Adaptation by alternative RNA splicing of slow troponin T isoforms in type 1 but not type 2 Charcot-Marie-Tooth disease

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Larsson L, Wang X, Yu F, Höök P, Borg K, Chong SM, Jin J-P. Adaptation by alternative RNA splicing of slow troponin T isoforms in type 1 but not type 2 Charcot-Marie-Tooth disease. Am J Physiol Cell Physiol 295: C722–C731, 2008. First published June 25, 2008; doi:10.1152/ajpcell.00110.2008.—Slow troponin T (TnT) plays an indispensable role in skeletal muscle function. Alternative RNA splicing in the NH2-terminal region produces multiple TnT isoforms. Normal adult slow muscle fibers express mainly HMW slow TnT. Charcot-Marie-Tooth disease (CMT) is a group of inherited peripheral polyneuropathies caused by various neuronal defects. We found in the present study that LMW slow TnT was significantly upregulated in demyelination form type 1 CMT (CMT1) but not axonal form type 2 CMT (CMT2) muscles. Contractility analysis showed an increased specific force in single fibers isolated from CMT1 but not CMT2 muscles compared with control muscles. However, an in vitro motility assay showed normal velocity of the myosin motor isolated from CMT1 and CMT2 muscle biopsies, consistent with their unchanged myosin isoform contents. Supporting a role of slow TnT isoform regulation in contractility change, LMW and HMW slow TnT isoforms showed differences in the molecular conformation in conserved central and COOH-terminal regions with changed binding affinity for troponin I and tropomyosin. In addition to providing a biochemical marker for the differential diagnosis of CMT, the upregulation of LMW slow TnT isoforms under the distinct pathophysiology of CMT1 demonstrates an adaptation of muscle function to neurological disorders by alternative splicing modification of myofilament proteins.

CHARCOT-MARIE-TOOTH DISEASE (CMT), also known as hereditary motor and sensory neuropathy, is a neurological disorder that causes damage to the peripheral nerves. The symptoms of CMT include muscle weakness and wasting and contractures. There are several forms of CMT. Depending on the genetic defect, the inheritance of CMT varies. CMT type 1 (CMT1) is a demyelinating form of the disease in which defects in axon myelination result in abnormalities in nerve conductional velocity; CMT type 2 (CMT2) is an axonal form of the disease in which the loss of axons reduces muscle innervations (42). Neurophysiological studies have shown an active muscle denervation-reinnervation process in CMT1 patients, whereas CMT2 patients had a low reinnervation capacity and thus compensated for the loss of motor units by increasing the amount of contractile myofibrils (5, 11).

Contraction of vertebrate skeletal muscle is regulated by Ca2+ through the troponin complex in the actin thin filament. Upon motor neuron stimulation, depolarization of the muscle cell membrane results in a rise of Ca2+ in the cytoplasm. Ca2+ binding to troponin induces a series of allosteric changes in the thin filament and activates the myosin motor ATPase and contraction (16). Troponin is a protein complex of three subunits: troponin C (TnC; the Ca2+-binding subunit), troponin I (TnI; the inhibitory subunit), and troponin T (TnT; the tropomyosin-binding subunit) (34). Anchoring the troponin complex to tropomyosin and actin, TnT is at a central position in the regulatory system of muscle (44). Higher vertebrates have evolved three types of striated muscle and three corresponding muscle type-specific TnT isoform genes: slow skeletal muscle TnT (TNT1), fast skeletal muscle TnT (TNT3), and cardiac TnT (TNT2) (6, 22, 27). These homologous TnT isoform genes have differentiated structures while each of the fiber type-specific TnT isoforms is conserved in the vertebrate phylum (29), suggesting differentiated functional roles. A nonsense mutation in the slow TnT gene that causes complete loss of slow TnT in Amish nemaline myopathy has a childhood lethal phenotype (33), demonstrating the indispensable role of slow TnT in skeletal muscle function.

Molecular imaging and structural studies have suggested that TnT is an extended protein (7, 31, 52). Studies using TnT fragments have identified the functional domains of TnT. The COOH-terminal (T2) region binds to tropomyosin and interacts with TnI, TnC, and F-actin (19, 40, 43). A crystal structure of the partial troponin complex confirmed the interaction of the TnT COOH-terminal domain with TnI and TnC (48). The central region of TnT has another tropomyosin-binding site (18, 23, 46). The NH2-terminal region of TnT is a variable region and does not bind any known myofilament protein. Alternative RNA splicing in the NH2-terminal region produces multiple TnT protein isoforms (34). The alternative splicing of cardiac and fast TnT isoforms is developmentally regulated to produce large to small, acidic to basic isoform switches (25, 49). Two alternatively spliced isoforms of slow TnT are found in embryonic and adult skeletal muscle (14, 29, 45). Normal adult slow muscles express mainly high-molecular-weight (HMW) slow TnT. The more acidic HMW and less acidic low-molecular-weight (LMW) slow TnT isoforms differ by inclusion or exclusion, respectively, of 11 amino acids in the NH2-terminal
region encoded by exon 5. TnT isoforms differing in the NH₂-terminal region show conformational and functional differences (4, 15). Different from cardiac and fast TnT isoform expressions, the alternative splicing of slow TnT is not clearly regulated during development, and the functional significance of HMW and LMW slow TnT isoforms is not understood.

