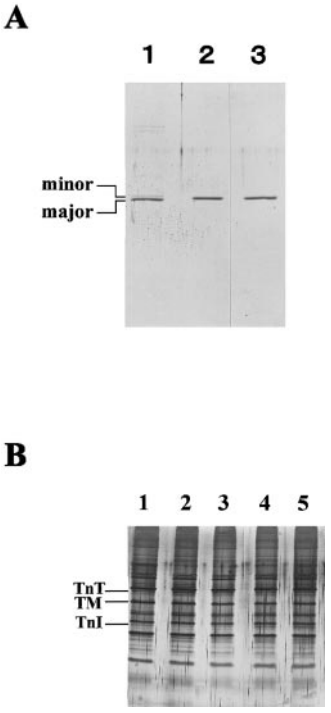


**FIG. 1.** (A) SDS–PAGE of purified recombinant wild-type and Arg278Cys mutant of human cardiac TnT. Lane 1, rabbit cardiac tissue-derived (native) TnT. Lane 2, human cardiac wild-type TnT. Lane 3, human cardiac Arg278Cys mutant TnT. The gel was stained with Coomassie Brilliant Blue R-250. (B) SDS–PAGE of skinned rabbit cardiac muscle fibers after treatment with recombinant human cardiac TnTs and after subsequent reconstitution with rabbit cardiac TnI and TnC. Lane 1, an untreated control fiber. Lane 2, a fiber treated with wild-type human cardiac TnT. Lane 3, a fiber treated with Arg278Cys mutant human cardiac TnT. Lanes 4 and 5, fibers reconstituted with rabbit cardiac TnI and TnC after treatment with human wild-type and Arg278Cys TnT, respectively. Individual skinned fibers were mounted to the mechanical apparatus and the TnT exchange was carried out under the same conditions as in the force measurements. The gel was stained with silver. Note that the amounts of proteins loaded on the gel are varied between lanes depending on the size of each skinned fiber.

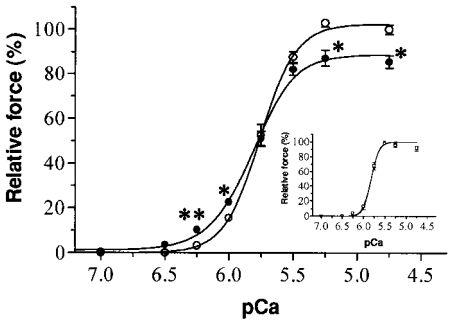


**FIG. 2.** Effect of exchanging Arg278Cys mutant TnT into skinned cardiac muscle fibers on force–pCa relationships. Force–pCa relationships determined after exchange of recombinant wild-type (○) and Arg278Cys mutant (●) of human cardiac TnT were compared; forces were normalized to the averaged maximum force in the fibers exchanged with wild-type TnT, and are expressed as means ± SE of measurements on 3 fibers. The inset shows force–pCa relationships determined in respective fibers before TnT exchange; forces were normalized to the maximum force developed by each fiber. *$P < 0.05$, **$P < 0.01$ vs wild-type TnT-exchanged fibers (unpaired *t*-test).

quantification of the TnI/TM ratio in each fiber by densitometric scans of the gel indicated the extent of TnT exchanged to be 50.5 and 53.7% by wild-type and Arg278Cys TnT treatment, respectively. The recombinant human cardiac TnT-treated fibers were next reconstituted with purified rabbit cardiac TnI and TnC, and re-incorporation of these troponin components into the fibers was directly demonstrated by SDS–PAGE analysis (Fig. 2B, lanes 4 and 5). A previous study demonstrated that the recombinant human cardiac wild-type TnT exhibits exactly the same function as the tissue-derived rabbit adult cardiac TnT when exchanged into rabbit skinned cardiac muscle fibers by these procedures (7).

