Leucine 135 of Tropomodulin-1 Regulates Its Association with Tropomyosin, Its Cellular Localization, and the Integrity of Sarcomeres*

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Tropomodulin-1 (Tmod-1) is a well defined actin-capping protein that interacts with tropomyosin (TM) at the pointed end of actin filaments. Previous studies by others have mapped its TM-binding domain to the amino terminus from amino acid 39 to 138. In this study, we have identified several amino acid residues on Tmod-1 that are important for its interaction with TM5 (a nonmuscle TM isoform). Glutathione S-transferase affinity chromatography and immunoprecipitation assays reveal that Tmod sense mutations of either amino acid 134, 135, or 136 causes various degrees of loss of function of Tmod TM-binding ability. The reduction of TM-binding ability was relatively mild (reduced 20–40%) from the G136A Tmod mutant but more substantially (reduced 50–100%) from the I134D, L135E, and L135V Tmod mutants. In addition, mutation at any of these three sites dramatically alters the subcellular location of Tmod-1 when introduced into mammalian cells. Further analysis of these three mutants uncovered a previously unknown nuclear trafficking site within a nuclear export signal motif. As a result, mutation on either residue 134 or residue 135 not only will cause a significant reduction of the Tmod-1 ability to bind to TM5 but also lead to predominant nuclear localization of Tmod-1 by crippling its nuclear export mechanism. The failure of the Tmod mutations to fully associate with TM5 when introduced into neonatal rat cardiomyocytes was also associated with an accelerated and severe fragmentation of sarcomeric structures compared with overexpression of wild type Tmod-1. The multiple losses of function of Tmod engendered by these missense mutations are most severe with the single substitution of residue 135.

Tropomodulins (Tmods)2 are highly conserved capping proteins for the pointed ends of the erythrocyte membrane and sarcomeric actin filaments (1). They are present in the membrane skeleton at levels of 1–2 copies per short actin filament (2, 3). Four different Tmod isoforms, Tmod-1 to -4 (often referred to as E-, N-, U- and Sk-Tmod), have been identified to date (2, 4–6). All Tmod isoforms have a similar number of amino acids and show very few deletions or insertions relative to one another (4–7). The regulatory role of Tmod in the polymerization and depolymerization of actin filaments has been well studied, and it is regarded as a major regulatory component for the architecture of the sarcomere in muscle cells and of the membrane skeleton in nonmuscle cells (3, 8, 9).

Tmod-1 was originally isolated from the erythrocyte membrane and was shown to bind to one end of erythrocyte tropomyosin (TM) (2, 10). Tmod-1 is also the dominant isoform expressed in vertebrate cardiac muscle and slow twitch muscle fibers, whereas Tmod-4 (Sk-Tmod) is the predominant isoform in fast twitch skeletal muscle (4, 11).

Subsequently, it has been shown that Tmod can completely inhibit elongation at, and depolymerization from, the pointed ends of TM-coated thin filaments and can function as an actin filament pointed end capping protein in vitro (12). Tmod requires TM for tight capping of actin filaments (Kd ≤ 1 nM); in the absence of TM, Tmod is a "leaky" cap and only partially inhibits actin monomer association and dissociation at the pointed end (Kd ~ 0.1–0.4 μM) (12). The ability of Tmod to cap the pointed ends of TM-actin filaments tightly is likely to be a consequence of binding of Tmod to both TM (Kd ~ 0.2–0.5 μM) (2, 13) and actin (Kd values, derived from the inhibition of elongation rates of pure actin filaments, range between 0.1 and 0.4 μM) (12). This interpretation is consistent with the presence of two distinct functional domains on Tmod. Conversely, the carboxyl-terminal portion of Tmod is required for binding to actin at the pointed filament end; a monoclonal antibody that binds to an epitope near the carboxyl terminus inhibits the ability of Tmod to cap the pointed ends of pure actin or TM-actin filaments but has no effect on binding of Tmod to TM (8, 14). Competition binding assays using recombinant Tmod fragments have shown that the amino-terminal half (residues 6–184) contains full binding activity for tropomyosin (13).