In the present study, we found that LMW slow TnT was significantly upregulated in CMT1 but not CMT2. CMT1 but not CMT2 muscle fibers increased specific force, whereas myosin motor function and myosin isoform contents were unchanged. Supporting a role of slow TnT isoform regulation in the modulation of muscle contractility, LMW and HMW slow TnT isoforms showed differences in molecular conformation and binding to TnI and tropomyosin. In addition to providing a biochemical marker for the differential diagnosis of CMT, the increase of the LMW slow TnT isoform in CMT1 demonstrates an adaptation of myofilament protein isoforms to a specific neurological disorder through alternative RNA splicing.

MATERIALS AND METHODS

All animal procedures were approved by Institutional Animal Care and Use Committees and were conducted in accordance with the “Guiding Principles in the Care and Use of Animals” as approved by the American Physiological Society. Human muscle samples. Biopsy samples were obtained from eight CMT1 and five CMT2 patients. Diagnosis was based on genetic analyses (chromosome 17 duplication or mutation in CMT1 patients), electrophysiology [motor and sensory neurographies, needle electromyography (EMG)], and clinical examination. In the CMT1 group, there were two men (49 and 52 yr) and six women (22–62 yr). In the CMT2 group, there were three men (46–69 yr) and two women (66 and 73 yr). Six tibialis anterior and two vastus lateralis biopsy samples from CMT1 patients with predominant slow fiber contents were analyzed. Two tibialis anterior and three vastus lateralis biopsy samples from CMT2 patients with predominant slow fiber contents were analyzed. Single muscle fiber contractile measurements were performed in muscle samples from two CMT1 patients (30 and 57 yr) and one CMT2 patient (73 yr). For comparison, contractile measurements were also performed on fibers from 1 patient with prior polio lesion (51 yr, 35) and 12 healthy controls (58 ± 6 yr; 18–84 yr). All of the patients were ambulatory and have previously been included in a study on macro EMG and muscle function in CMT1 and CMT2 patients (11). The muscle strength according to the Hendall scale did not differ between the two groups, although the average peak isokinetic torque at 30°/s was lower in CMT1 patients than in CMT2 patients (11). Informed consent was obtained from the patients and control subjects enrolled in this study. The Karolinska Institute ethics committee approved the protocol, and the experiments were carried out according to the guidelines of the Declaration of Helsinki.

Each biopsy was dissected free of fat and connective tissues and divided into two portions. One portion was frozen in isopentane cooled by liquid nitrogen and stored at −80°C for later analyses. Small bundles of 25–50 fibers were dissected from the biopsy material. These small bundles were tied to glass capillary tubes and stretched to ~110% of the resting length. Muscle bundles were then treated with skinnning solution [relaxing solution containing glycerol, 50:50 (vol/vol) for 24 h at 4°C, after which they were transferred to −20°C]. In addition, muscle bundles were treated with sucrose, a cryoprotectant, within 1–2 wk for long-term storage (52). For contractile experiments, permeabilized muscle bundles were removed from the capillary tube to isolate single muscle fibers.

Force measurement of skinned single muscle fibers. The experimental procedure has been described in detail elsewhere (13, 35). Skinned muscle fibers used in this work had an average segment length of 2.02 ± 0.51 mm (mean ± SD, range 1.05–3.60 mm) and were exposed to solution between connectors of the force transducer. The sarcomere length (SL) of the single fiber segment was set between 2.75 and 2.85 µm by adjusting the overall segment length. SL was measured routinely in the fibers during maximal activation. Fiber depth was measured by recording the vertical displacement of the microscope nosepiece while focusing on the top and bottom surfaces of the fiber. The fiber cross-sectional area (CSA) was calculated from the width and depth, assuming an elliptical circumference. Every width and depth value represented the average of three different measurements. The accuracy of the measurements done by the same observer was verified by comparing 200 average values for depth calculated at two different SLs (r² = 0.99). Specific tension was calculated as maximum tension (P₀) normalized to CSA and was corrected for the 20% swelling that is known to occur during skinnning (41). CSA, maximum force, and specific tension were calculated in a total of 223 fibers expressing the type I isoform of myosin heavy chain (MHC), i.e., in 110, 81, and 32 control, CMT1, and CMT2 fibers, respectively. A total of 107 fibers fulfilled the criteria for maximum unloaded shortening velocity (V₀) measurements, i.e., 54, 34, and 19 control, CMT1, and CMT2 fibers, respectively.