Figure 2 compares the force-pCa relationship of the fibers exchanged with the missense mutant Arg278Cys with that of the fibers exchanged with wild-type. Table 1 summarized the $Ca^{2+}$ sensitivity ($pCa_{50}$) and cooperativity ($n_H$) in the force-pCa relationships, as well as the maximum force. The Arg278Cys mutant TnT-exchanged fibers developed a significantly lower level of maximum force and a significantly higher level of sub-half-maximum force than the wild-type TnT-exchanged fibers (Fig. 2 and Table 1). The Arg278Cys mutant TnT also conferred a slightly higher $Ca^{2+}$ sensitivity and a lower cooperativity than wild-type TnT, as demonstrated by a slight but significant increase in the $pCa_{50}$ and a significant decrease in the $n_H$ (Table 1). Before TnT exchange, there were no statistically significant differences in these parameters between these two fiber groups (inset to Fig. 2).

## DISCUSSION

In a previous biochemical study, the Arg278Cys mutation in human cardiac TnT was found to increase the $Ca^{2+}$ sensitivity of the ATPase activity in isolated cardiac myofibrils (14), consistent with the present study performed under physiological conditions using skinned cardiac muscle fibers. In the present study, this mutation was also found to decrease the maximum force and cooperativity in the $Ca^{2+}$-activated force generation of skinned cardiac muscle fibers; these effects was not observed on the myofibrillar ATPase activity in the previous study, probably due to the difference in the experimental conditions or the accuracy of measurement.

TnT has an important structural role in anchoring other components of the troponin complex, TnI and TnC, to TM in the thin filament (1). Previous studies on rabbit fast skeletal TnT molecule have shown that a highly $\alpha$-helical N-terminal region of 81 amino acid residues constitutes a primary, strong TM-binding site and a C-terminal region of the molecule constitutes a second, weak TM-binding site (4, 15). Chymotryptic digestion splits the fast skeletal TnT into two subfragments designated as $TnT_1$ and $TnT_2$ (or $TnT_2\alpha$); $TnT_1$ is the N-terminal three-fifths of TnT containing the primary TM-binding region, and $TnT_2$ is the remaining C-terminal two-fifths of TnT containing the second TM-binding region and retaining an essential part of the regulatory function of TnT (16, 17). Further digestion by chymotrypsin of $TnT_2\alpha$ into a smaller subfragment lacking C-terminal 17 residues designated as $TnT_2\beta$ greatly reduces the binding affinity for TM and results in a significant increase in the activities of ATPase and superprecipitation of an actomyosin preparation at low $Ca^{2+}$ and a slight decrease in these activities at high $Ca^{2+}$ (3–5), indicating that the C-terminal 17 residues of rabbit fast skeletal TnT plays an important role through its binding to TM in allowing the troponin complex to regulate the muscle contraction in a $Ca^{2+}$-dependent manner. The present study demonstrates that the C-terminal missense mutation Arg278Cys in human cardiac TnT has qualitatively the same effects on the force development of skinned fibers as those exerted on the ATPase or superprecipitation by removal of the C-terminal 17 residues from rabbit fast skeletal TnT. Since Arg-278 is in the C-terminal region of human cardiac TnT that is highly homologous to the C-terminal 17 residues of rabbit fast skeletal TnT, the present results strongly suggest that the residue Arg-278 plays a critical role in the $Ca^{2+}$ regulatory function performed by the C-terminal region of human cardiac TnT through the interaction with TM.

We have previously shown that the HCM-causing N-terminal missense mutations Ile79Asn and Arg92Gln in human cardiac TnT increase the $Ca^{2+}$ sensitivity in the force development of skinned fibers without affecting the maximum force and cooperativity (7). In the present study, the C-terminal missense mutation Arg278Cys was found to show quite different effects on

the force development of skinned fibers; a much smaller increase in the $Ca^{2+}$ sensitivity, and significant decreases in the maximum force and cooperativity. However, it should be noted that the mutation Arg278Cys, as well as the N-terminal missense mutations, causes a significant elevation of the force at sub-half-maximum $Ca^{2+}$ concentrations. Because the intact cardiac muscle is considered to never be activated beyond the half-maximum level (18), an enhancement of the myofilament response to $Ca^{2+}$ and thus an enhanced cardiac performance might be a common phenomenon involving the pathogenesis of HCM associated with these N- and C-terminal missense mutations in human cardiac TnT.

## ACKNOWLEDGMENTS

This study was supported in part by grants-in-aid (07458222, 0868089, 08770071, 109670771, 10557073, and 11670045) from the Ministry of Education, Science, Sports and Culture of Japan; the Ministry of Health and Welfare of Japan; and the Uehara Memorial Foundation.

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