Initial studies by Babcock and Fowler (13) elegantly revealed that the TM-binding domains of Tmod-1 differ between muscle and nonmuscle TM isoforms. These authors identified amino acid residues 6–94 on Tmod-1 as containing a skeletal muscle TM binding site and residues 90–184 on Tmod-1 as containing an erythrocyte TM-binding domain (13). Better refined sites of interaction of Tmod-1 with TM have now been mapped (15–18).

In this study, we applied several mutagenesis techniques to map and analyze the functional domain(s) of Tmod-1 in single residue detail. In addition to defining at least three amino acid residues that contribute to TM (nonmuscle isoform) binding by Tmod-1, we were surprised to discover that these same residues were those responsible for nuclear cytoplasmic transport of Tmod (19), and the integrity of at least one residue, leucine 135, is crucial for sarcomere stability in cardiomyocytes.

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2 The abbreviations used are: Tmod, tropomodulin-1; TM, tropomyosin; HA, hemagglutinin; GST, glutathione S-transferase; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; LMW, low molecular weight/nonmuscle isoform; GFP, green fluorescent protein.
Novel Functional Domain of Tropomodulin-1

EXPERIMENTAL PROCEDURES

Plasmid Construction

Yeast Two-hybrid System—Various Tmod cDNAs, wild type or mutants, were generated by specifically designed primers (Table 1) and subcloned into either pGBT9 or pGADGH vectors (Clontech) through the EcoRI site. The Tmod-1 cDNA template used for all of the assays in this study was originally isolated from a mouse muscle cDNA library (20). All internal deletion constructs were made by a two-step PCR. In brief, specific primer pairs that targeted the deletion areas were generated and are shown in Table 1. These primer pairs were used separately and paired with another primer that matched either the N or C terminus for the first PCR (Table 1 (top), wild type Tmod-1). The products from the first PCR were then mixed and used as templates for the second PCR to create the final internal deletion constructs. Primers used to generate wild type TM5 cDNAs for subcloning into pGEMT and pGADGH vectors are also shown in Table 1. Both subclonings were done through the EcoRI sites of the vectors. The original TM5 template was kindly provided by Dr. David Helfman (21). All nucleotide sequences for the constructs were verified either by the sequencer version 2.0 T7 DNA polymerase sequencing method (U.S. Biochemical Corporation) for the E(−) psi cyclist DNA sequencing kit (Stratagene). Dideoxynucleotides were labeled by [35S]dATP (Amersham Biosciences). Sequencing gels were made by Ultra Pure Sequaged-6 ready-to-use sequencing gel solution (National Diagnostics).

Glutathione S-Transferase (GST) Fusion Protein Expression—All Tmod-1 cDNAs (wild type or mutants) and TM5 cDNA were subcloned into the pGEX3X vector (Amersham Biosciences) through the EcoRI site. Single mutation Tmod/pGEX3X constructs were created by introducing the mutation using a QuickChange site-directed mutagenesis method (Stratagene). PCR conditions were carried out following the supplier’s suggestions, and the primers used for the reactions are shown in Table 1. Sequences verification was carried out as described above.

In Vitro Transcription/Translation Assays—All Tmod cDNAs, wild type or mutants, TM5, TM5a, TM5b (nonmuscle TM isoforms), α-TM, and β-TM (muscle TM isoforms) cDNAs were subcloned into the pCDNA3 vector (Invitrogen) through the EcoRI site. The original TM5a and TM5b cDNA templates were also gifts from Dr. David Helfman (22). The α- and β-TM PCR templates were from LK clones 504 and 417, respectively. All constructs were sequenced by DNA sequence analysis (ABI PRISM 377 DNA sequencer, Applied Biosystems).

Transfection Assays—A FLAG epitope tag, DYKDDDK, was added at the N terminus of wild type and the single mutation Tmod-1 mutants by PCR. The Tmod-1 cDNAs (wild type or mutants) and TM5 cDNA templates were from LK clones 504 and 417, respectively. All constructs were sequenced by DNA sequence analysis (ABI PRISM 377 DNA sequencer, Applied Biosystems).

