Alteration of Tropomyosin Function and Folding by a Nemaline Myopathy-Causing Mutation

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ABSTRACT Mutations in the human *TPM3* gene encoding γ-tropomyosin (α-tropomyosin-slow) expressed in slow skeletal muscle fibers cause nemaline myopathy. Nemaline myopathy is a rare, clinically heterogeneous congenital skeletal muscle disease with associated muscle weakness, characterized by the presence of nemaline rods in muscle fibers. In one missense mutation the codon corresponding to Met-8, a highly conserved residue, is changed to Arg. Here, a rat fast α-tropomyosin cDNA with the Met8Arg mutation was expressed in *Escherichia coli* to investigate the effect of the mutation on in vitro function. The Met8Arg mutation reduces tropomyosin affinity for regulated actin 30- to 100-fold. Ca^{2+}-sensitive regulatory function is retained, although activation of the actomyosin S1 ATPase in the presence of Ca^{2+} is reduced. The poor activation may reflect weakened actin affinity or reduced effectiveness in switching the thin filament to the open, force-producing state. The presence of the Met8Arg mutation severely, but locally, destabilizes the tropomyosin coiled coil in a model peptide, and would be expected to impair end-to-end association between TMs on the thin filament. In muscle, the mutation may alter thin filament assembly consequent to lower actin affinity and altered binding of the N-terminus to tropomodulin at the pointed end of the filament. The mutation may also reduce force generation during activation.

INTRODUCTION

Nemaline myopathy is a rare, clinically heterogeneous congenital skeletal muscle disease with associated muscle weakness, characterized by the presence of nemaline rods in skeletal muscle fibers, and within the nucleus in severe cases (Shy et al., 1963; reviewed in North et al., 1997; Laing, 1999). Nemaline rods originate at the Z-line and are formed of actin filaments and α-actinin, which presumably cross-links the filaments (Yamaguchi et al., 1978, 1982; Jockusch et al., 1980; Morris et al., 1990; Wallgren-Pettersson et al., 1995) implying that the mutations may affect thin filament assembly or stability. Congenital nemaline myopathy has, so far, been mapped to three genetic loci: the *NEM1* locus is the *TPM3* gene that encodes γ-TM (α-TM-slow, Laing et al., 1995), the *NEM2* locus is the nebulin gene (Pelin et al., 1999), and *ACTA1* is the skeletal muscle α-actin gene (Nowak et al., 1999). In the present study we have investigated the effect of a missense mutation on tropomyosin (TM) function.

Tropomyosin is a coiled coil protein that binds head-to-tail along the length of actin filaments. The striated muscle thin filament contains actin, TM, and troponin (Tn), and is cooperatively activated by two ligands: Ca^{2+}, which binds to TnC, and myosin, which binds to and whose ATPase is activated by actin. The cooperativity depends on TM (reviewed in Lehrer, 1994; Tobacman, 1996). Tropomyosins form a highly conserved family of proteins (reviewed in Pittenger et al., 1994). Three forms are expressed in human striated muscles. The most abundant forms are α-TM, a product of the *TPM1* gene; β-TM (*TPM2* gene), and γ-TM (*TPM3* gene, also referred to as the nmTM gene). α-TM and γ-TM have similar sequences, being 92.6% identical in humans (Reinach and MacLeod, 1986; Clayton et al., 1988; MacLeod and Gooding, 1988). The isoforms are expressed in developmental and fiber-specific patterns (Salviati et al., 1984; reviewed in Schiaffino and Reggiani, 1996). For example, type 2, fast fibers (as well as cardiac muscles) may have αα-TM or αβ-TM, whereas type 1, slow fibers contain γ-TM, and the other two isoforms. For this reason, α-TM is sometimes referred to as α-TM-fast, and γ-TM as α-TM-slow.

Three independent mutations have been identified in the *TPM3* gene that cause nemaline myopathy: a missense mutation, Met9Arg (Laing et al., 1995), recently referred to as Met8Arg (Laing, 1999), is dominant; a nonsense mutation at codon 31, resulting in premature termination, is recessive (Tan et al., 1999); and a missense mutation of the termination codon 285 to Ser, resulting in 57 additional amino acids encoded at the C-terminus (A. Beggs, personal communication), causes recessive but severe disease. The two mutations that would result in expressed protein are at the ends of TM, the site of head-to-tail association of TM molecules along the actin filament. It is well-established that the ends of TM are critical determinants of actin affinity and cooperative TM function (e.g., Lehrer et al., 1997; Moraczewska et al., 1999). The N-terminal sequence of

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284-residue TMs is highly conserved throughout phylogeny, and Met-8 is invariant.

In the present work we have introduced the Met8Arg mutation into a rat α-TM-fast cDNA, and studied the effect of the mutation on the in vitro function of recombinant protein expressed in *Escherichia coli.* Actin affinity is greatly reduced. Ca^{2+}-sensitive regulatory function is normal, although activation of the actomyosin S1 ATPase in the presence of Ca^{2+} is reduced. The presence of the Met8Arg mutation severely, but locally, destabilizes the TM coiled coil in a model peptide. We suggest that the mutation would severely alter end-to-end association of TM molecules on the thin filament. The functional consequence of the mutation could be lower actin affinity and thin filament assembly, stability, and reduced force generation during activation.

MATERIALS AND METHODS

Nemaline myopathy mutant construction

General DNA recombination methods were carried out as described by Sambrook et al. (1989) or as recommended by the supplier. Plasmid DNA was purified using Quiagen DNA preparation kits (Chatsworth, CA).

The nemaline myopathy mutation was introduced using oligonucleotide-directed mutagenesis into rat striated muscle α-TM cDNA (gift of Dr. B. Nadal-Ginard; Ruiz-Opazo and Nadal-Ginard, 1987) that was previously cloned into pET11d (Studier et al., 1990; Hammell and Hitchcock-DeGregori, 1996). Two oligonucleotide primers were synthesized on an Applied Biosystems DNA synthesizer, purified using a NENSORB cartridge (UMDNJ-DNA Synthesis and Sequencing Facility at Robert Wood Johnson Medical School, Piscataway, NJ) and phosphorylated enzymatically using phage T4 polynucleotide kinase. One oligonucleotide (a 25-mer) was complementary to pET11d plasmid and cDNA coding sequence 5′ to the Met8Arg mutation: 3′-GTGGTACCTGCGGTAGTTCTTCTTC-5′. The second oligonucleotide (a 27-mer) was the coding sequence starting at codon 8 which was changed from ATG (Met) to CGC (Arg): 5′-*CGC*CAGATGCTGAAGCTCGACAAAGAG-3′. The synthesis was carried out using a Stratagene ExSite PCR-based site-directed mutagenesis kit (La Jolla, CA) with Taq DNA polymerase. The PCR product was ligated using T_4 ligase and used for transformation of Stratagene Epicurean Coli XL1-Blue Supercompetent Cells (La Jolla, CA). The transformed cells were plated for colony isolation. Single colonies were reisolated and then used for DNA preparation. The mutation was confirmed by sequencing of the entire cDNA on an ABI Perkin-Elmer-Cetus 277 PRISM automatic DNA sequencer by the UMDNJ-DNA Synthesis and Sequencing Facility at Robert Wood Johnson Medical School, Piscataway, NJ.

