A Mutant Tropomyosin That Causes Hypertrophic Cardiomyopathy Is Expressed In Vivo and Associated With an Increased Calcium Sensitivity


Abstract—Mutant contractile protein genes that cause familial hypertrophic cardiomyopathy (FHC) are presumed to encode mutant proteins that interfere with contractile function. However, it has generally not been possible to show mutant protein expression and incorporation into the sarcomere in vivo. This study aimed to assess whether a mutant α-fast tropomyosin (TM) responsible for FHC is actually expressed and determines abnormal contractile function. Since α-fast TM is expressed in heart and skeletal muscle, samples from vastus lateralis muscles were studied from two FHC patients carrying an Asp\textsuperscript{175}Asn α-fast TM mutation and two healthy control subjects. TM isoforms from whole biopsy samples and single fibers were identified by gel electrophoresis and Western blot analysis. An additional faster-migrating TM band was observed in both FHC patients. The aberrant TM was identified as the Asp\textsuperscript{175}Asn α-fast TM by comigration with purified recombinant human Asp\textsuperscript{175}Asn α-fast TM. Densitometric quantification of mutant and wild-type α-fast TMs suggested equal expression of the two proteins. Contractile parameters of single skinned muscle fibers from FHC patients and healthy control subjects were compared. Calcium sensitivity was significantly increased in muscle fibers containing Asp\textsuperscript{175}Asn α-fast TM compared with fibers lacking the mutant TM. No discernible difference was found regarding cooperativity, maximum force, and maximum shortening velocity. This is the first demonstration that the mutant TM that causes FHC is indeed expressed and almost certainly incorporated into muscle in vivo and does result in altered contractile function; this confirms a dominant-negative, rather than null allele, action. Since the mutant TM was associated with increased calcium sensitivity, this mutation might be associated with an enhancement and not a depression of cardiac contractile performance. If so, this contrasts with the hypothesis that FHC mutations induce contractile impairment followed by compensatory hypertrophy. (Circ Res. 1998;82:106-115.)

Key Words: familial hypertrophic cardiomyopathy  ■ tropomyosin  ■ Ca\textsuperscript{2+} sensitivity  ■ skinned muscle fiber

Familial hypertrophic cardiomyopathy is an autosomal dominant inherited heart disorder characterized by cardiac hypertrophy in the absence of increased workload. FHC has been linked to point mutations in certain cardiac contractile proteins: more frequently to \( \beta \)-MHC and cardiac MyBP-C mutations,\textsuperscript{1,2} less frequently to troponin T and α-fast TM,\textsuperscript{3,4} and very rarely to cardiac MLC mutations.\textsuperscript{5} It has been suggested that cardiac hypertrophy is actually caused by such mutations through a dominant-negative effect; ie, all mutant proteins would in some way interfere with the function of WT proteins impairing cardiac performance. Such impairment would give rise to compensatory cardiac hypertrophy.\textsuperscript{3}

The above hypothesis has been mainly derived from studies of \( \beta \)-MHC mutations. For one such \( \beta \)-MHC mutation (Arg\textsuperscript{403}Gln), it has been shown that in FHC patients the mutant gene is transcribed in the heart and in skeletal muscle\textsuperscript{6,7} and that the mutant protein is expressed in skeletal muscle.\textsuperscript{8} Functional studies\textsuperscript{8,9} have shown that several mutant MHCs have abnormal function; ie, these studies have determined a slowing of actin-myosin kinetics and decrease in force. Studies of mouse models of FHC have supported such findings.\textsuperscript{10}

Mutations in troponin T, α-fast TM, and MyBP-C have been less studied, and their pathogenetic role is much less understood. This is mostly due to the fact that, apart from the Arg\textsuperscript{403}Gln \( \beta \)-MHC mutation, the identification of the mutant form of the protein in skeletal and/or cardiac samples of affected individuals has not been possible. A possible role for troponin T mutations in the pathogenesis of FHC has been suggested by the demonstration of abnormal function in two mutations expressed in vitro.\textsuperscript{11,12} One such mutation (equivalent to Ile\textsuperscript{79}Aln in human troponin T) has been shown to result in an increase, not a decrease, in the velocity of actin-troponin-TM filaments in an in vitro motility assay; thus, it has been suggested that not all mutations necessarily act through a
common mechanism of functional impairment and compensatory hypertrophy.\(^1\)\(^1\) Certain mutations might determine a “hypercontractile” state, which would directly induce cardiac hypertrophy.\(^1\)\(^1\) Interestingly, the very rare mutations in cardiac MLCs have also been associated with an increase in the velocity of actin-myosin interaction.\(^5\)

Very little is known of the mechanism underlying the rare cases of FHC linked to \(\alpha\)-fast TM mutations. Five kindreds carrying one of the three known TM mutations (Asp\(^{175}\)Asn, Glu\(^{176}\)Gly, and Ala\(^{178}\)Val) have been identified, and their genetic, clinical, and histopathological features have been studied.\(^1\)\(^3\)\(^\)\(^\)\(^4\)\(^\)\(^5\)\(^6\) Since three of the five kindreds carried the same Asp\(^{175}\)Asn mutation, nucleotide residue 579 in the \(\alpha\)-TM gene was considered a “hot spot” for mutation.\(^8\) No information is available on whether mutant TM is actually expressed in human skeletal and cardiac muscle.