Relaxing and activating solutions were prepared as previously described (35), and the apparent dissociation constants for Ca²⁺-EGTA were corrected for temperature and ionic strength (12). V₀ was measured by the slack test procedure (9). Fibers were activated at pCa 4.5, and, once steady tension had been reached, various amplitudes of slack (ΔL) were rapidly introduced (within 1–2 ms) at one end of the fiber. The time (∆t) required to take up the imposed slack was measured from the onset of the length step to the beginning of tension redevelopment. For each amplitude of ∆L, the fiber was reextended while relaxed to minimize the nonuniformity of SL. A straight line was fitted to the plot of ∆L versus ∆t, using least-squares regression, and the slope of the line normalized to muscle fiber length was recorded as V₀ for that fiber.

P₀ was calculated as the difference between the total tension in the activating solution (pCa 4.5) and the resting tension measured in a control, CMT1, and CMT2 fibers, respectively. Contractile measurements were carried out at 15°C. Contractile recordings were accepted in subsequent analyses if a V₀ value was based on linear regressions including four or more data points, and data were discarded if r for the fitted line was <0.97, if P₀ changed >10% from first to final activation, or if SL during isometric tension development changed by >0.10 µm compared with SL when the fiber was relaxed (41).

After contractile analysis, fibers were detached from the force transducer and snap frozen with either Freon or isopentane cooled by liquid nitrogen and stored at −80°C for SDS-PAGE and Western blot analyses. SDS-PAGE and Western blot analysis. Muscle samples, bacterial extracts, or purified slow TnT proteins were prepared in SDS-PAGE sample buffer as described above and resolved by SDS-PAGE using the Laemmli buffer system. Gels were stained with silver or Coomassie brilliant blue R250 to show the resolved protein bands. Duplicated gels were electrically transferred to nitrocellulose membranes as previously described (50). After being blocked in Tris-buffered saline (TBS) containing 0.5% Triton X-100, 0.05% SDS, and 1% BSA, nitrocellulose membranes were incubated with monoclonal antibodies (mAb) [CT3 against slow and cardiac TnT but not fast TnT (30), TnI-1 against all three TnI isoforms (32), and FA2 against cardiac β-MHC/skeletal muscle type I, but not type II, MHC (26)] or a polyclonal anti-TnT antibody (RATnT) (50). Membranes were then washed in TBS containing 0.5% Triton X-100 and 0.05% SDS, incubated with alkaline phosphatase-labeled anti-mouse IgG second antibody (Sigma Chemical), and washed again to remove the unbound

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second antibody. Blots were developed in 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate solution as previously described (50) to detect TnT or Tnl isoform bands.

Two-dimensional gel electrophoresis. Total protein extracts from mouse soleus muscle were analyzed by two-dimensional gel electrophoresis as described previously (2). The first dimension was isoelectric focusing in Bio-Rad Lab mini tube gels containing pH 3.5–10 ampholine (Amersham). The second dimension was 14% Laemmli SDS-PAGE, and the resolved gel was transferred to a nitrocellulose membrane for Western blot analysis using the CT3 mAb as described above.

In vitro motility assay. The unregulated F-actin used throughout this study was purified from rabbit skeletal muscle and fluorescently labeled with rhodamine-phalloidin (Molecular Probes, Eugene, OR). The single fiber in vitro motility assay has been described in detail elsewhere (20, 21). Briefly, a short muscle fiber segment was placed on a glass slide between two strips of grease, and a mica overslip was placed on top, creating a flow cell of ~2 μl volume. Myosin was extracted from the fiber segment through the addition of a high-salt buffer (0.5 M KCl, 25 mM HEPES, 4 mM MgCl2, 1 mM EGTA, and 1% 2-mercaptoethanol; pH 7.6). After 30 min of incubation on ice, a low-salt buffer (25 mM KCl, 25 mM HEPES, 4 mM MgCl2, 1 mM EGTA, and 1% 2-mercaptoethanol; pH 7.6) was applied, followed by 0.1% BSA in the same buffer. Nonfunctional myosin molecules were blocked with fragmentized F-actin, and rhodamine-phallolidin-labeled actin filaments were subsequently infused into the flow cell, followed by motility buffer (2 mM ATP, 0.1 mg/ml glucose oxidase, 23 μg/ml catalase, 2.5 mg/ml glucose, and 0.4% methyl cellulose) to initiate movement. The pH of the buffers was adjusted with KOH, and the final ionic strength of the motility buffer was 71 mM. The flow cell was placed on the stage of an inverted epifluorescence microscope (Olympus IX 70). The movements of F-actin were filmed with an image-intensified SIT camera (SIT 66, DAGE-MITCO) and recorded on tape with a videocassette recorder.

From each single fiber preparation, 13 to 20 actin filaments moving with constant speed in an oriented motion were selected for speed analysis. Recordings and analysis were only performed from preparations in which >90% of the filaments moved bidirectionally. A filament was tracked from the center of mass, and the speed was calculated from 10 to 20 frames at an acquisition rate of 5 or 1 frames/s, depending on the fiber type, using image-analysis software (OPTIMAS 6.0, Optimas). The average speed and SD of 13–20 filaments were calculated. The average motility speed of each fiber was taken as representative for the muscle fiber.