Protein expression and purification

Recombinant TM was expressed in *E. coli* BL21(DE3)pLysS expression cells (Studier et al., 1990) and purified as previously described (Hitchcock-DeGregori and Heald, 1987; Hammell and Hitchcock-DeGregori, 1996) except the $(NH_4)_2SO_4$ fractionation was 35–70% saturation instead of 35–60%. The N-terminal Met is unacetylated when expressed in *E. coli*. The mutation was further confirmed by sequencing of the N-terminal 12 amino acids on Procise cLC amino acid sequencer at the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT).

Actin was isolated from White Leghorn chicken pectoral muscle acetone powder (Hitchcock-DeGregori et al., 1982), except that actin was polymerized by addition of KCl and $MgCl_2$ to 20 mM and 0.7 mM, respectively, and incubated at 37°C for 10 min before polymerization at room temperature. Myosin S1 was prepared by papain digestion of chicken pectoral myosin (Margossian and Lowey, 1982). Troponin was purified from chicken pectoral muscle (gift of Dr. J. Fagan, Rutgers University, New Brunswick, NJ) according to the method of Potter (1982), with modifications described in Moraczewska et al. (1999).

The concentrations of actin, myosin S1, and Tn were spectrophotometrically determined using the extinction coefficients at 280 (0.1%) of 1.1, 0.83, and 0.45, respectively. Concentrations of recombinant TM were determined by differential absorption spectra of tyrosine as previously described (Edelhoch, 1967; Lehrer, 1975; Hammell and Hitchcock-DeGregori, 1996).

Actin binding assays

TM binding to F-actin was measured using a cosedimentation assay as previously described (Heald and Hitchcock-DeGregori, 1988) with modifications (Urbancikova and Hitchcock-DeGregori, 1994). The amount of bound and free TM in the pellets and supernatants, respectively, were quantified by densitometry of SDS-polyacrylamide gels (Laemmli, 1970) using Molecular Dynamics Model 300A computing densitometer (Sunnyvale, CA). The apparent K_a of TM for F-actin and Hill coefficient (α^H) were determined by fitting the experimental data to the equation using SigmaPlot (Jandel Scientific, San Rafael, CA):

$$v = n[\mathrm{TM}]^{nH}K_{app}^{nH}/(1 + [\mathrm{TM}]^{nH}K_{app}^{nH}) \quad (1)$$

where v = fraction maximal TM binding to actin, n = maximal TM bound, $[TM] = [TM]_{free}$. The TM/actin ratio was normalized to 1.0 by dividing the TM/actin ratio obtained from densitometry by the TM/actin maximal ratio (n) from each experiment calculated using Eq. 1. The TM/actin density ratio at saturation was the same for all TM isoforms, indicating that the same mass of TM binds to actin independent of the length of the TM. We have previously shown that the observed density ratio at saturation reflects stoichiometric binding of TM to actin (Cho and Hitchcock-DeGregori, 1991). We have normalized the data because the intensity of the staining is somewhat variable from experiment to experiment.

Myosin S1-induced tropomyosin binding to actin

Actin (3 μM) and TM (1 μM) in 30 mM NaCl, 0.5 mM $MgCl_2$, 1 mM DTT, 10 mM imidazole, pH 7.0, were mixed with myosin S1 (0–4.2 μM). The mixture was incubated at room temperature for 30 min to ensure hydrolysis of residual ATP from F-actin and then centrifuged in a TLA-100 rotor for 25 min, at 60,000 rpm, 20°C, in a Beckman TL-100 ultracentrifuge (Fullerton, CA). The pellets were washed with assay buffer and then solubilized in actin extraction buffer (5 mM imidazole, pH 7.0, 0.5 mM DTT, 0.1 mM $CaCl_2$, 0.1 mM ATP) by sonication in an ultrasonic cleaner. Pellets were electrophoresed on 12% SDS-PAGE gels (Laemmli, 1970). Proteins were visualized with Coomassie brilliant blue. The composition of proteins sedimented in pellets was analyzed by densitometry. The results were plotted as the TM/actin and S1/actin ratio obtained from intensities of protein bands on the gel versus the initial S1/actin molar ratio. The data were fit to Eq. 2, modified from Eq. 1, using SigmaPlot (Jandel Scientific, San Rafael, CA):

$$v = n[\mathrm{X}]^{nH}K^{nH}/(1 + [\mathrm{X}]^{nH}K^{nH}) + C \quad (2)$$

where v = TM/actin ratio; [X] = S1/actin molar ratio, and C = TM/actin ratio without S1. The S1/actin ratio necessary for half-maximal saturation of actin with TM = $1/K$. The maximal TM/actin ratio was the same for both tropomyosins, indicative of complete saturation.

Acto-myosin S1 MgATPase assay

The acto-S1 ATPase activity was measured as a function of TM concentration using 5 μM actin, 1 μM myosin S1, 1 μM Tn, and 0 to 1 μM TM in 40 mM NaCl, 5 mM $MgCl_2$, 5 mM imidazole, pH 7.0, 0.5 mM DTT, and either 0.1 mM $CaCl_2$ or 0.2 mM EGTA. Assays were carried out in 96-well microtiter plates at 28°C in a thermoequilibrated Molecular Devices ThermoMax microtiter reader (Menlo Park, CA). The reaction was initiated by adding MgATP to final concentration 2 mM and terminated after 15 min by adding SDS and EDTA to final concentration 3.3% and 30 mM, respectively. The amount of inorganic phosphate released was determined colorimetically (White, 1982). The plates were read in a Molecular Devices ThermoMax plate reader with a 650 nm filter (Menlo Park, CA). Specific activity was expressed as nanomoles Pi/s/nmol S1.

The Ca^{2+}-dependence of the acto-myosin S1 ATPase was carried out under the same conditions except that wild-type TM = 0.8 μM, Met8Arg TM = 5 μM, and in the presence of 0.45 mM CaEGTA. The ratio of Ca^{2+}/EGTA was varied to determine the desired free Ca^{2+}, as previously described (Mukherjea et al., 1999). The data were fit to Eq. 2, where v = specific activity, n = maximal specific activity, [X] = $[Ca^{2+}]$, K_{app} = apparent K_a of Ca^{2+} for activation, and C = constant for activity at $[Ca^{2+}] = 0$.