The present study aimed to assess whether the mutant TM is actually expressed in FHC patients and has abnormal function. Two subjects belonging to two different kindreds carrying the same Asp\(^{175}\)Asn TM mutation\(^1\)\(^5\) and two healthy control subjects were enrolled in the study. Because of the ethical reasons that prevent cardiac biopsy and the favorable prognosis of this form of FHC, analysis was performed on needle-biopsy samples of the vastus lateralis muscle.\(^1\)\(^0\)\(^7\)\(^8\) Needle biopsy samples (~100 mg), taken from the vastus lateralis muscle,\(^2\)\(^0\) were divided in two portions of similar size: one was put in sample buffer\(^2\)\(^1\) and stored at \(-20^\circ\)C for subsequent TM isoform identification, and the other was further divided in small bundles of ~50 fibers. From such bundles, which were stored at \(-20^\circ\)C in skinning solution with 50% glycerol added for up to 4 weeks before experiment, single fiber segments 2 to 4 mm long were dissected. To ensure complete removal of the plasma membrane, fibers were bathed in skinning solution with Triton X-100 1% (Sigma Chemical Co) for 1 hour. Light aluminum clips were used to attach the fiber segments to the beams of the force transducer (AE 801 SensoNor) and of the isotonic lever (model 101 vibrator, Ling Dynamic System) in the experimental setup.

**Experimental Setup for Single Muscle Fiber Analysis**

The experimental setup used was very similar to that previously described in detail;\(^2\)\(^0\)\(^7\) however, there were seven instead of three muscle chambers. The setup allowed quick transfer of the muscle fibers from the first (larger) chamber to the other six (smaller) chambers (70 \(\mu\)L) containing different solutions (relaxing, preactivating, and activating solutions at maximal or submaximal calcium concentrations). The electromagnetic pulser could either keep the length of the fiber segment constant to elicit isometric contractions or impose on the specimen quick releases of preset amplitude completed in 2 milliseconds. A stereomicroscope was fitted over the apparatus to view the fiber at \(\times\)20 to \(\times\)60 magnification during the mounting procedure and during the experiment. The setup was placed on the stage of an inverted microscope (Axiovert 20, Zeiss). Since the floors of the muscle chambers were made with coverslips, specimens could be viewed at \(\times\)320 magnification through the eyepieces of the microscope. A video camera (MICAM HRS, System Sud), fitted to the camera tube of the microscope and connected through an analog-to-digital converter (Cyclope, System Sud) to a computer ( Olivetti M24), allowed viewing on a TV screen at \(\times\)1000 magnification and storage of digitized images of the specimen during the experiment. The signals from the force and displacement transducer were visualized on the screen of a storage oscilloscope (model 5113, Tektronix) and on a chart recorder (WR3701, Graphtec). The signals after analog-to-digital conversion (Interface CED 1401 Plus) were fed into a personal computer and stored on the hard disk. For data storage, recall and analysis were performed using the software Spike 2 (CED) implemented on the computer (DEX 486,66).

**Materials and Methods**

**Subjects**

Two subjects, one male (aged 55 years) from an Italian family and one female (aged 40 years) from an American family, carrying the Asp\(^{175}\)Asn \(\alpha\)-fast TM mutation and two healthy male control subjects (aged 40 and 55 years) were enrolled in the study. The clinical, genetic, and histopathological features of the two FHC patients have been previously described in detail (Coviello et al\(^1\)\(^9\) in 1997). Both patients had virtually normal functional capacity, exercise levels, and peripheral circulation; ie, skeletal muscle changes from disuse were not expected. The study was approved by the ethics committee of the Department of Medicine of the University of Genoa (Italy), and informed consent was obtained.

**Needle Biopsy and Fiber Dissection**

Needle biopsy samples (~100 mg), taken from the vastus lateralis muscle,\(^2\)\(^0\) were divided in two portions of similar size: one was put in sample buffer\(^2\)\(^1\) and stored at \(-20^\circ\)C for subsequent TM isoform identification, and the other was further divided in small bundles of ~50 fibers. From such bundles, which were stored at \(-20^\circ\)C in skinning solution with 50% glycerol added for up to 4 weeks before experiment, single fiber segments 2 to 4 mm long were dissected. To ensure complete removal of the plasma membrane, fibers were bathed in skinning solution with Triton X-100 1% (Sigma Chemical Co) for 1 hour. Light aluminum clips were used to attach the fiber segments to the beams of the force transducer (AE 801 SensoNor) and of the isotonic lever (model 101 vibrator, Ling Dynamic System) in the experimental setup.