Transduction Assays—pHR-IRES-GFP (provided by Dr. Nori Kasahara, UCLA) was used to subclone both wild type and mutant Tmods through the EcoRI site for the generation of lentiviruses. All constructs were sequenced by DNA sequence analysis (ABI PRISM 377 DNA sequencer, Applied Biosystems).

Yeast Two-hybrid System

Yeast strains SFY and HF7c were used for the assays. β-Galactosidase activities of the lacZ reporter gene were measured by the liquid culture assay using o-nitrophenylgalactoside as substrate. We used the detailed experimental procedures provided by the Clontech yeast two-hybrid product protocol (www.ClonTech.com).

Briefly, after resuspending the overnight SFY yeast culture in 1.5 ml of 1 × TE/LiAc (0.01 M Tris-HCl, 1 mM EDTA, pH 7.5, and 0.1 mM lithium acetate, pH 7.5) solution, the cells were stored on ice until ready for co-transformation. 0.1 μg of each DNA plasmid was used for each designed reaction (pGEMT, pGADGH, Tmod/pGBT9, TM5/pGADGH, or Tmod mutants/pGEMT) together with 100 μg of salmon carrier DNA. 100 μl of the SFY yeast competent cells was then added to each reaction and mixed well. 600 μl of sterile PEG/LiAc (40% PEG 4000 and 0.1 mM lithium acetate in TE buffer) was added subsequently to each reaction and was vortexed to mix. The reactions were incubated at 30 °C with shaking for 30 min. After the incubation, 70 μl of Me2SO was added to each reaction and mixed gently. After heating for 15 min at 42 °C, the transformed yeast cells were spun and resuspended with a small volume of TE buffer for plating on appropriate medium (SD medium minus Trp, Leu, and His for SFY or SD medium minus Trp and Leu for HF7c). Inoculated plates were cultured at 30 °C for 3–7 days until colonies appeared.

Yeast Two-hybrid Liquid Culture Assay for β-Galactosidase

Four colonies from each transformation reaction were picked and cultured into appropriate synthetic medium containing glucose. Liquid cultures were incubated at 30 °C until they reached midlog phase. 0.7 ml of Z buffer with β-mercaptoethanol (16 g/liter Na2HPO4·7H2O, 5.5 g/liter NaH2PO4·H2O, 0.75 g/liter KCl, and 0.246 g/liter MgSO4·7H2O, pH 7.0; β-mercaptoethanol was added to Z buffer freshly at a concentration of 0.27 ml/100 ml of Z buffer) was added to 0.1 ml of each culture together with 50 μl of CHC3 and 50 μl of 0.1% SDS. After vortexing for 30 s, 0.16 ml of o-nitrophenylgalactoside solution (4 mg/ml in 0.1 M phosphate buffer, pH 7.0) was added to the lysed cells. The reactions were then incubated at 37 °C for 1 h and were stopped by adding 0.4 ml of 1 M Na2CO3. Levels of o-nitrophenol released from the substrate o-nitrophenylgalactoside by the lacZ gene product, β-galactosidase, were measured by reading the samples at A420. The β-Galactosidase activity was calculated by formula, β-galactosidase units = 1000 × (A420/t × V × A600), where t represents time (min) of incubation, and V is volume (ml) of culture added to Z buffer.

The β-galactosidase activity and S.D. were calculated from each transformation reaction repeated at least three times.

Random Mutagenesis

Degenerate primers 96 bases long encoding the region from amino acid 108 to 138 on Tmod were synthesized by introducing random mutations through the addition of a dNTP mixture during oligonucleotide generation following synthesis of the first three bases (Norris Cancer Center Core Facility, USC). After the PCRs, a pool of Tmod templates with lower numbers (~6–12) of random mutations were purified and then subcloned into the pCDNA3 vector for large scale screening assays. All Tmod mutants of interest were subsequently examined by DNA sequence analysis (ABI PRISM 377 DNA sequencer, Applied Biosystems).