Peptide synthesis and circular dichroism measurements

TMZip was synthesized as previously described (Greenfield et al., 1998). Met8Arg TMZip was synthesized on a Rainin Symphony instrument (HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory, Yale University School of Medicine). The N-terminal methionine was acetylated. The molecular weight, 3913.6, determined by MALDI mass spectrometry, was within the limits of accuracy of the technique of the weight of expected from the amino acid sequence, 3912.0.

CD measurements were made and analyzed using an Aviv model 62 DS spectropolarimeter (Lakeview, NJ) as previously described (Greenfield et al., 1998).

RESULTS AND DISCUSSION

To gain an understanding of how the nemaline myopathy-causing mutation of Met9Arg in γ-TM encoded by *TPM3* causes disease, recently referred to as Met8Arg in Laing (1999), we investigated the effect of the mutation on TM structure and function. In γ-TM Met-9 corresponds to Met-8 in other TMs. It is conserved throughout phylogeny in 284-residue TMs or their equivalents. The first nine residues of TM have been proposed to overlap with the C-terminal nine residues of the adjacent TM on the actin filament. The N-terminal overlap region forms a coiled coil structure in a model peptide (Greenfield et al., 1998) although the structure of the complex is unresolved in the TM x-ray structure (Whitby and Phillips, 2000). Met-8 is at an *a* position at the interface between the two chains of the coiled coil (Fig. 1).

Since the striated muscle α-TM (α-TM-fast) has been extensively analyzed, we introduced the Met8Arg mutation into the rat striated α-TM cDNA. There are 19 amino acid differences between rat striated α-TM and human striated γ-TM, spaced throughout the sequence. In the overlap region, residue 2 is Asp in rat α-TM and Glu in human γ-TM, as it is in some vertebrate β-TMs. The sequences are then identical until residue 28 (D28E, K29Q, A31Q, D34E). The mutation was initially referred to as Met9Arg because of an additional Met at the N-terminus in the original cDNA sequence (MacLeod and Gooding, 1988; Laing, 1999). More recently, the mutation has been called Met8Arg (Laing, 1999) because the first Met is presumably removed after translation. Rat Met8Arg TM expressed well in *E. coli*; the N-Met is unacetylated.

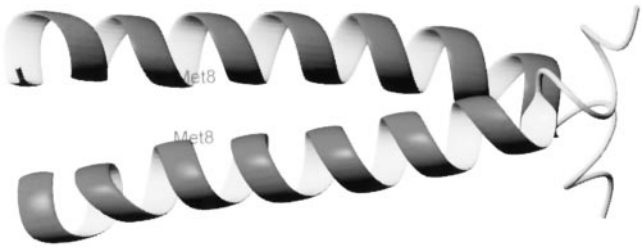

FIGURE 1 Ribbon diagram of the structure of the N-terminus of tropomyosin in a model peptide with the site of the Met8Arg mutation. The model shows the coiled coil structure of TMZip, a synthetic peptide with the first 14 residues of rat striated α-TM and the last 18 residues of the yeast GCN4 leucine zipper solved at atomic resolution using 2D-NMR (Greenfield et al., 1998). The model is an average of the 15 best structures from the NMR data. Residues 1–29 form a continuous coiled coil α-helix, while the last three residues (GlyGluArg) are disordered. The interface position of the mutation is indicated. The model was prepared with MOLMOL (Koradi et al., 1996) using the coordinates deposited in the Protein Data Bank (1TMZ, accession number 7983). The N-terminus is at the left.

Actin affinity

A universal TM function is the ability to bind cooperatively to F-actin. In skeletal muscle, TM and Tn assemble with F-actin to form regulated actin filaments. Fig. 2 *A* shows that Met8Arg TM bound to actin with Tn ($+Ca^{2+}$), but the affinity was at least 30-fold weaker than wild type, at the limit of sensitivity for our assay. With actin alone, binding of Met8Arg TM, like wild-type TM, was too weak to measure, a consequence of the unacetylated N-terminal Met (Heald and Hitchcock-DeGregori, 1988; Urbancikova and Hitchcock-DeGregori, 1994). In the absence of Ca^{2+}, the actin binding was stronger and cooperative, but Met8Arg TM still bound with ~100-fold weaker affinity than wild-type TM (Fig. 2 *B*). Clearly, the single amino acid change dramatically affects the assembly of Met8Arg TM onto regulated actin filaments.

Myosin S1-induced binding of Met8Arg tropomyosin to actin

Myosin S1 increases the affinity of TM for actin (Eaton, 1976; Cassell and Tobacman, 1996; Lehrer and Geeves, 1998). In terms of the Geeves and Lehrer model for thin filament regulation, the binding of myosin heads (myosin S1 or myosin S1-ADP) to actin shifts the equilibrium of

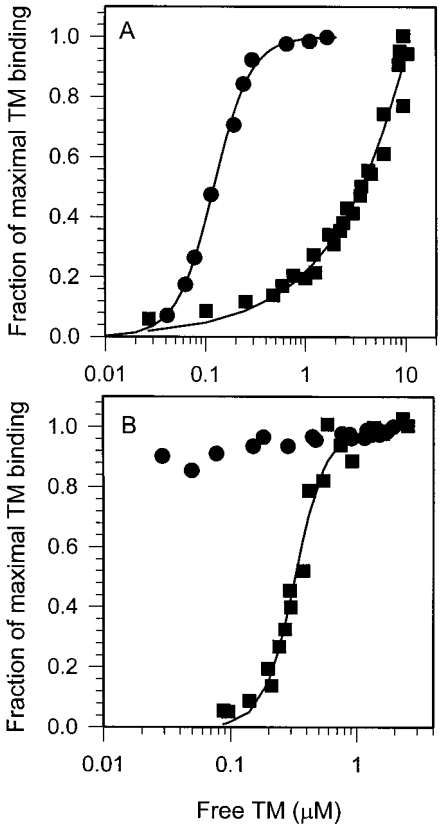

FIGURE 2 Binding of wild-type (●) and M8R tropomyosin (■) to actin in the presence of Tn, $+Ca^{2+}$ (*A*) and Tn, $-Ca^{2+}$ (*B*). Tropomyosin at increasing concentrations was cosedimented with 5 μM actin in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM $CaCl_2$ or 0.2 mM EGTA, as described in Materials and Methods. The troponin concentration was at 1.2 molar excess over TM. The TM binding curves were fit to the data using the Hill equation (Table 1).