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**Solutions**

Skinning, relaxing, preactivating (pCa 9.0), and maximally activating (pCa 4.45) solutions were prepared according to Bottinelli et al.\(^2\)\(^0\) Activating solutions of intermediate calcium concentrations were obtained by mixing appropriate quantities of two solutions having the same ionic composition as the maximally activating solution but differing in calcium concentration: one at pCa 4.45 solutions were prepared according to Bottinelli et al.\(^2\)\(^0\) Activating solutions of intermediate calcium concentrations were obtained by mixing appropriate quantities of two solutions having the same ionic composition as the maximally activating solution but differing in calcium concentration: one at pCa 9.0 and one at pCa 5.05. Free ionic concentration and ionic strength of all solutions were calculated using a computer program designed by Fabiato.\(^2\)\(^2\)
Experimental Plan

Force-pCa relationships, Po, and Vo of a large population of fibers from FHC patients (FHC fibers, n = 150) and from control subjects (control fibers, n = 110) were determined. After the experiment, all single fibers were characterized on the basis of MHC isoform composition by SDS-PAGE and divided in five types: types 1, 2A, and 2B (pure types) and types 1–2A and 2A–2B (mixed types). Calcium sensitivity (pCa50%), Hill’s slope coefficient, Po, and Vo of corresponding fiber types from FHC and control subjects were compared. A subset of type 2A and type 2A-2B FHC fibers and of type 2A control fibers whose force-pCa relationship had been determined were studied for TM isoform composition. After TM isoform identification, a more selective comparison between FHC fibers actually containing the Asp175Asn fast TM and control fibers was performed for all functional parameters studied. Finally, the relative amounts of the Asp175Asn fast TM and of the WT fast TM were determined by densitometric scanning using SDS-PAGE and Western blots of whole biopsy samples and of single fibers.

Experimental Procedure

The following procedures were used in all mechanical experiments: temperature was set at 12°C; the fiber segment was mounted in a chamber containing relaxing solution; sarcomere length and fiber diameter were measured at three different locations along the length of the specimen at X320 magnification; sarcomere length, which was determined by counting striations in segments of known length, was set at 2.5 μm; and fiber length was measured using a stereomicroscope fitted over the apparatus at X40 magnification. Cross-sectional area of the specimen was determined assuming a circular shape from the mean of the three diameters measured at X320 magnification. Normalization to tension developed at the initial length, was applied to carefully and reliably measure shortening, followed after 7 milliseconds by a fast reelongation to relative tension (K values. The resulting sigmoid curve was fitted by Hill’s equation: log K, corresponding to pCa50%, and n, were determined. The resulting sigmoid curve was fitted by Hill’s equation: relative tension = [K(X/C)]/[1 + K(X/C)], where C = [Ca2+]. The parameters pK = -log K, corresponding to pCa50%, and n, corresponding to the slope (Hill’s slope) of the curve, were determined. pCa50% and Hill’s slope give an indication of sensitivity to calcium and of cooperativity between calcium binding sites, respectively. An example of an experimental force-pCa curve is given in the inset of Fig 5.

Fiber Typing and Myosin Isoform Identification

Since the three main human fiber types (types 1, 2A, and 2B) are known to differ in contractile properties (ie, Vo, Po, and calcium sensitivity), a comparison of functional properties of isolated fibers is meaningful only between fibers of the same type.

At the end of the mechanical experiment, each fiber segment was immersed in a small test tube containing 20 μL of sample buffer and stored at -20°C. The composition in MHC isoforms, which is the most precise marker of fiber type, of each segment was determined later by PAGE (acylamide concentration, 6%) after denaturation in SDS (SDS-PAGE). The method was derived from that of Danieli-Betto et al and was described in detail by Steinen et al. In the MHC region, three bands corresponding to three MHC isoforms (MHC-1, MHC-2A, and MHC-2B) could be separated. In relation to the presence of one or two bands in the MHC region, fibers were classified into one of the following five types: 1, 2A, and 2B (or pure fibers) and 1–2A and 2A–2B (or mixed fibers). Only 20 μL of sample buffer containing the fiber (total protein content, <4 μg) was used for MHC identification.

Overexpression and Purification of Recombinant Human α-TM

A PCR product containing the human α-TM cDNA sequence29 was generated using primers that incorporate unique restriction sites: HTMF (5’-ACTCGAGAAGATATGCTAGACGACGCA-3’) and HTR (5’-GGAGGACGCTCTTTATATGGAAGTCATATCGTT-3’). The amplified product was digested using the enzymes NdeI and HindIII and subcloned into the Escherichia coli expression plasmid, pMW172,29 cut with the same enzymes. The HTMF primer also encodes a Met-Ala-Ser N-terminal extension, such that the final construct encodes human α-TM as a fusion protein. This two-residue extension functionally mimics the N-terminal acetylation that occurs in vivo; the expressed WT α-TM is indistinguishable on SDS-PAGE from α-TM extracted from tissue. The expression plasmid encoding the Asp175Asn mutant was assembled by PCR amplification of a 382-bp fragment containing the mutation using the primers HTMF and D17SN (5’-TCTCTAGAGCTCACGCGCGTCTCCATGTTCAAGTCATATCGTT-3’) (mutated base is underlined). This fragment was then digested using NdeI and SacI and ligated into a WT construct cut with the same enzymes. All PCR-generated fragments were sequenced in full. Both constructs were used to transform the E. coli strain, BL21(DE3)LyS. Cultures were grown, induced for 3 hours, and harvested, and the bacteria were lysed according to standard protocols.21 Both WT and mutant TM were expressed at a high level and were purified by a modification of the method described by Monteiro et al.31

TM Isoform Identification

TM isoform identification was performed by SDS-PAGE on 15% acrylamide concentration gels according to a procedure described by Giulian et al (Fig 1A) and subsequent Western blot analysis (Fig 1B). Identification of TM bands on gels was confirmed by coelectrophoresis and subsequent Western blot analysis (Fig 1D) of biopsy samples (Fig 1D; lanes 1, 4, and 5) with the two anti-TMs (Fig 1D) was achieved by coelectrophoresis and subsequent Western blot analysis (Fig 1D) of biopsy samples (Fig 1D; lanes 1, 4, and 5) with the two purified recombinant human α-fast TMs (WT α-fast TM [lane 6] and Asp175Asn α-fast TM [lanes 2 and 3]).