Glycercol-SDS-PAGE analysis of MHC isoforms. As described previously (53), total protein was extracted by homogenizing isolated human skeletal muscle single fibers in Laemmli SDS-PAGE sample buffer that contained 2% SDS to inactivate proteases. Samples were heated at 80°C for 5 min and clarified by spinning in a microcentrifuge at top speed and room temperature for 5 min. MHC isoforms were resolved by glycerol-SDS-PAGE. The resolving gel contained 8% acrylamide with an acrylamide-bisacrylamide ratio of 50:1, 30% glycerol, 200 mM Tris base, 100 mM glycine (pH 8.8), and 0.4% SDS. The stacking gel contained 4% acrylamide, 70 mM Tris-HCl (pH 6.7), 4 mM EDTA, and 0.4% SDS. Gels were cast in a Bio-Rad mini-Protean II system and run at 70 V in an icebox for 24 h. The upper running buffer was composed of 100 mM Tris base, 150 mM glycine, and 0.1% SDS. The lower running buffer was a 50% dilution of the upper running buffer. The gel was stained with Coomassie brilliant blue R250 to detect protein bands. The MHC I band was confirmed by transferring parallel gels to nitrocellulose membranes for Western blot analysis using mAb FA2 as described above.

Expression and purification of human slow TnT isoforms. cDNAs encoding HMW and LMW isoforms of human slow skeletal muscle TnT cloned in the pAED4 prokaryotic expression vector were used for protein expression in Escherichia coli as previously described (51). The two slow TnT isoforms proteins expressed in bacterial culture were purified by identical procedures involving ammonium sulfate fractionation, anion-exchange, and gel filtration chromatographies as previously described (51).

mAb epitope analysis of the molecular conformation of slow TnT isoforms. The binding affinity between an antibody and its antigenic epitope depends on its three-dimensional structural fit. Therefore, epitope affinity analysis can detect conformational differences of protein isoforms (3). ELISA epitope analysis (4) was used to examine conformational differences between HMW and LMW TnT isoforms. Abs against CT3 and 2C8 against epistopes in the central region and I9 against a COOH-terminal epitope (8) were used to examine global conformational changes in slow TnT due to alternative splicing in the NH2-terminal region.

Purified HMW and LMW slow TnT isoform proteins were dissolved in buffer A [0.1 M KCl, 3 mM MgCl2, and 10 mM PIPES (pH 7.0) or 10 mM Tris-HCl (pH 8.0)] at 2 μg/ml to coat microtiter plates at 100 μl/well and 4°C incubation overnight. After the removal of any unbound TnT and two washes with buffer A containing 0.05% TWEEN 20 (buffer T), plates were blocked with 150 μl/well buffer T containing 1% BSA at room temperature for 1.5 h. The immobilized TnT was incubated with 100 μl/well of serial dilutions of CT3, 2C8, or I9 mAb in buffer T containing 0.1% BSA at room temperature for 2 h. Following three washes with buffer T in a 10-min period to remove any unbound primary antibody, plates were incubated with 100 μl/well of horseradish peroxidase-conjugated anti-mouse immunoglobulin second antibody (Sigma) in buffer T containing 0.1% BSA (buffer B) at room temperature for 1 h. Unbound second antibody was removed by three washes as described above. The binding of mAb to its epitopes on MHW and LMW slow TnT isoforms within the thin filament regulatory system, ELISA solid-phase protein binding experiments (50) were performed to compare their binding affinity to TnT and tropomyosin.

HMW and LMW slow TnT proteins were dissolved individually at 5 μg/ml in buffer A and coated on triplicate wells of microtiter plates by an incubation at 4°C overnight. After washes with buffer T to remove unbound TnT, plates were blocked with buffer T containing 1% BSA. Plates were then incubated with serial dilutions of bovine cardiac TnI, purified from adult ventricular muscle as previously described (4), in buffer B at room temperature for 2 h. After washes with buffer T, bound TnI was quantified via anti-TnI mAb TnI-1 (32), horseradish peroxidase-conjugated goat anti-mouse immunoglobulin second antibody (Sigma), and H2O2/2′-azinobis-(3-ethylbenzthiazolinesulfonic acid) substrate reaction. The enzymatic reaction in each assay was monitored at a series of time points using an automated microplate reader (Bio-Rad Benchmark). Absorbance at 405 nm (A405 nm) values in the linear course of the color development were used to plot antibody titration curves for the quantification of the binding affinity of each mAb to its specific epitope to compare the difference due to NH2-terminal alternative splicing. All experiments were done in triplicate.

TnT and tropomyosin binding assays. To compare the functions of HMW and LMW slow TnT isoforms within the thin filament regulatory system, ELISA solid-phase protein binding experiments (50) were performed to compare their binding affinity to TnT and tropomyosin.