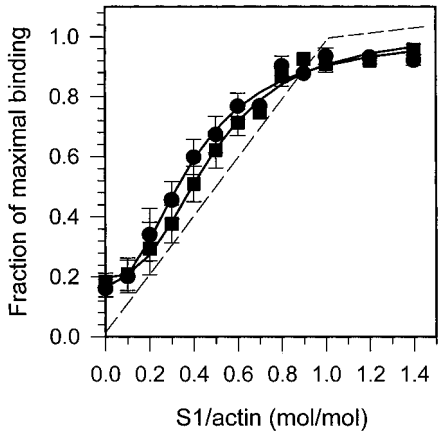

FIGURE 3 Myosin S1-induced binding of wild-type tropomyosin (●) and M8RTM (■) to actin. Binding of TM (1 μM) and S1 to actin (3 μM) was measured as a function of S1 concentration (0–4.2 μM) in 30 mM NaCl, 0.5 mM $MgCl_2$, 1 mM DTT, 10 mM imidazole, pH 7.0, as described in Materials and Methods. The TM binding curves were fit to the data from three experiments using the Hill equation (Table 1). The dashed line shows myosin S1 binding to actin, fit by linear regression to the experimental points for the S1/actin ratios from 0–1.0. The line at saturation was drawn manually.

actin-TM from the closed state to the open state, in which TM binds to actin with higher affinity and myosin binds strongly and develops force (McKillop and Geeves, 1993; reviewed in Lehrer, 1994; Lehrer and Geeves, 1998). We have monitored myosin S1-induced binding of TM to actin in the open state using a direct cosedimentation assay (Eaton, 1976; Moraczewska et al., 1999). Myosin S1 induced Met8Arg TM to bind to actin, but somewhat more myosin S1 was consistently required for half-maximal binding (Fig. 3, Table 1). The difference reflects the weaker actin affinity of Met8Arg TM, a major determinant of the cooperativity of myosin S1 in inducing TM binding to actin (Eaton, 1976; Moraczewska et al., 1999).

Regulation of the actomyosin S1 ATPase with troponin

Another measure of TM's regulatory function is its ability to regulate the actomyosin ATPase with Tn in a Ca^{2+}-dependent fashion. Inhibition in the absence of Ca^{2+} and activation in the presence of Ca^{2+} are analogs of relaxation and contraction, respectively. Fig. 4 shows that Met8Arg TM could inhibit the actomyosin S1 ATPase to the same extent as wild-type TM, but there was little activation in the presence of Ca^{2+}, even at much higher TM concentrations (5 μM) where Met8Arg TM would partially saturate the thin filament (Fig. 2, Table 1). The lower activation in the presence of Ca^{2+} may indicate reduced effectiveness in switching the thin filament to the open, force-producing state related to reduced affinity or dissociation of TM. We could not determine the actin affinity of Met8Arg TM for actin in the conditions of the ATPase assay because Tn binds non-specifically to actin at low ionic strength and may carry TM with it (Hitchcock, 1975).

The Ca^{2+}-dependence of the regulated acto-myosin S1 ATPase was similar with wild-type and Met8Arg TM (Fig. 5). Although the cooperativity with Met8Arg TM may be slightly greater than with wild type, the Ca^{2+}-dependence was the same. The main difference, as in Fig. 4, was the reduced activation in the presence of Ca^{2+}. In contrast, Michele et al. (1999a) reported a slightly lower Ca^{2+} sensitivity of steady-state isometric force of adult cardiac myocytes expressing Met8Arg TM compared to wild type. As our steady-state ATPases are in solution, the conditions would relate, at best, to unloaded shortening velocity in an intact muscle.

The functional assays all required different ionic conditions, for practical reasons. The myosin-S1 induced binding was assayed in conditions where TM alone binds poorly to actin. The higher ionic strength (150 mM NaCl) used to measure actin affinity was chosen to optimize TM binding

TABLE 1 Binding of wild-type and mutant TM to actin in the presence of Tn or myosin S1

	TM-actin binding constant K_{app} (M^{-1})			
	Tn, + Ca^{2+}	Tn, − Ca^{2+}	S1/actin molar ratio*	Number of S1 per 7 actin subunits*
wt TM	$8.2 \pm 0.22 \times 10^6$	$>10^8$	0.38 ± 0.08	2.7 ± 0.5
M8R TM	$\approx 3 \times 10^5$	$3.1 \pm 0.10 \times 10^6$	0.47 ± 0.07	3.3 ± 0.5

*Myosin S1/actin ratio for half-maximal saturation of F-actin with TM ± SE ($n = 3$). The values are statistically different ($p < 0.005$, paired t-test).

while minimizing actin binding of Tn alone to actin (Hitchcock, 1975). The ATPases were at lower ionic strength to allow higher acto-myosin S1 ATPase, while the high Mg^{2+} concentration promotes actin binding. The TM affinity would be expected to be at least as high as that in the cosedimentation experiment.

Michele et al. (1999a) have expressed Met8Arg TM in cultured adult cardiac myocytes using adenoviral-mediated gene transfer. The mutant TM is incorporated into the myofibrils and the myocytes are capable of normal force generation with reduced Ca^{2+} sensitivity. It is difficult to compare their results to ours for several reasons. Most important is that their recombinant TMs carry an eight-residue, highly charged acidic FLAG epitope on the C-terminus to allow localization using immunocytochemistry. The effect of the epitope on TM function has not been investigated in vitro, but it is attached to a functionally critical end of TM known to bind to Tn (Hammell and Hitchcock-DeGregori, 1996, 1997). A mutation resulting in a C-terminal extension results in nemaline myopathy (Beggs, personal communication). Also, the C-terminal nine amino acids overlap with the highly conserved first nine amino acids at the N-terminus to form a complex that is critical for actin binding and regulatory function, and is the site of the Met8Arg mutation. The expressed FLAG-TMs (mutant or wild-type) are not incorporated along the full I band in myocytes used for force measurements after 5–6 days in culture (Michele et al., 1999a, b). The published gels show that the FLAG-TMs may be selectively extracted upon cell permeabilization; wild-type FLAG-TM does not seem to replace the endogenous TM. The endogenous TM is

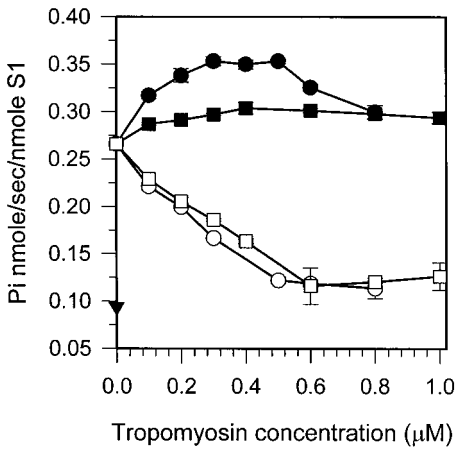

FIGURE 4 Acto-myosin S1 ATPase regulation by wild-type (*circles*) and M8R tropomyosin (*squares*). Actin (5 μM), S1 (1 μM), Tn (1 μM), and tropomyosin (0–1 μM) were incubated at 28°C in 5 mM imidazole, pH 7.0, 40 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, and either 0.1 mM $CaCl_2$ (*closed symbols*) or 0.2 mM EGTA (*open symbols*). The triangle indicates ATPase activity of myosin S1 alone.