TM isoform identification was performed on biopsy samples from the two FHC patients and the two control subjects. Attempts were also made to characterize TM isoform composition and the presence of the mutant Asp175Asn α-fast TM in all FHC fibers (types 1, 2A, and 2A–2B) and in all type 2A control fibers whose force-pCa relationships had been studied. TM isoform analysis was successfully achieved only for 48 (21 type 2A FHC fibers, 12 type 2A-2B FHC fibers, and 15 type 2A control fibers) of the 97 fast fibers analyzed. Because segments of fibers used for experiments were kept short (2 mm) to optimize mechanical behavior and because a fraction of this segment was used for MHC identification, the low recovery of single fibers with TM...
isoform identification was likely due to a combination of technical difficulties and the small amount of proteins available. Determination of TM isoform distribution in type I fibers used for the experiment was less successful, possibly because of the smaller size and amount of protein in such fibers, and was abandoned. However, TM isoform composition was successfully determined in longer (5-mm) segments of 43 slow fibers, which were dissected for this purpose and not used for mechanical experiments.

To quantify the relative amount of Asp$^{175}$Asn and WT $\alpha$-fast TM, densitometric scanning of SDS-PAGE TM gels (Fig 1E, upper trace) and Western blots of whole biopsy samples (Fig 1E, lower trace) and single fibers (Fig 1F, upper and lower traces) were also done. Densitometry and quantification of the area under densitometric peaks were performed by a computerized densitometer. Images of the gels were fed to a personal computer (Laris P-100 Computer Discount) through a digital video camera (MICAM, HRS, System-Sud) and a frame grabber (Matrox Graphics Inc), and densitometry was performed by an especially designed software (Eidosoft). Quantification of the relative amount of Asp$^{175}$Asn and WT $\alpha$-fast TM, performed on SDS-PAGE (Fig 1E, upper trace) and on Western blots bands (Fig 1E, lower trace) of the same biopsy samples, gave very similar results. The latter finding suggested that under the conditions used, such quantification could be reliably performed on Western blots, presumably because Asp$^{175}$Asn $\alpha$-fast TM and WT $\alpha$-fast TM, which differ by only one amino acid, very likely have the same affinity for the antibody. This is important because quantification of the relative content in Asp$^{175}$Asn and $\alpha$-fast TM of single fibers was possible only on Western blots (Fig 1F).

Statistical Analysis

Data were expressed as mean±SE. Statistical significance of the differences between means was assessed by ANOVA followed by the Student-Newman-Keuls test. A probability of <5% (P<.05) was considered to be statistically significant. Statistical analysis, linear regression analysis for slack-test determination, and nonlinear regression for force-pCa curve fitting were performed using a computer program (Prism, GraphPad software). Slopes were considered significantly different from zero at P<.05.

Results

TM Isoform Composition of Whole Biopsy Samples and Single Fibers

Fig 1 shows representative SDS-PAGE runs (Fig 1A) and Western blot analysis (Fig 1B) of whole biopsy samples of FHC patients and control subjects. In Fig 1A, whole biopsy samples of a control subject (lane 1) and of a patient (lane 3) are comigrated in an SDS-PAGE gel. Purified TM is also comigrated with $\alpha$-fast TM and that was stained by the antibody against TM in Western blot. In Fig 1B, Western blot analysis of whole biopsy samples of the two patients (lanes 1 and 4) and of the two control subjects (lanes 2 and 6) are shown. It can be seen that (1) in control subjects, the three main TM isoforms ($\beta$, $\alpha$-slow, and $\alpha$-fast) could be separated, and no other band was stained by the antibody against TM in Western blot, and (2) in both patients, besides the three known TM isoforms, a fourth electrophoretic band that migrated faster than $\alpha$-fast TM and that was stained by anti-TM antibody in Western blot analysis was identified.