HMW and LMW slow TnT proteins were dissolved individually at 5 μg/ml in buffer A and coated on triplicate wells of microtiter plates by an incubation at 4°C overnight. After washes with buffer T to remove unbound TnT, plates were blocked with buffer T containing 1% BSA. Plates were then incubated with serial dilutions of bovine cardiac TnI, purified from adult ventricular muscle as previously described (4), in buffer B at room temperature for 2 h. After washes with buffer T, bound TnI was quantified via anti-TnI mAb TnI-1 (32), horseradish peroxidase-conjugated goat anti-mouse immunoglobulin second antibody (Sigma), and H2O2/2′-azinobis-(3-ethylbenzthiazolinesulfonic acid) substrate reactions using ELISA procedures as previously described (50). ELISA results were recorded using an automated microplate reader (Bio-Rad Benchmark), and A405 nm values from the linear range of color development were used to plot the binding curves of TnI to HMW and LMW slow TnT isoforms. BSA-coated wells were used to produce a control curve.

Tropomyosin binding was analyzed similarly except that serial dilutions of α-tropomyosin purified from the rabbit heart, as previously described (50), and anti-tropomyosin mAb CH1 (39), a gift from Dr. Jim Lin (University of Iowa), were used.

Chronically denervated rat muscle samples. Adult rats underwent an operation under surgical conditions to remove ~1.0 cm of the trunk portion of the left tibial nerve. Animals were allowed to recover from anesthesia and maintained without movement restrictions. Animals were euthanized 2 wk after the operation, and gastrocnemius muscles
CMT1 muscle fibers had a unique increase in protein profiles in CMT2 and control muscle fibers, whereas \( A_1 \) protein band. The representative Western blots shown in Fig. 1 demonstrated similar protein profiles in CMT2 and normal skeletal muscle fibers. In accordance with previous observations, predominantly MHC-I fibers were present in CMT muscles (5, 10). In fact, all the single fibers analyzed for contractility in the present study were type I slow fibers as identified by Western blots using FA2 mAb as well as glycerol-SDS-PAGE.

RESULTS

Upregulation of alternatively spliced LMW slow TnT in muscle of CMT1 but not CMT2 patients. Normal human tibialis anterior and vastus lateralis muscles contain both fast and slow fibers, although fibers expressing the slow myosin isoform dominate in the tibialis anterior muscle (24). In accordance with previous observations, predominantly MHC-I fibers were present in CMT muscles (5, 10). In fact, all the single fibers analyzed for contractility in the present study were type I slow fibers as identified by Western blots using FA2 mAb as well as glycerol-SDS-PAGE. SDS-PAGE and Western blot analysis of total myofilament proteins extracted from a large number of skinned single fibers isolated from tibialis anterior muscle biopsies and frozen sections of tibialis anterior and vastus lateralis muscle biopsies revealed an upregulation of the LMW slow TnT isoform. The representative SDS gel shown in Fig. 1A demonstrated similar protein profiles in CMT2 and control muscle fibers, whereas CMT1 muscle fibers had a unique increase in a \( \sim 30 \)-kDa protein band. The representative Western blots shown in Fig. 1A revealed that CMT1 muscle fibers, but not CMT2 muscle fibers, had a significant increase in the level of LMW slow TnT and decrease in the level of HMW slow TnT, whereas the expression of slow TnI and MHC-I isoforms was unchanged. The identification of HMW and LMW slow TnT isoforms in CMT muscle samples was confirmed by their gel mobility in Western blots along with control human slow TnT isoform proteins expressed in \( E. \ coll i \) from cloned cDNA (51) (Fig. 1B).

Table 1 shows quantification data summarized from densitometry analysis of CT3 mAb Western blots of muscle samples from CMT1, CMT2, and control subjects. The results confirmed that the LMW slow TnT isoform had a significantly increased percentage of expression in CMT1 muscle compared with control muscle. In contrast, the expression pattern of HMW and LMW slow TnT isoforms in CMT2 patients was not different from the control, in which the HMW slow TnT was predominant.

The physical properties of HMW and LMW slow TnT isoforms were analyzed by Western blot of two-dimensional gel electrophoresis-resolved mouse soleus muscle homogenates. The mAb CT3 blot shown in Fig. 2A demonstrated that the two alternatively spliced slow TnT isoforms were different in their size and isoelectric points. The linear structural map of slow TnT deduced from amino acid sequences demonstrated the difference between HMW and LMW slow TnT isoforms due to alternative splicing of exon 5, which encodes an 11-residue acidic peptide in the NH\(_2\)-terminal region (Fig. 2B). Consistent with their two-dimensional gel mobility, the calculated molecular weights and isoelectric points of HMW and LMW human slow TnT isoforms (Fig. 2B) demonstrated that the splice out of exon 5 in LMW slow TnT reduces the NH\(_2\)-terminal negative charge. As shown in Fig. 2C, TnT occupies a central position in the thin filament Ca\(^{2+}\)-regulatory system of striated muscle. The NH\(_2\)-terminal variable region of TnT is a modulatory structure. The alternative splicing of exon 5 in the NH\(_2\)-terminal region may affect the protein binding sites in central and COOH-terminal regions, conferring a specific functional adaptation by the increase in LMW slow TnT in CMT1 muscle.