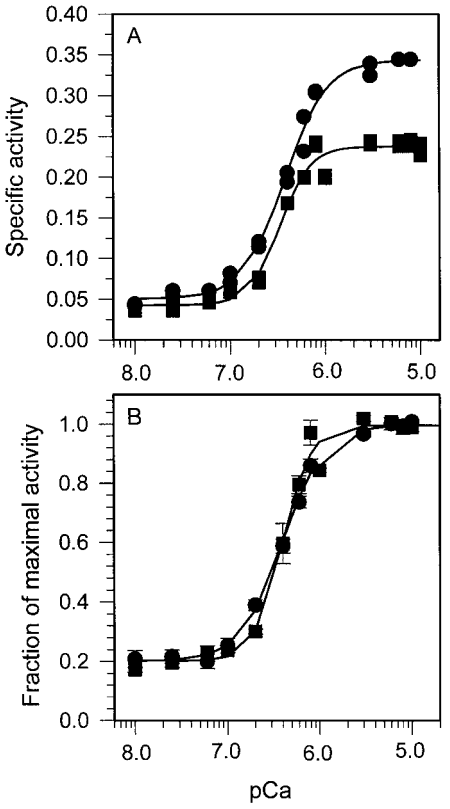

FIGURE 5 Calcium dependence of the acto-myosin S1 ATPase. The conditions are as for Fig. 4 except wild-type TM was 0.8 μM (●) and Met8Arg TM was 5 μM (■) and with 0.45 mM CaEGTA. The free Ca^{2+} concentration was controlled using CaEGTA buffer (see Materials and Methods). (*A*) A representative experiment showing specific activity (nmol P_i/nmol S1/s). (*B*) Normalized data from three (wt TM) or two (Met8Arg TM) independent experiments. Each set of data was normalized individually to its own maximal activity. The points are averages (±SE) of normalized data. The Ca^{2+} concentration for half-maximal ATPase activation was the same for both TMs ($p > 0.95$, t-test): $3.98 \pm 0.19 \times 10^{-7}$ ($n = 3$) for wild-type TM, and $4.03 \pm 0.68 \times 10^{-7}$ for Met8Arg TM ($n = 2$). The Hill coefficient was 1.79 ± 0.16 ($n = 3$) for wild-type TM and 2.96 ± 0.26 ($n = 2$) for Met8Arg TM. These values are marginally different ($p = 0.07$, t-test).

not down-regulated unless the myocytes redifferentiate (Michele et al., 1999b).

Local destabilization of TM by Met8Arg

Conformational analysis of Met8Arg TM using circular dichroism showed that the effect of the mutation on the TM conformation is local and that it did not affect the overall folding and conformation of TM (Fig. 6 *A*). The mutation caused a decrease in T_M of ~0.6°C (T_M of wild-type TM = 42.9 ± 0.5°C, n = 6; Met8Arg TM = 42.3 ± 0.2°C, n = 3; 1.5 μM TM). There was also a small change in the mean residue ellipticity of the fully folded proteins at 0°C, but the difference was within the experimental error of the determination of the protein concentration. The lack of effect would be anticipated since TM is a linear molecule and the mutation represents only one residue out of 284.

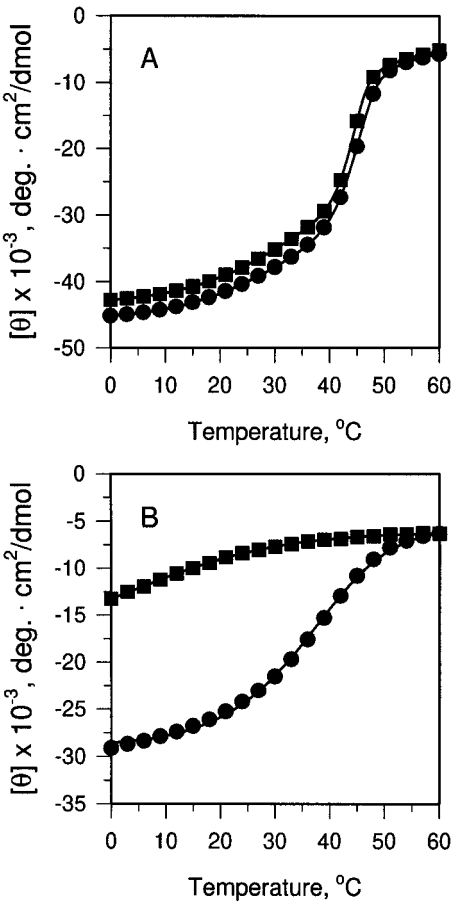

FIGURE 6 Effect of the nemaline myopathy mutation on the folding of a tropomyosin and a tropomyosin model peptide. (*A*) Circular dichroism of wild-type TM (●) and Met8ArgTM (■). Mean residue ellipticity at 222 nm as a function of temperature in 500 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM sodium phosphate, pH 7.5, 1.5 μM protein. (*B*) Circular dichroism of TMZip and M8R TMZip. Mean residue ellipticity at 222 nm as a function of temperature in 100 mM NaCl, 10 mM $NaPO_4$, pH 6.9. TMZip (●) and M8RTMZip (■) were 1.1 mg/ml (150 μM).

Although the Met8Arg mutation had little effect on the overall stability of TM, its location at an *a* interface position of the coiled-coil heptapeptide repeat predicts a local destabilization effect (Greenfield and Hitchcock-DeGregori, 1995). To test this idea, we introduced the mutation into a model peptide, TMZip, whose structure had been determined using 2D-NMR (Greenfield et al., 1998). TMZip consists of the first 14 N-terminal residues of rat striated α-TM and the last C-terminal 18 residues of the GCN4 leucine zipper (Landschulz et al., 1988; O'Shea et al., 1991) to allow formation of a stable two-chained coiled coil. The N-terminal Met is acetylated. TMZip forms a continuous coiled coil α-helix from residues 1–29; the C-terminus (GlyGluArg) is disordered in TMZip as well as in the parent GCN4 peptide (O'Shea et al., 1991; Greenfield et al., 1998). Fig. 1 illustrates the interface position of the Met8Arg mutation in the structure.