With the TM bands in whole biopsy samples used as standards, TM isoform composition was successfully analyzed...
in a subset of fibers from FHC patients (FHC fibers) and control subjects (control fibers) whose force-pCa relations were determined. Fig 1C shows Western blot analysis of two single FHC fibers (lanes 2 and 3) and of a whole biopsy sample from a patient (lane 1); not only in the biopsy sample but also in both single fibers the aberrant TM band can be seen. The aberrant TM isoform is referred to as Asp\textsuperscript{175}Asn α-fast TM because it has been shown to correspond to the mutant TM (see below), and the WT α-fast TM is referred to as WT α-fast TM. The Table shows the number of 2A and 2A-2B FHC fibers and control fibers in which TM identification was successfully achieved and the number of fibers that contained each of the four TMs (Asp\textsuperscript{175}Asn α-fast, WT α-fast, α-slow, and β). It can be seen that the newly identified extra TM band was present in the large majority of single type 2A and type 2A-2B fibers from FHC subjects and in none of the control fibers. α-Fast TM was expressed virtually in all type 2A and 2A-2B FHC fibers and in all 2A control fibers. Type 2A FHC and control fibers did not have the same α-slow and β-TM isoform composition. FHC fibers containing the mutant TM did not contain α-slow TM and mostly contained β-TM. Control fibers contained α-slow TM more frequently (9 of 15 fibers) and β-TM less frequently (8 of 15 fibers) than did FHC fibers. This point is relevant for functional comparisons and will be considered below. Type 2A-2B FHC fibers contained mostly Asp\textsuperscript{175}Asn α-fast and WT α-fast TM and β-TM and did not contain α-slow TM.

Because attempts to determine TM isoform composition in slow fibers whose force-pCa relation was studied were mostly unsuccessful (see “Materials and Methods”), TM isoform composition was determined in 43 fibers dissected for this purpose and not used for mechanical experiments (Table). Interestingly, on average, 30% (13 of 43) of the slow fibers contained the aberrant TM isoform; such fibers always contained the α-fast TM and β-TM and never the α-slow TM. On the other hand, the slow fibers that did not contain the aberrant TM contained α-slow TM and β-TMs but not α-fast TM.

Identification of the Mutant Asp\textsuperscript{175}Asn α-Fast TM

To assess whether the extra TM expressed in whole biopsy samples and in most fast single fibers of the FHC patients was the Asp\textsuperscript{175}Asn α-fast TM, two purified recombinant human α-fast TMs, one WT and one carrying the Asp\textsuperscript{175}Asn mutation, were prepared (see “Materials and Methods”). Both purified proteins were run in SDS-PAGE with the biopsy samples. Fig 1D shows that the extra TM band of the FHC samples (fastest migrating band in lanes 1 and 4 where biopsy samples of the two patients were loaded) comigrated with the purified recombinant Asp\textsuperscript{175}Asn TM (lanes 2 and 3), whereas the WT α-fast purified TM (lane 6) comigrated with the native α-fast TM (fastest migrating band in lane 5 where a control biopsy sample was loaded). Confirmation that the aberrant migration of the recombinant Asp\textsuperscript{175}Asn TM protein reflects the impact of only the single residue substitution was provided by identical observations on SDS-PAGE electrophoresis of recombinant WT and Asp\textsuperscript{175}Asn mutant chicken α-TM (data not shown).

Quantification of the Relative Amount of WT and Mutant TM

To determine the relative amount of the Asp\textsuperscript{175}Asn and WT α-fast TM in whole biopsy samples, densitometric scanning was performed on three different SDS-PAGE gels of each of the two patients. An example of a densitometric trace of a gel of a whole biopitic sample from an FHC patient is shown in Fig 1E (upper trace). Approximately equal amounts of Asp\textsuperscript{175}Asn (51.5 ± 0.5\% [mean ± SE]) and WT (48.5 ± 0.50\% [mean ± SE]) α-fast TMs were found in both patients. On the basis of considerations reported in “Materials and Methods,” quantification was also performed on Western blots (Fig 1E, lower trace), and very similar results were obtained (49.35 ± 2.65\% for Asp\textsuperscript{175}Asn α-fast TM versus 50.15 ± 3.15\% for WT α-fast TM) (note that upper and lower traces of Fig 1E refer to same sample). This supports the quantification of the relative amount of Asp\textsuperscript{175}Asn and WT α-fast TMs in single fibers expressing the mutant TM on Western blots, which also showed approximately equal amounts of Asp\textsuperscript{175}Asn (53.31 ± 1.29\%) and WT (46.79 ± 1.29\%) α-fast TMs. Examples of densitometric scanning of Western blots of single fibers are shown in Fig 1F (upper and lower traces).

Functional Properties of Single Skeletal Fibers

Calcium sensitivity (pCa50\%) was found to be significantly higher in type 1 and type 2A fibers from FHC patients (FHC fibers) than in corresponding fiber types from control subjects.
(control fibers) (Fig 2A and 2B). Since few pure 2B fibers were found in control subjects (n = 1), comparison was performed on mixed fibers containing both 2A and 2B MHC isoforms. Calcium sensitivity was again higher in FHC than control 2A-2B fibers, but this difference did not reach statistical significance (Fig 2C).

Differences in Hill’s slope between type 1, 2A, and 2A-2B fibers of FHC and control subjects were not statistically significant (Fig 3D through 3F).

Fig 3 shows the mean values of Po (Fig 3A through 3C) and Vo (Fig 3D through 3F) of muscle fibers from FHC patients and CTRL subjects. None of the differences between FHC and CTRL subjects were statistically significant. The number of fibers studied is reported above each bar.