In Vitro Transcription/Translation

One μg of various supercoiled DNA plasmids was transcribed in vitro and then translated in the presence of [35S]methionine with the TNT coupled reticulocyte lysate system (Promega) according to the manufacturer’s instructions.
Glutathione S-transferase Affinity Chromatography Assay

All GST and GST fusion proteins used in this study were recombinant proteins prepared from a bacterial expression system. Expression and purifications of GST and GST fusion proteins were performed as described previously (23). The GST proteins were analyzed on SDS-polyacrylamide gels for integrity and to normalize the amount of each protein. To perform protein-protein interaction analysis, glutathione beads (Sigma) coated with GST or the GST fusion protein (~5 μg) were reacted with 10 μl of [35S]methionine-labeled translation product in 200 μl of binding buffer containing 100 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40 detergent (Nonidet P-40), and 5 μg of ethidium bromide. The reaction was allowed to proceed for 1 h at 4 °C with gentle rocking, after which the glutathione beads were collected by brief centrifugation and subjected to extensive washing with the binding buffer. The beads were resuspended in 20 μl of SDS-PAGE sample buffer and boiled for 5 min. Eluted proteins were resolved by SDS-PAGE; the gel was dried and subjected to autoradiography.

Site-directed Mutagenesis

Single amino acid mutation Tmod mutants were generated using custom designed primers (Invitrogen) that are specific to the site of mutation (Table 1). The reactions were carried out using a QuickChange site-directed mutagenesis kit (Stratagene). PCR conditions were carried out following the supplier’s suggestions. The integrity of the coding capability of all Tmod mutants was confirmed by DNA sequence analysis (ABI PRISM 377 DNA sequencer; Applied Biosystems).

Immunoprecipitation

Human embryonal kidney cells (293T) were cultured with Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin (penicillin/streptomycin) (Sigma) both before and after transfection. Except for control plates, a total of 4 μg of DNA plasmid was transfected by calcium phosphate precipitation onto each 10-cm culture plate with cells at ~50% confluence as described previously (24). Cells were washed and collected in PBS 48 h after transfection. After centrifugation, the cell pellets were resuspended with 0.3 ml of 1× protein buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (Sigma), 10 μg/ml aprotinin (Sigma), 10 μg/ml leupeptin (Sigma), 10 μg/ml pepstatin A (Sigma)). Cells were lysed by pipetting and then centrifuged at full speed (~14,000 rpm) for 15 min at 4 °C. Supernatants were collected as total protein extracts and stored at −80 °C until ready for further analysis.

The resin for immunoprecipitation was prepared as follows. 1 μl (~3 μg) of anti-FLAG M2 antibody was preabsorbed with 15 μl of protein A/G Plus-agarose (50% slurry) for 45 min at room temperature and washed extensively. Antibody-bound protein A/G Plus-agarose was resuspended with 10 μl of 1× protein buffer. An aliquot containing 1 mg of total protein extracted from each transfection reaction (range from 2.5 to 3.5 μg/ml) was incubated with 20 μl of anti-FLAG M2 antibody-bound A/G protein beads and 250 μl of 1× protein buffer. All reactions were rocked for 2 h at 4 °C and then washed extensively with protein buffer. The beads were pelleted by centrifugation, and the

### Table 1

| Primer design for generation of wild type or mutant Tmod-1 and TM5 cDNAs | Forward | Reverse |
|---------------------------|---------|---------|
| pGBT9 vector              |         |         |
| Wild type                 | ccc gaa ttc aca att cgg atg atg tcg tac aga cga | ggc gaa ttc caa ttc ggc ttc tag aca |
| T1–211                    | gag tca ttc atg cct gat aat gca ctg c | cat gaa ttc gtt cat gcc |
| T73–138                   | gca tta gac aca ctc atg | ctt gaa ttc gtt gag aat att cgg g |
| T39–138                   | gaa cta gac ac a ctc atg | cat gag tgt gtc tag ttc atc tag c |
| TM5                       | ggt gaa ttc atg gcc ggg agc acc acc atc g | tgg gaa ttc cta cat ctc gtt gtc gac gac g |