The folding properties of TMZip and Met8Arg TMZip were also compared using CD spectroscopy. The mutation profoundly decreased the stability of TMZip. At 150 μM, the apparent T_M folding was decreased from 36°C to ~1°C (Fig. 6 *B*, Table 2). To better estimate the mean residue ellipticity of the Met8Arg peptide, and the value of T_M at K = 1, CD data were collected over a wide concentration range and were globally fit to the Gibbs-Helmholtz equation for the unfolding of a two-stranded dimer to monomer. The mutation decreased the enthalpy of unfolding from 38.5 to 22.6 Kcal/mol, and the mean residue ellipticity at 222 nm decreased from −27,800 to −15,900 deg · cm^2/dmol, although the extrapolated T_M value of folding at K = 1 was almost unchanged. The results show that the mutation greatly destabilizes the folding of TMZip, although it can still partially fold to form a coiled coil at very low temperatures. The folding is probably mainly due to the C-terminal, GCN4 portion of the peptide. The stability of Met8ArgTMZip is similar to that of a 24-residue GCN4 peptide corresponding to the C-terminal portion of TMZip (Lumb et al., 1994). We suggest that the decrease in the enthalpy and loss of ellipticity are caused by the loss of approximately four helical turns of coiled coil (13–16 residues) that correspond to the N-terminal TM portion of the peptide.

The recombinant TM expressed in *E. coli* is unacetylated. We have shown that N-acetylation stabilizes the coiled coil conformation of an N-terminal peptide (Greenfield et al., 1994) and increases actin affinity of striated α-TM (Urbancikova and Hitchcock-DeGregori, 1994). The results here with the peptides show that the mutation is deleterious to the TM structure even when the N-terminal Met is acetylated.

Competition experiments

In *TPM3* the Met8Arg mutation exhibits autosomal dominant inheritance (Laing et al., 1995). Tropomyosin is a two-chained coiled coil and in heterozygotes half of the

TABLE 2 Effect of the Met8Arg mutation on the thermodynamics of folding of a chimeric N-terminal peptide, TMZip

Peptide	$\theta_{Max} \times 10^3$ deg · cm^2 · dmol^{-1}	$\theta_{Min} \times 10^3$ deg · cm^2 · dmol^{-1}	$\Delta H_{unfolding}$ Kcal/mol	$T_{M(apparent)}$, °C 150 μM	T_M, °C $K = 1$, $\Delta G = 0$
TMZip	−27.8 ± 0.1	−6.2 ± 0.1	38.5 ± 0.2	36.1 ± 0.1	82.0 ± 0.4
M8R TMZip	−15.9 ± 0.1	−6.6 ± 0.1	22.6 ± 0.3	1.3 ± 0.3	84.0 ± 0.9

The sequence of TMZip (Greenfield et al., 1998) is: Ac-MDAIKKK (M/R)QMLKLD NYHLENE VARLKKL VGER where the first 14 residues are from TM and the last 18 are from the GCN4 leucine zipper (Landschulz et al., 1988). In γ-TM residue 2 is Glu instead of Asp. Circular dichroism data were collected from 0 to 60°C in 100 mM NaCl, 10 mM sodium phosphate pH 6.9 at 222 nm. The concentrations of TMZip were 1.5, 7.7, 15, and 150 μM, and of Met8Arg TMZip were 15, 38, 150, and 224 μM. Data were fit globally (± SE) to a two-state transition between a folded dimer and an unfolded monomer as described in Greenfield et al. (1998).

expressed γγ-TM molecules should have one normal chain and one mutant chain, given the similar stabilities in vitro of the full-length wild-type and mutant TMs. γ-Tropomyosin could also form heterodimers with the products of other TM genes. Since the mutation has only a local effect on the stability of the TM coiled coil, mutant TM could be dominant by negatively influencing N-terminal-associated functions of the wild-type TM chain in heterodimers: actin affinity, head-to-tail association of neighboring TM molecules on the actin filament, binding to tropomodulin, and interaction with Tn at TM's molecular ends. The mutation could also have a dominant effect in homodimers by cooperatively influencing the functions of neighboring TM molecules, such as end-to-end association. To address the latter question, we compared the actin binding (with Tn, $+Ca^{2+}$) of wild-type-Met8Arg TM mixtures to the binding of the two forms alone over a concentration range where wild-type TM binds well and Met8Arg TM binds poorly. The binding of the mixed samples was indistinguishable from the binding of the sum of the two forms alone (results not shown). The failure of Met8Arg homodimers to compete with wild type may reflect its low affinity and the absence of end-to-end association in solution (versus on the filament) in the conditions of our experiment. We obtained similar negative results in previous mixing experiments with TMs carrying N-terminal modifications (Heald and Hitchcock-DeGregori, 1988). We could not evaluate the function of heterodimers. Since two TMs comigrate in SDS-PAGE and in urea gels, we were unable to confirm the presence of heterodimers in unfolded and refolded mixtures of wild-type and mutant TM. Michele et al. (1999a) did not address the question of whether the FLAG-TMs form heterodimers with endogenous TM.

CONCLUSIONS

The effect of the nemaline myopathy-causing mutation in TM, Met8Arg, is consistent with the mutation affecting thin filament and sarcomere assembly, and cooperative functions within the thin filament, rather than affecting the ability of TM to form a stable coiled coil. Our in vitro experiments suggest that if Met8Arg TM can assemble into a regulated actin filament it can function quite normally. When bound, the extent of inhibition of the regulated acto-myosin S1 ATPase by Met8Arg TM in the absence of Ca^{2+} and the Ca^{2+}-dependence of the acto-myosin S1 ATPase are normal. The mutation impairs myosin S1-induced TM binding as well as activation of the acto-myosin S1 ATPase in the presence of Ca^{2+}. These results imply that the mutation impairs the switch of the thin filament to the full open, force-producing state and may cause associated muscle weakness. However, the main effect of the mutation is the weaker actin affinity. Impaired end-to-end interactions of the TMs along the length of the thin filament may make thin filament assembly more difficult and result in less stable actin filaments. The N-terminus of TM is oriented toward the "pointed" or "minus" end of the thin filament, away from the Z-line, where the thin filament is capped by tropomodulin (Weber et al., 1994, 1999; Fowler et al., 1993). The Met8Arg mutation may alter the dynamics of the actin filament during initial assembly and in the mature myofibril. Failure of tropomodulin to properly cap thin filaments may result in long filaments that prevent normal sarcomere assembly. Weakened TM binding may allow these filaments to be cross-linked by α-actinin. It would be valuable to obtain information on the myofibrillar organization and the levels of expression and localization of Met8Arg TM in the muscles of individuals harboring the mutation.