Functional Properties of Single Fibers Related to Mutant TM Expression

The study of TM isoform composition of single fibers (see above) allowed division of type 2A and 2A-2B fibers from FHC patients into two subgroups according to the presence or absence of the Asp175Asn TM band and a more selective comparison between only those FHC fibers containing the mutant TM and control fibers. Both type 2A (Fig 4A) and type 2A-2B (Fig 4B) fibers containing the mutant TM had significantly higher calcium sensitivity than did the corresponding fiber types from control fibers (Fig 4A and 4B). Differences in pCa50% between FHC and control fibers were larger (0.09 pCa for type 2A and 0.10 pCa for type 2A-2B) when only FHC fibers expressing the aberrant fast migrating TM were considered (Fig 4A and 4B) than when all fibers from FHC patients were pooled together regardless of their TM composition (0.06 pCa for type 2A and 0.09 pCa for type 2A-2B, Fig 2B and 2C). Interestingly, pCa50% of the FHC type 2A fibers that did not express the Asp175Asn α-fast TM (Fig 4A) was not distinguishable from pCa50% of control fibers and significantly lower than pCa50% of FHC type 2A fibers that expressed the mutant TM (Fig 4A). The shift in the force-pCa curve that corresponds to the reported changes in pCa50% can be appreciated in Fig 5, where mean force-pCa curves of type 2A fibers expressing Asp175Asn α-fast TM and type 2A control fibers are shown.

Type 2A FHC fibers that contained the Asp175Asn α-fast TM and type 2A control fibers did not have identical α-fast,
Increased Calcium Sensitivity in FHC

α-slow, and β-TM content. To verify that the increased calcium sensitivity of FHC fibers containing the mutant α-fast TM was actually due to Asp175Asn mutation and not to coexpression of specific TM isoforms, two approaches were used. First, control fibers (all type 2A fibers) were grouped on the basis of the presence of β-TM, α-slow TM, and α-fast TM, and their calcium sensitivities were compared. No statistically significant differences were found among fibers containing β-TM (pCa50% = 5.68 ± 0.02, n = 8), α-slow TM (pCa50% = 5.69 ± 0.02, n = 9), and α-fast TM (pCa50% = 5.72 ± 0.02, n = 14) and between each group and the mean calcium sensitivity of all control fibers (pCa50% = 5.71 ± 0.02, n = 28) pooled together, whereas all groups had statistically lower pCa50% than did FHC 2A fibers containing the Asp175Asn α-fast TM (pCa50% = 5.80 ± 0.02, n = 15). Second, because FHC fibers containing Asp175Asn α-fast TM always contained WT α-fast TM, almost always the β-TM, and never the α-slow TM, the following, more selective, comparisons were also done: (1) the whole group of FHC fibers containing the Asp175Asn α-fast TM was compared only with control fibers containing the α-fast TM (pCa50% = 5.72 ± 0.02, n = 14) and with a subset of such fibers expressing the α-fast but not the α-slow TM (pCa50% = 5.70 ± 0.03, n = 6); (2) FHC fibers containing Asp175Asn α-fast TM, WT α-fast TM, and β-TM (pCa50% = 5.80 ± 0.04, n = 6) were compared only with control fibers containing both α-fast TM and β-TM (pCa50% = 5.70 ± 0.03, n = 6). In all cases, FHC fibers expressing the Asp175Asn α-fast TM had significantly higher calcium sensitivity than did the corresponding control groups.

No significant differences were observed between fibers containing the extra TM band and fibers that did not contain such a band regarding Hill’s slope, Po, and Vo (data not shown).

Discussion

We have taken advantage of the expression of α-fast TM in skeletal, as well as heart, muscle to determine whether the mutant Asp175Asn α-fast TM that causes FHC is expressed and incorporated and whether it alters contractile function. Abluent electrophoretic migration of both mutant TMs extracted from patient samples and purified recombinant TM allowed direct identification and quantification of the mutant peptide in a way that has not previously been possible in FHC mutant proteins. The primary results of the present study are as follows: (1) the mutant TM is expressed in skeletal muscle of FHC patients carrying a Asp175Asn α-fast TM mutation; (2) Asp175Asn and WT TM are expressed and almost certainly incorporated in approximately equal amounts; (3) Asp175Asn α-fast TM is associated with an increased calcium sensitivity, whereas cooperativity between calcium binding sites, Po, and Vo were not detectably modified.

Asp175Asn TM Expression

Although it is generally thought that in FHC, mutant contractile proteins are expressed and incorporated in the sarcomere, this is still a hypothesis based mainly on in vitro experiments. Resolution of mutant from WT protein has typically not been possible because of the subtle nature of most FHC mutations and the limited access to affected cardiac tissue. The incorporation of mutant protein in vivo has been demonstrated for only a single β-MHC mutation (Arg403Gln), since the mutant protein could be distinguished in extracts from skeletal muscle through loss of an endoprotease Arg-C digest site. In the present study, besides the three main TM isoforms, a fourth TM band was found in skeletal muscle of patients carrying an Asp175Asn TM mutation. This aberrant TM could be identified as the Asp175Asn α-fast TM, since it comigrated with a purified recombinant human Asp175Asn α-fast TM. The SDS-PAGE separation of the mutant TM from the WT TM is not easily explained purely on the change in charge that follows the substitution of Asp with Asn. Presumably, the substitution in
some way alters the conformation of the protein under the denaturing conditions of SDS-PAGE. Of note, a similar effect has been seen with the Glu180Gly TM mutation that also causes FHC (Drs Redwood and Watkins, unpublished data, 1997).