Increase in Ca\(^{2+}\)-activated tension in skinned single muscle fibers from CMT1 but not CMT2 patients. Figure 3 shows the results from contractility experiments using skinned single muscle fibers isolated from tibialis anterior muscle biopsies selected from tibialis anterior muscle biopsies in the present study were type I slow fibers as identified by Western blots using FA2 mAb as well as glycerol-SDS-PAGE.
The NH2-terminal region on functional regions in slow TnT modulates the molecular conformation and function of TnT (4, C). The results shown in Fig. 6B demonstrated different binding affinities of these site-specific mAb to HMW and LMW isoforms of slow TnT, indicating a conformational difference in central and COOH-terminal regions. The data suggest that the presence or absence of exon 5 in the NH2-terminal region of HMW and LMW slow TnT isoforms has a functional significance.

Table 1. Increased expression of the LMW slow TnT isoform in CMT1 but not CMT2 patients

<table>
<thead>
<tr>
<th>Number of Subjects</th>
<th>LMW Slow TnT Isoform, % total slow TnT</th>
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<tbody>
<tr>
<td>Control</td>
<td>12</td>
</tr>
<tr>
<td>CMT1</td>
<td>8</td>
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<tr>
<td>CMT2</td>
<td>5</td>
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Data are means ± SD. Data are summarized from a densitometry analysis of CT3 monoclonal antibody Western blots of single muscle fibers or frozen sections of muscle biopsies from Charcot-Marie-Tooth disease (CMT) type 1 (CMT1), CMT type 2 (CMT2), and control subjects, which demonstrated that the low-molecular-weight (LMW) slow tropinin T (TnT) isoform had a significantly increased expression in CMT1 muscle compared with control or CMT2 muscles (\(P < 0.005\)). In contrast, the expression pattern of high-molecular-weight and LMW slow TnT isoforms in CMT2 was not different from the control (\(P > 0.75\)).

muscle fibers isolated from 2 CMT1, 1 CMT2, and 12 control subjects. The maximum Ca\(^{2+}\) activated force of CMT1 muscle fibers normalized by CSA (specific tension) was higher than those of CMT2 and control muscle fibers. No differences were observed between CMT2 and control fibers. The average CSA of individual MHC-I fibers was 3,640 ± 150 \(\mu\)m\(^2\) in controls, 2,670 ± 140 and 7,650 ± 300 \(\mu\)m\(^2\), respectively, in fibers from two CMT1 patients, and 3,860 ± 160 \(\mu\)m\(^2\) in CMT2 fibers. Thus, a significant muscle fiber hypertrophy was observed in one, but not the other, CMT1 patient, consistent with previous observations of a large muscle fiber size variability among CMT1 patients (11). The fibers used in the contractility analysis were recovered and examined by glycerol-SDS-PAGE and FA2 mAb Western blots. The results showed that all fibers used for the contractility analysis were MHC-I fibers. Representative data are shown in Fig. 1C.

\(V_0\) in single muscle fibers increased in both CMT1 and CMT2 patients. Although the single muscle fibers examined in the present study were all MHC-I fibers, \(V_0\) values were significantly different between groups (Fig. 4). Both CMT1 and CMT2 fibers showed higher \(V_0\) values compared with those of control fibers. Although a trend of difference was seen between the two CMT1 patients, no statistical significance was established.

Unchanged function of myosin isolated from CMT1 and CMT2 single muscle fibers. Figure 5 shows the results from a single muscle fiber in vitro motility assay using unregulated F-actin. Actin filament speed propelled by myosin from single muscle fibers in the absence of tropomyosin-troponin regulation did not differ among CMT1, CMT2, and control muscle myosins.

Consistently, CMT1 and CMT2 single muscle fibers randomly selected for examination in the present study were all MHC-I fibers, as shown by glycerol-SDS-PAGE and mAb FA2 Western blots (Fig. 1C). The results indicate that the differences in the Ca\(^{2+}\)-activated specific tension and \(V_0\) in CMT muscle fibers was not due to an alteration of the intrinsic activity of the myosin motor.

Effect of the NH2-terminal variation in HMW and LMW slow TnT on the molecular conformation of remote functional regions. The NH2-terminal variable region is a structure that modulates the molecular conformation and function of TnT (4, 34). The conformational effect of alternative splicing of exon 5 in the NH2-terminal region on functional regions in slow TnT was analyzed by ELISA epitope affinity titration using mAb CT3, 2C8, and 1G9 against epitopes in the central and COOH-terminal regions that bind tropomyosin, TnC, and TnI (Fig. 6A). The results suggested that the presence or absence of exon 5 in the NH2-terminal region of HMW and LMW slow TnT isoforms has a functional significance.
MHC-I fibers. ML, muscle lengths.

the HMW slow TnT isoform (Fig. 7A). The LMW slow TnT isoform further showed a clearly higher binding affinity for tropomyosin than the HMW slow TnT isoform, and the difference was greater than that of their Tnl-binding affinity (Fig. 7B). This observation indicates that the NH2-terminal alternative splicing of TnT more effectively affects the adjacent central region containing a tropomyosin-binding site, whereas the Tnl-binding site is in the COOH-terminal region (Fig. 6A). The results suggest that the presence or absence of the NH2-terminal 11 amino acids differentially alters slow TnT conformation and function in the central and COOH-terminal regions, supporting a notion that the change in the Ca2+-activated Ca2+ pump of CMT1 muscle fibers is due to the change in slow TnT isoform regulation.