Neither our study, nor that of Michele et al. (1999a) can yet address the mechanism of dominant inheritance of the Met8Arg mutation. We would anticipate that the mutated protein is expressed and that it is dominant because it *can* form a coiled coil TM with wild-type striated muscle γ-TM or another isoform. The mutation would likely destabilize the N-terminal overlap region in homodimers or heterodimers and result in altered end-to-end association and Tn binding on the actin filament. Met-8 is conserved throughout phylogeny in 284-residue TMs, and is frequently found at the interface *a* position in the heptapeptide repeat of the coiled coil. In contrast, Arg is rare at this position, being absent from any *a* position in most TMs. One may deduce that it would impair the stability of the coiled coil, especially at the end of the molecule, whether in a homodimer or heterodimer.

The relatively mild progression of the disease is likely because *TPM3* is expressed only in type 1, slow fibers, and it is not the only TM expressed in many slow fibers. Furthermore, γ-TM muscle TM is the only long isoform expressed by *TPM3*. Were the comparable mutation to occur at Met-8 in the genes encoding α or β-TMs that encode the major muscle TM isoforms as well as TMs expressed in most non-muscle cells, the effects would likely be dominant and lethal.

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REFERENCES

Cassell, M., and L. S. Tobacman. 1996. Opposite effects of myosin subfragment 1 on binding of cardiac troponin and tropomyosin to the thin filament. *J. Biol. Chem.* 271:12867–12872.

Cho, Y. J., and S. E. Hitchcock-DeGregori. 1991. Relationship between alternatively spliced exons and functional domains in tropomyosin. *Proc. Natl. Acad. Sci. U.S.A.* 88:10153–10157.

Clayton, L., F. C. Reinach, G. M. Chumbley, and A. R. MacLeod. 1988. Organization of the hTMnm gene. Implications for the evolution of muscle and non-muscle tropomyosins. *J. Mol. Biol.* 201:507–515.

Eaton, B. L. 1976. Tropomyosin binding to F-actin induced by myosin heads. *Science.* 192:1337–1339.

Edelhoch, H. 1967. Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry.* 6:1948–1954.

Fowler, V. M., M. A. Sussmann, P. G. Miller, B. E. Flucher, and M. P. Daniels. 1993. Tropomodulin is associated with the free (pointed) ends of the thin filaments in rat skeletal muscle. *J. Cell Biol.* 120:411–420.

Greenfield, N. J., and S. E. Hitchcock-DeGregori. 1995. The stability of tropomyosin, a two-stranded coiled-coil protein, is primarily a function of the hydrophobicity of residues at the helix-helix interface. *Biochemistry.* 34:16797–16805.

Greenfield, N. J., G. T. Montelione, R. S. Farid, and S. E. Hitchcock-DeGregori. 1998. The structure of the N-terminus of striated muscle alpha-tropomyosin in a chimeric peptide: nuclear magnetic resonance structure and circular dichroism studies. *Biochemistry.* 37:7834–7843.

Greenfield, N. J., W. F. Stafford, and S. E. Hitchcock-DeGregori. 1994. The effect of N-terminal acetylation on the structure of an N-terminal tropomyosin peptide and alpha alpha-tropomyosin. *Protein Sci.* 3:402–410.

Hammell, R. L., and S. E. Hitchcock-DeGregori. 1996. Mapping the functional domains within the carboxyl terminus of alpha-tropomyosin encoded by the alternatively spliced ninth exon. *J. Biol. Chem.* 271: 4236–4242.

Hammell, R. L., and S. E. Hitchcock-DeGregori. 1997. The sequence of the alternatively spliced sixth exon of alpha-tropomyosin is critical for cooperative actin binding but not for interaction with troponin. *J. Biol. Chem.* 272:22409–22416.

Heald, R. W., and S. E. Hitchcock-DeGregori. 1988. The structure of the amino terminus of tropomyosin is critical for binding to actin in the absence and presence of troponin. *J. Biol. Chem.* 263:5254–5259.

Hitchcock, S. E. 1975. Regulation of muscle contraction: bindings of troponin and its components to actin and tropomyosin. *Eur. J. Biochem.* 52:255–263.

Hitchcock-DeGregori, S. E., and R. W. Heald. 1987. Altered actin and troponin binding of amino-terminal variants of chicken striated muscle alpha-tropomyosin expressed in *Escherichia coli*. *J. Biol. Chem.* 262: 9730–9735.

Hitchcock-DeGregori, S. E., S. Mandala, and G. A. Sachs. 1982. Changes in actin lysine reactivities during polymerization detected using a competitive labeling method. *J. Biol. Chem.* 257:12573–12580.

Jockusch, B. M., H. Veldman, G. W. Griffiths, B. A. van Oost, and F. G. Jennekens. 1980. Immunofluorescence microscopy of a myopathy. Alpha-Actinin is a major constituent of nemaline rods. *Exp. Cell. Res.* 127:409–420.

Koradi, R., M. Billeter, and K. Wuthrich. 1996. MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph.* 14: 51–55, 29–32.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.

Laing, N. G. 1999. Inherited disorders of sarcomeric proteins. *Curr. Opin. Neurol.* 12:513–518.

Laing, N. G., S. D. Wilton, P. A. Akkari, S. Dorosz, K. Boundy, C. Kneebone, P. Blumbergs, S. White, H. Watkins, and D. R. Love. 1995. A mutation in the alpha tropomyosin gene TPM3 associated with autosomal dominant nemaline myopathy *Nat. Genet.* 9:75–79.

Landschulz, W. H., P. F. Johnson, and S. L. McKnight. 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science.* 240:1759–1764.

Lehrer, S. S. 1975. Intramolecular crosslinking of tropomyosin via disulfide bond formation: evidence for chain register. *Proc. Natl. Acad. Sci. U.S.A.* 72:3377–3381.

Lehrer, S. S. 1994. The regulatory switch of the muscle thin filament: Ca2+ or myosin heads? *J. Muscle Res. Cell Motil.* 15:232–236.

Lehrer, S. S., and M. A. Geeves. 1998. The muscle thin filament as a classical cooperative/allosteric regulatory system *J. Mol. Biol.* 277: 1081–1089.

Lehrer, S. S., N. L. Golitsina, and M. A. Geeves. 1997. Actin-tropomyosin activation of myosin subfragment 1 ATPase and thin filament cooperativity. The role of tropomyosin flexibility and end-to-end interactions. *Biochemistry.* 36:13449–13454.

Lumb, K. J., C. M. Carr, and P. S. Kim. 1994. Subdomain folding of the coiled coil leucine zipper from the bZip transcription activator GCN4. *Biochemistry.* 33:7361–7367.

MacLeod, A. R., and C. Gooding. 1988. Human hTM alpha gene: expression in muscle and nonmuscle tissue. *Mol. Cell Biol.* 8:433–440.

Margossian, S. S., and S. Lowey. 1982. Preparation of myosin and its subfragments from rabbit skeletal muscle. *Methods Enzymol.* 85:55–71.