### Table 2

| Measurements of β-galactosidase activities of wild type Tmod-1 and Tmod-1 deletion mutants when cotransformed with either a GalAD or GalAD-TMS construct in the yeast two-hybrid liquid culturing assays | GalBD: GalAD-GalBD-p300 | GalAD: GalAD-TMS-GalAD-GRIP |
|---------------------------------------------------------------------------------------------------------------------------------|-------------------------|-----------------------------|
| Wild type                                                           | 2.8 ± 0.3               | 9.9 ± 2.7                   |
| T1–211                                                              | 9.61 ± 3.63             | 16.5 ± 2.93                 |
| T73–211                                                             | 0.86 ± 0.32             | 2.4 ± 0.02                  |
| T39–138                                                             | 1.56 ± 0.2              | 5.27 ± 0.8                  |
| T39–211                                                             | 0.78 ± 0.32             | 2.95 ± 0.63                 |
| T439–138                                                            | 0.53 ± 0.18             | 0.42 ± 0.1                  |
| T73–72                                                              | 19.33 ± 4.32            | 23.47 ± 3.93                |
| T439–72                                                             | 0.39 ± 0.07             | 0.3 ± 0.05                  |
| T73–107                                                             | 12.43 ± 1.6             | 17.6 ± 1.3                  |
| T73–108–138                                                        | 1.27 ± 0.36             | 1.55 ± 0.65                 |
| GalBD-p300                                                          | 0.44 ± 0.14             | 8.4 ± 0.82                  |
supernatants were removed. The proteins bound by the beads were eluted by exposure to 15 mM phenyl phosphate. The eluted proteins were loaded directly onto an SDS acrylamide gel for Western blot analysis. Aliquots containing 10 μl of the total protein extracts were also examined by SDS-PAGE to determine the efficiency of transgene expression.

Primary antibodies used for immunoblotting were anti-HA polyclonal antibodies (1:250; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-FLAG M2 monoclonal antibody (1:500; Sigma). Secondary antibodies used for immunoblotting were alkaline phosphatase-conjugated goat anti-mouse IgG (1:25,000; Sigma) and alkaline phosphatase-conjugated goat anti-rabbit IgG (1:25,000; Sigma). Proteins were detected by chemifluorescence (ECF kit; Amersham Biosciences) according to the supplier’s instructions. Probed and washed membranes were incubated with Vistra ECF substrate solution (Amersham Biosciences) at a ratio of 1 ml/24 cm² for 5 min. Membranes were scanned directly with a Storm PhosphorImager, and signals emitted by targeted proteins were measured with the ImageQuant version 1.1 program (Amersham Biosciences).

**Transfection Assays and Microscopy**

C3H10T1/2 cells were grown at 50 – 60% confluence and were transfected with various pEGFP constructs using calcium phosphate precipitation as previously described. Cells were washed with phosphate-buffered saline ~16 h after transfection and replaced with fresh medium to grow for an additional 8 – 10 h before fixing or harvesting for further analysis. C3H10T1/2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen) before and after transfection.

Cells were fixed 24 h after transfection with 1% formaldehyde for 10 min at ambient temperature followed by 100% methanol at −20 °C for 5 min. After extensive washing and counterstaining with ToPro3 (Molecular Probes) at 1 μM final concentration for 10 min, slides were washed again and mounted with mounting medium. Photomicrographs were taken with a 20× objective on a Zeiss LSM 5 Pascal confocal system, and images were processed by LSM 5 Pascal software version 2.8 WS. 300 cells from separate experiments were examined to calculate the percentage of cells possessing the representative phenotype.

**Generation of Lentiviruses**

Lentivirus-based vectors encoding IRES-GFP (pHR′-IRES-GFP) were generated by transient co-transfection of 293T cells with a three-plasmid combination as described previously (25). All viral stocks used in the transduction of the neonatal rat cardiomyocytes were from unfractionated culture media. Titers were determined by infecting 293T cells with an aliquot of each viral stock in a dilution series. Control cells were examined at the time of infection and at the time of harvesting. The percentages of GFP-positive cells were determined by flow cytometry by the USC Pathology Reference Laboratory.