Muscle denervation or overuse did not affect the ratio of HMW and LMW slow TnT isoforms. It is very intriguing that CMT1 but not CMT2 results in the change in slow TnT isoform expression. Considering that the pathology of CMT2 is axon loss, we examined TnT isoform expression in muscle denervation. The results shown in Fig. 8A demonstrated that rat gastrocnemius muscles 2 wk after removal of the main trunk of the tibial nerve showed atrophy but no change in alternatively spliced slow TnT isoforms compared with innervated normal muscle. We have previously demonstrated that during skeletal muscle adaptation, the change in alternatively spliced TnT isoforms is a rapid response and can be detected as early as 3 days after unloading (53). Therefore, the lack of detectable change in TnT isoform expression after 2 wk of denervation indicates no detectable fiber type switching. Consistently, the expression of fast TnT and Tnl isoforms also did not change.

The results shown in Fig. 8B further demonstrated that normal human tibialis anterior muscle had detectable expression of fast TnT, whereas a muscle sample from a prior polio patient (36) had predominantly slow TnT, similar to that found in CMT patient muscles. The switching to slow fibers after overuse of healthy muscle in prior polio patient suggests a transcriptional regulation that might also be responsible for the predominant slow fibers found in CMT muscles. However, the overuse conditions did not change the alternative splicing pattern of the HMW versus LMW slow TnT isoforms, which may reflect a unique adaptation to the qualitative changes in neurological stimulation in CMT1.

DISCUSSION

Regulation of TnT isoforms by alternative RNA splicing in muscle diseases and adaptation. Skeletal muscle is a highly plastic tissue that undergoes dramatic changes in contractile protein gene regulation during development and adaptation to functional demands and stress conditions (47). For example, we and others have found that the expression of slow versus fast isoforms of MHC, Tnl, and TnT was highly sensitive to muscle unloading in simulated weightlessness (53). It is worth noting that these adaptations are by regulating the transcription of different genes encoding the protein isoforms. On the other hand, alternative RNA splicing has been found to regulate TnT isoforms during cardiac and fast skeletal muscle development (27, 49), primarily under the control of a systemic biological clock (28).
Nonetheless, we previously reported primary changes in cardiac TnT alternative splicing in dilated cardiomyopathy (2, 3). Alternatively spliced cardiac TnT variants have also been reported in hypertrophic and failing human hearts (1). Changes in the alternative splicing of fast TnT isoforms have been observed during hindlimb skeletal muscle unloading (5). There was no clear developmental regulation of HMW and LMW slow TnT isoforms found in mice and sheep (29). Our finding of the slow TnT isoform switch in the muscle of CMT1 patients provides a novel evidence for the role of alternatively spliced slow TnT isoforms in the modification of skeletal muscle function under disease conditions.

Specific adaptation by upregulation of LMW slow TnT in muscle of CMT1 patients with axon demyelination. A previous study (37) has observed the role of innervations in the expression of muscle fiber type-specific TnT isoforms. A neurophysiological study (5) on muscle biopsy specimens showed an active denervation-reinnervation process in

Fig. 6. Effect of the NH$_2$-terminal variation in HMW and LMW sTnT isoforms on the molecular conformation of remote functional regions. A: the effect of alternative splicing of exon 5 in the NH$_2$-terminal region on functional regions in sTnT, which has an extended structure, was analyzed by ELISA epitope affinity titration using mAb CT3, 2C8, and 1G9 against epitopes in the central and COOH-terminal regions that bind tropomyosin (Tm), troponin C (TnC), and TnI. B: titration curves of mAb CT3 and 2C8 detected different binding affinities to HMW and LMW sTnT isoforms at pH 7.0 ($P < 0.05$ for both epitopes), indicating a conformational difference in the central region. Significant affinity difference was also found for mAb 1G9 at pH 8.0 ($P < 0.01$), demonstrating a conformational difference between HMW and LMW sTnT isoforms in the COOH-terminal region. Data are presented as means $\pm$ SD. The conformational differences in functional regions due to the presence or absence of exon 5 in the NH$_2$-terminal region suggest a functional significance of the differential regulation of HMW and LMW sTnT isoforms.