McKillop, D. F., and M. A. Geeves. 1993. Regulation of the interaction between actin and myosin subfragment 1: evidence for three states of the thin filament. *Biophys. J.* 65:693–701.

Michele, D. E., F. P. Albayya, and J. M. Metzger. 1999a. A nemaline myopathy mutation in alpha-tropomyosin causes defective regulation of striated muscle force production [In Process Citation]. *J. Clin. Invest.* 104:1575–1581.

Michele, D. E., F. P. Albayya, and J. M. Metzger. 1999b. Thin filament protein dynamics in fully differentiated adult cardiac myocytes: toward a model of sarcomere maintenance. *J. Cell Biol.* 145:1483–1495.

Moraczewska, J., K. Nicholson-Flynn, and S. E. Hitchcock-DeGregori. 1999. The ends of tropomyosin are major determinants of actin affinity and myosin subfragment 1-induced binding to F-actin in the open state. *Biochemistry.* 38:15885–15892.

Morris, E. P., G. Nneji, and J. M. Squire. 1990. The three-dimensional structure of the nemaline rod Z-band. *J. Cell Biol.* 111:2961–2978.

Mukherjea, P., L. Tong, J. G. Seidman, C. E. Seidman, and S. E. Hitchcock-DeGregori. 1999. Altered regulatory function of two familial hypertrophic cardiomyopathy troponin T mutants. *Biochemistry.* 38: 13296–13301.

North, K. N., N. G. Laing, and C. Wallgren-Pettersson. 1997. Nemaline myopathy: current concepts. *The ENMC International Consortium and Nemaline Myopathy J. Med. Genet.* 34:705–713.

Nowak, K. J., D. Wattanasirichaigoon, H. H. Goebel, M. Wilce, K. Pelin, K. Donner, R. L. Jacob, C. Hubner, K. Oexle, J. R. Anderson, C. M. Verity, K. N. North, S. T. Iannaccone, C. R. Muller, P. Nurnberg, F. Muntoni, C. Sewry, I. Hughes, R. Sutphen, A. G. Lacson, K. J. Swoboda, J. Vigneron, C. Wallgren-Pettersson, A. H. Beggs, and N. G.

Laing. 1999. Mutations in the skeletal muscle alpha-actin gene in patients with actin myopathy and nemaline myopathy. *Nat. Genet.* 23: 208–212.

O'Shea, E. K., J. D. Klemm, P. S. Kim, and T. Alber. 1991. X-ray structure of the GCN4 leucine zipper, a two stranded, parallel coiled coil. *Science.* 254:539–544.

Pelin, K., P. Hilpela, K. Donner, C. Sewry, P. A. Akkari, S. D. Wilton, D. Wattanasirichaigoon, M. L. Bang, T. Centner, F. Hanefeld, S. Odent, M. Fardeau, J. A. Urtizberea, F. Muntoni, V. Dubowitz, A. H. Beggs, N. G. Laing, S. Labeit, A. de la Chapelle, and C. Wallgren-Pettersson. 1999. Mutations in the nebulin gene associated with autosomal recessive nemaline myopathy. *Proc. Natl. Acad. Sci. U.S.A.* 96:2305–2310.

Pittenger, M. F., J. A. Kazzaz, and D. M. Helfman. 1994. Functional properties of non-muscle tropomyosin isoforms. *Curr. Opin. Cell Biol.* 6:96–104.

Potter, J. D. 1982. Preparation of troponin and its subunits. *Methods Enzymol.* 85:241–263.

Reinach, F. C., and A. R. MacLeod. 1986. Tissue-specific expression of the human tropomyosin gene involved in the generation of the trk oncogene. *Nature.* 322:648–650.

Ruiz-Opazo, N., and B. Nadal-Ginard. 1987. Alpha-tropomyosin gene organization. Alternative splicing of duplicated isotype-specific exons accounts for the production of smooth and striated muscle isoforms. *J. Biol. Chem.* 262:4755–4765.

Salviati, G., R. Betto, D. Danieli-Betto, and M. Zevani. 1984. Myofibrillar protein isoforms and sarcoplasmic reticulum Ca2+-transport activity. *Biochem. J.* 224:215–225.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, NY.

Schiaffino, S., and C. Reggiani. 1996. Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiol. Rev.* 76: 371–423.

Shy, G., W. Engel, J. Sorners, and T. Wanko. 1963. Nemaline myopathy. A new congenital myopathy. *Brain.* 86:793–810.

Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185:60–89.

Tan, P., J. Briner, E. Boltshauser, M. R. Davis, S. D. Wilton, K. North, C. Wallgren-Petterson, and N. G. Laing. 1999. Homozygosity for a nonsense mutation in the alpha-tropomyosin slow gene TPM3 in a patient with a severe infantile nemaline myopathy. *Neuromusc. Dis.* 9:573–579.

Tobacman, L. S. 1996. Thin filament-mediated regulation of cardiac contraction. *Annu. Rev. Physiol.* 58:447–481.

Urbancikova, M., and S. E. Hitchcock-DeGregori. 1994. Requirement of amino-terminal modification for striated muscle alpha- tropomyosin function. *J. Biol. Chem.* 269:24310–24315.

Wallgren-Pettersson, C., B. Jasani, G. R. Newman, G. E. Morris, S. Jones, S. Singhrao, A. Clarke, I. Virtanen, C. Holmberg, and J. Rapola. 1995. Alpha-actinin in nemaline bodies in congenital nemaline myopathy: immunological confirmation by light and electron microscopy. *Neuromusc. Dis.* 5:93–104.

Weber, A., C. R. Pennise, G. G. Babcock, and V. M. Fowler. 1994. Tropomodulin caps the pointed end of actin filaments. *J. Cell Biol.* 127:1627–1635.

Weber, A., C. R. Pennise, and V. M. Fowler. 1999. Tropomodulin increases the critical concentration of barbed end-capped actin filaments by converting ADP.P(i)-actin to ADP-actin at all pointed filament ends. *J. Biol. Chem.* 274:34637–34645.

Whitby, F. G., and G. N. Phillips, Jr. 2000. Crystal structure of tropomyosin at 7 Angstroms resolution. *Proteins.* 38:49–59.

White, H. D. 1982. Special instrumentation and techniques for kinetic studies of contractile systems. *Methods Enzymol.* 85:698–708.

Yamaguchi, M., R. M. Robson, M. H. Stromer, D. S. Dahl, and T. Oda. 1978. Actin filaments form the backbone of nemaline myopathy rods. *Nature.* 271:265–267.

Yamaguchi, M., R. M. Robson, M. H. Stromer, D. S. Dahl, and T. Oda. 1982. Nemaline myopathy rod bodies. Structure and composition. *J. Neurol. Sci.* 56:35–56.