This is the first demonstration that mutant contractile proteins other than myosin are in fact expressed in FHC in vivo. In addition, given the direct way in which the mutant protein can be distinguished, these observations allow the first accurate quantification of the ratio of mutant and WT protein. The observation that Asp175Asn and WT $\alpha$-fast TM were expressed in virtually identical amounts suggests that in vivo Asp175Asn $\alpha$-fast TM expression is not downregulated.

Formal demonstration of incorporation of Asp175Asn $\alpha$-fast TM in the sarcomere is not possible with immunohistological techniques in the absence of an antibody that distinguishes mutant from WT TM. However, our observations indicate that the Asp175Asn $\alpha$-fast TM is almost certainly functionally incorporated in the thin filament. In skinned fibers, the plasma membrane has been removed, and all proteins that are soluble and/or not firmly bound to cytoplasmic structures leave the cell. In the FHC biopsies, the ratio of mutant-to-WT TM was the same in skinned fibers as in whole biopsy samples, indicating that all of the mutant TM is firmly bound. The observation of a functional effect corresponding to the presence of the mutant TM makes it extremely unlikely that mutant TM is unincorporated (and just attached to some contractile or cytoskeletal protein) but indicates that the protein is incorporated and functional.

The observation that some fast fibers that expressed WT did not express Asp175Asn $\alpha$-fast TM is difficult to interpret given that overall expression was so close to 50% (and no purely mutant fibers were observed to make up the balance). Although this might suggest that a minority of fibers Asp175Asn $\alpha$-fast TM is not expressed and/or incorporated in the sarcomere, the apparent lack of mutant TM may simply reflect some technical limitations in single-fiber analysis.

**Asp175Asn $\alpha$-Fast TM Function**

Type 1, type 2A, and type 2A-2B fibers from FHC patients had higher calcium sensitivity than did corresponding fiber types from control subjects. Differences were small, $\approx 0.05$ U of pCa, but statistically significant for type 1 and type 2A fibers. Interestingly, differences almost doubled ($\approx 0.1$ U of pCa) and became more statistically significant when only FHC fibers containing the mutant TM were compared with control fibers. It is also of note that the few type 2A FHC fibers not expressing the mutant $\alpha$-fast TM were indistinguishable from control fibers and had significantly lower calcium sensitivity than did the type 2A FHC fibers expressing the mutant TM. These observations strongly suggest that the expression of Asp175Asn $\alpha$-fast TM causes the increased calcium sensitivity. This conclusion is in agreement with a preliminary report that suggested an increased calcium sensitivity of myofilaments from transgenic mice overexpressing Asp175Asn $\alpha$-fast TM compared with myofilaments from control mice expressing WT $\alpha$-fast TM.\(^{36}\)

Our data suggest that it is very unlikely that the increased calcium sensitivity in the presence of Asp175Asn $\alpha$-fast TM reflects secondary changes in expression of other contractile protein isoforms and is not a functional property of the mutant TM itself. Within fibers with homogeneous MHC isoform composition, calcium sensitivity is thought to be modulated by the expression of at least three main TM isoforms and several troponin isoforms.\(^{17,18}\) Although troponin isoforms were not studied, major changes in troponin expression induced by mutant TM expression seem unlikely given that expression of the mutant $\alpha$-fast TM appeared to determine just subtle, if any, changes even in the expression of the other TM isoforms. Because the comparisons reported here between FHC and control fibers grouped on the basis of TM isoform composition (to control for the effects of differences in other TM isoforms) showed that in all cases FHC fibers expressing the Asp175Asn $\alpha$-fast TM had significantly higher calcium sensitivity than did the corresponding control groups, the presence of Asp175Asn $\alpha$-fast TM itself is by far the most likely determinant of the increased calcium sensitivity observed in FHC fibers. However, a minor role of possibly subtle TM isoform shifts cannot be ruled out.

TM isoform composition of type 1 fibers used for determination of calcium sensitivity was not studied because of technical limitations (small fiber size; see “Materials and Methods”), and the association between mutant TM and higher calcium sensitivity in slow fibers cannot be directly shown for fast fibers. However, the finding that 30% of type 1 FHC fibers contained the mutant TM demonstrates that the mutant TM is expressed in a subset of slow FHC fibers and suggests that its presence is the most likely explanation of the higher calcium sensitivity not only of type 2A and type 2A-2B FHC fibers but also of type 1 FHC fibers. Interestingly, Asp175Asn $\alpha$-fast TM was much more frequent in the slow fibers of one patient (50%) than in the slow fibers of the other (10%), and calcium sensitivity was also significantly higher in the slow fibers of the former patient than in the slow fibers of the latter patient (data not shown). The fact that mutant and WT $\alpha$-fast TM s were also found in slow FHC fibers is unlikely to be a feature of FHC patients themselves, since a specific pattern of association between fiber type and TM isoform expression has not been clearly shown in human muscle fibers so far.\(^{37,38}\) According to the only previous work in which $\alpha$-fast TM, $\alpha$-slow TM, and $\beta$-TM isoforms have been possibly identified in human muscle fibers,\(^{39}\) it is also not surprising that type 2A control fibers were found to contain all three TM isoforms.