**Viral Transduction in Rat Cardiac Myocytes and Immunofluorescence Staining**

Neonatal rat cardiac myocytes from 2–4-day-old Sprague-Dawley rats were prepared by using a previously described protocol (26) and
TABLE 3
Derived amino acid sequences of Tmod-1 mutants with loss of TM5 binding

| Tmod Mutants | GST | 2-hyb |
|--------------|-----|-------|
| 78           |     | L I   |
| 84           | I   | V G A |
| 104          |     | D E A |
| 164          |     | T A I |
| 165          | V   | T L D G |

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The results of these assays are shown in Table 2 and in Fig. 1 (right panel). These data suggest that there is a region of Tmod-1 between residues 39 and 138 that is both necessary and sufficient to interact well with TM5. Several subdeletions on Tmod-1 between residues 39 and 138 (T Δ39–72, T Δ73–138, and T Δ108–138) all showed a complete or nearly complete elimination of TM5-binding ability in the two-hybrid assays (Fig. 1). These results are consistent with the possibility that several subdomains located between amino acids 39 and 72, between amino acids 73 and 107, and between amino acids 108 and 138 contribute to the binding of the two proteins. However, only the T Δ73–138 deletion construct showed a complete loss of [35S]TM5 pull-down signal when we submitted the same constructs for further confirmation by using GST affinity chromatography assays (data not shown). Accordingly, we focused on this region as probably comprising the key binding residues. Random mutations were then introduced within residues 108–138 in order to identify specific amino acids that are required for the strong interaction between Tmod-1 and TM5.

Identification of Tmod Mutants with Loss of TM5-binding Function by Random Mutagenesis Assays—More than 200 Tmod-1 clones created by random mutagenesis were examined, and 85 of them were selected for GST affinity chromatography screening. Five of the clones showed a significant reduction of binding interaction with TM5 in the GST assay, and the same five clones were then examined using the yeast two-hybrid liquid culturing analysis to confirm the reduction of the TM5-binding affinity (Table 3). All five clones had, as expected, multiple amino acid missense mutations. We sequenced all 85 clones at the region between residues 108–138 and T Δ108–138 deletion constructs showed a complete loss of [35S]TM5 pull-down signal when we submitted the same constructs for further confirmation by using GST affinity chromatography assays (data not shown). Accordingly, we focused on this region as probably comprising the key binding residues. Random mutations were then introduced within residues 108–138 in order to identify specific amino acids that are required for the strong interaction between Tmod-1 and TM5.
tial for maintaining the strong interaction between Tmod-1 and TM5, the missense residues in clone 104 (Table 3) were selected, and clones with single missense mutations were created to further analyze the roles of residues 134, 135, and 136 in Tmod-1.

**TM5-binding Abilities of Tmod-1 Altered at Residue 134, 135, or 136**

Examined by Affinity Chromatography—GST fusion proteins of three Tmod-1 mutants (I134D, L135E, and G136A) were produced with a bacterial expression system (Fig. 2A) and then tested for their binding affinities to in vitro translated TM5 by GST affinity chromatography (Fig. 2B). No TM5 binding was detected with the L135E mutant. The I134D mutant had significant reduction of TM5, whereas the G136A mutant had only a relatively small reduction when compared with the wild type Tmod-1. To eliminate the possibility that the change of TM5-binding ability of the L135E mutant was solely charge-related, we created a second missense mutation, L135V. This mutation also significantly reduced interaction with TM5, comparable with the reduction engendered by the I134D mutation (Fig. 2 and Table 4).

**Cellular Analysis of Binding Affinities of Tmod Mutants with TM5 Using an Immunoprecipitation Assay**—To verify that the variable interactions we measured among the Tmod-1 missense mutants and TM5 reflect events in cells, we tested cell lysates using an immunoprecipitation assay. We co-transfected cells with FLAG epitope-tagged Tmod-1 (wild type or mutants) and HA-tagged TM5. Total cell lysates from cells transfected with FLAG Tmod-1 evinced strong accumulation of the FLAG-bearing 43-kDa Tmod-1 but had no reactivity with anti-HA antisera (Fig. 3B, lane 3). Conversely, lysates from cells transfected solely with HA-tagged TM5 showed significant quantities of 29-kDa HA reactivity but no anti-FLAG signal (Fig. 3B, lane 2). Lysates from cells transfected with control empty vector had no signals with either antisera (Fig. 3B, lane 1). Lysates from all cells transfected with a combination of a Tmod-1 and a TM5 fusion construct fully expressed both recombinant FLAG-Tmod-1 (Fig. 3A, top, lanes 4–8) as well as HA-TM5 (Fig. 3B, bottom, lanes 4–8).