Fig. 7. Different binding affinities of HMW and LMW sTnT isoforms to TnI and Tm. Solid-phase protein binding assays were used to assess the binding of sTnT isoforms with TnI and Tm. A: normalized titration curves showed that the LMW sTnT isoform has a higher binding affinity for TnI than that of the HMW sTnT isoform ($P < 0.01$). B: normalized titration curves further showed that the LMW sTnT isoform had a higher binding affinity to Tm than that of the HMW sTnT isoform ($P < 0.002$). Data are presented as means $\pm$ SD.

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CMT1 (demyelinating form) patients, whereas CMT2 (axonal form) patients had a low reinnervation capacity and thus compensated for the loss of motor units by increasing contractile tissue. Since neuropathic abnormalities were found in CMT1 and myopathic changes due to the loss of innervation occur in CMT2, it was noted that the muscle fiber abnormalities in CMT1 patients would reflect abnormalities due to the primary nerve dysfunction and that the abnormalities seen in CMT2 patients would be secondary changes as a compensation for loss of muscle strength with an increase of contractile material (5).

Consistently, we demonstrated that the axonal loss associated with peripheral denervation in experimental animal models or in patients with prior polio lesion did not change the ratio of TnT isoforms, similar to that observed in CMT2 patients with axon loss. Furthermore, we (36) have previously reported an approximately twofold higher V0 and average CSA in muscle fibers from the prior polio patient compared with healthy control MHC-I fibers without an increase in specific tension, reflecting an adaptation to chronic overuse. Therefore, the results demonstrate that axon loss and compensatory overuse have mainly quantitative structural and functional consequences at the muscle level. In contrast, the altered splicing of slow TnT isoforms is a specific adaptation to the pathophysiology of CMT1 in which the changed quality, rather than quantity, of neurological stimuli induces adaptive changes in muscle contractile function. This novel correlation between neurogenic stimulation and/or muscle activation pattern and muscle contractile protein isoform regulation needs to be further investigated for the understanding of neuromuscular plasticity and functional interactions.

Potential effect of the increased LMW slow TnT isoform in CMT1 patients on muscle function. In the present study, we demonstrated an increase in the Ca2+-activated tension normalized to the CSA of CMT1 muscle fibers compared with CMT2 or control fibers. This difference occurred on the basis of identical myosin isoforms in muscle fibers and, therefore, cannot be attributed to myosin motor function. Consistently, the activity of unregulated myosin isolated from CMT1, CMT2, and control muscles was not different in in vitro motility experiments and MHC isoform expression was identical in these muscle fibers. By excluding the role of changes in intrinsic myosin function, the correlation between the upregulation of the LMW slow TnT isoform and the increase in Ca2+-activated contractile force production suggests a plausible mechanism of functional adaptation.

A previous study (15) has shown that TnT isoforms with less NH2-terminal negative charge confer lower Ca2+ sensitivity in the activation of cardiac muscle thin filaments. The observation that the less acidic LMW slow TnT isoform correlates to higher myofibril force production in CMT1 provides a new lead to further understand the physiological and pathological significance of the NH2-terminal regulation of TnT structure and function.

V0 values were significantly increased in single muscle fibers expressing the slow (type I) MHC isoform from CMT patients irrespective of the etiology of the denervation, i.e., demyelinating or axonal type. The expression of essential myosin light chain isoforms has been shown to have a significant modulatory impact on V0 in single muscle fibers from young rats and rabbits (17, 38), although this relationship has not been confirmed in old rats or in single muscle fibers from humans (35, 38). In the original study (17) reporting the modulatory influence of essential myosin light chains on V0, it was also observed that the variability in myosin light chain isoform expression covaried with TnT isoform expression. The results from the present study did not show a significant modulatory influence of the alternatively spliced HMW and LMW slow TnT isoforms on V0, i.e., increased V0 values were observed in MHC-I fibers from both CMT1 and CMT2 patients. However, the increased V0 in MHC-I fibers is consistent with previous observations of a positive impact of chronic overuse on V0 in MHC-I fibers in previously denervated muscles, such as in patients with a prior polio lesion (36), whereas the molecular mechanisms underlying this adaptation remain to be established.

Increases in the LMW slow TnT isoform may aid the differential diagnosis of CMT1. The diagnosis and classification of CMT are currently based on clinical, electrophysiological...
(EMG and electroneurography), and genetic evaluations. In the present study, we demonstrated the unique increase of the LMW slow TnT isoform in CMT1 but not CMT2 muscles as well as the feasibility of determining the pattern of slow TnT isoform expression on as little biopsy materials as a single skinned muscle fiber. Therefore, a clinical test to confirm the diagnosis can be done on a spare portion of regular needle biopsy muscle samples. The Western blot procedure for such determination can be done in less than a day, making the application in routine clinical diagnosis practical. The use of standard Western blot techniques does not require specialized equipment and allows almost any diagnostic laboratory to adopt this new examination. More importantly, a differential diagnosis of CMT by the patterns of slow TnT isoform expression is based on the adaptation of muscle to the neuropathology of the disease. Therefore, this phenotype-based diagnosis will be of unique value in guiding functional evaluation and clinical management.

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