At present, little can be concluded about the molecular mechanism at the basis of the observed increase in calcium sensitivity. The Asp175Asn mutation and one other (Glu180Gly) of the three known TM mutations linked to FHC occur at residues that have been highly conserved through evolution and that are in the region of the putative calcium-dependent troponin-T binding domain.\(^{39}\) Two recent articles have reported in vitro studies on purified Asp175Asn and Glu180Gln mutant $\alpha$-TMs expressed in E. coli. Bing et al\(^{40}\) have shown that in the in vitro motility assay in the presence of troponin and activating Ca\(^{2+}\) (pCa5), the mutant TMs give an increase in actin filament velocity compared with WT $\alpha$-TMs. This suggests that the mutations in TM may be causing a change in the “on” state of the thin filament mediated by altered interaction of TM either with the troponin complex or actin.
Golitsina et al. have used myosin subfragment-1 to induce the binding of pyrene-labeled WT or FHC mutant TM to actin in the "on" state. The change in excimer fluorescence for WT TM was found to be different from that observed using either Asp175Asn or Glu180Gln TM, thus also suggesting that the FHC mutations affect the conformation of the TM in the "on" state, possibly because of an increase in local flexibility of the TM molecule at the level of the mutation. The Asp175Asn mutation, possibly supported by the head-to-tail assembly of adjacent TM molecules, apparently does not interfere with the TM role in determining cooperativity, since fibers containing the Asp175Asn α-fast TM were not different from control fibers regarding Hill’s slope. The Asp175-Asn mutation does not appear to severely hinder the structural role of TM either, since force and shortening velocity, which heavily depend on the integrity of myofilament structure, were not impaired in fibers expressing the mutation. It should be pointed out, however, that some subtle change in force cannot be excluded on the basis of the present data because of the large variability in force of corresponding fiber types of healthy control subjects. Indeed, there were appreciable, but nonsignificant, trends toward increased velocity, but decreased force, in the FHC fibers.

Pathogenetic Mechanism of FHC

The present findings support the hypothesis that FHC is caused by mutations in contractile proteins that are expressed and incorporated in the sarcomere and, having abnormal function, interfere with the function of the WT proteins, ie, are consistent with a dominant-negative action of the mutation. However, at variance with most functional studies so far, the present results did not show an impairment of actin-myosin interaction and/or some major structural alteration following expression of the FHC mutation. Po and Vo in FHC fibers expressing Asp175Asn TM were not distinguishable from normal fibers. On the contrary, an increased calcium sensitivity was certainly associated with, and very likely determined by, the TM mutation. Such an increase should be even more pronounced in the heart, since α-fast TM is the dominant cardiac TM. Because the heart is not fully activated in vivo, the increase in calcium sensitivity could potentially enhance and not depress cardiac contractility. Therefore, it is not straightforward to attribute to the Asp175Asn mutation the pathogenetic mechanism of functional impairment and compensatory hypertrophy suggested for most mutations studied so far.

A recent study has suggested that not all FHC-linked mutations necessarily act through this common mechanism but that some might determine a "hypercontractile" state that could directly induce cardiac hypertrophy. In that study, in fact, recombinant troponin T carrying an Ile35Aln mutation determined an increase, and not the generally observed decrease, in the velocity of actin-TM filaments in an in vitro motility assay. Interestingly, the very rare cardiac MLC mutations have also been shown to cause an increase in velocity in a sliding filament assay. The present findings involving the Asp175Asn α-fast TM mutation might be taken to support the possibility of a mutation-induced hypercontractile state. However, it should be pointed out that not only the "hypercontractile" hypothesis but also the more common and better defined “hypercontractile” hypothesis are still somewhat tentative and incomplete. For technical reasons, in most studies the motility assay technique has been used to assess function of mutant proteins. Because velocity is determined in the absence of load and the heart always works under load in vivo, it is not clear what a decrease or increase in velocity in motility assays necessarily means in terms of force generation, or power output, under load. Force was found depressed only in two of the three β-MHC mutations studied by Lankford et al. and in one troponin-T mutation (troponin-T truncation). Moreover, care must be taken to transfer to a complex in vivo situation all findings reported so far that were not obtained in strictly physiological conditions. Even results involving human skinned fibers from living muscles (Reference 9 and present results), which can be considered the most physiological of the specimens used to date, should be considered with some caution, because the experiments were performed at low temperature (12°C to 15°C).

In conclusion, the present work provides the first quantitative evidence of equal expression and apparent incorporation of mutant and WT contractile protein into muscle in subjects with FHC. These findings strongly support the dominant-negative role of mutant TM in determining FHC. The Asp175Asn mutant TM interferes with the function of the WT protein and results in increased calcium sensitivity. This might imply an enhancement in cardiac performance.

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References


A Mutant Tropomyosin That Causes Hypertrophic Cardiomyopathy Is Expressed In Vivo and Associated With an Increased Calcium Sensitivity


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