We immunoprecipitated the cell protein lysates with anti-FLAG antiserum bound to agarose beads. Following washing and elution from the beads, the proteins were electrophoresed on gels and Western blotting was performed (Fig. 3A). Wild type Tmod-1 is able to co-immunoprecipitate significant amounts of recombinant TM5 (Fig. 3A, lane 4). Both mutations of Tmod at residue 135 abrogated this strong interaction (Fig. 3A, lanes 6 and 8). Mutations at residue 134 or 136 also interfered with the interaction but less so (lanes 5 and 7, respectively). The relative order of interaction by the mutant Tmods assayed by immunoprecipitation paralleled the results obtained with the in vitro GST binding assays.

**Comparisons of Relative Interactions between TMS and Tmod Missense Mutants**—To more definitively compare the relative amounts of TM5 interacting with wild type and missense mutant Tmod-1 proteins, we measured the signals obtained with the GST pull-down and immunoprecipitation assays. TM5 signals from the GST affinity chromatography assay were determined by comparing signal intensity with the 10% input signal by AlphaImager analysis. V, unit of labeled protein signal intensity measured by a Storm PhosphorImager.

| GST affinity chromatography | Immunoprecipitation |
|-----------------------------|----------------------|
| %                          | %                    | %                          |
| WT Tmod                    | 12                   | 100                        |
| TL135E                     | 0                    | 0                          |<10^4 |
| TL135V                     | 4.2                  | 35                         |0 0 |
| TI134D                     | 6.4                  | 57.6                       |6.8 × 10^4 |
| TG136A                     | 9.1                  | 82                         |47.6 × 10^4 |

Order of TM5-binding affinity

WT Tmod > TL136A > TI134D > TL135V ≈ TL135E

The interaction but less so (lanes 5 and 7, respectively). The relative order of interaction by the mutant Tmods assayed by immunoprecipitation paralleled the results obtained with the in vitro GST binding assays.

**Summary of TM5 binding activities of the single amino acid mutation Tmod-1 mutants**

Quantitative values from both GST affinity chromatography and immunoprecipitation assays and a comparison of the TM5-binding ability with wild type (WT) Tmod and the point mutation mutants are shown. Actual, raw data of the signal intensity from each reaction. Relative, the relative percentage of signal intensity from each reaction when the amount of TM5 protein recovered by wild type Tmod was set at 100%. Actual percentages of TM5 pulled down in the GST affinity chromatography assay were determined by comparing signal intensity with the 10% input signal by AlphaImager analysis. V, unit of labeled protein signal intensity measured by a Storm PhosphorImager.

**TABLE 4**

| GST affinity chromatography | Immunoprecipitation |
|-----------------------------|----------------------|
| %                          | %                    | %                          |
| WT Tmod                    | 12                   | 100                        |
| TL135E                     | 0                    | 0                          |<10^4 |
| TL135V                     | 4.2                  | 35                         |0 0 |
| TI134D                     | 6.4                  | 57.6                       |6.8 × 10^4 |
| TG136A                     | 9.1                  | 82                         |47.6 × 10^4 |

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**Comparisons of Relative Interactions between TMS and Tmod Missense Mutants**—To more definitively compare the relative amounts of TM5 interacting with wild type and missense mutant Tmod-1 proteins, we measured the signals obtained with the GST pull-down and immunoprecipitation assays. TM5 signals from the GST affinity chromatography assay were measured with an AlphaImager Imaging System (Alpha Innotech), and the TM5 signals from the immunoprecipitation assays were measured using a Storm PhosphorImager Imaging System (Amersham Biosciences). The signals from the mutant Tmod interactions were measured relative to the strong signal detected from wild type Tmod set to 100%. The results of both GST pull-down and immunoprecipitation are compared in Table 4. The L135V and L135E missense mutations strongly or completely interfered with the normal interaction with TM5 in both assays. The results with the G136A and I134D mutations were intermediate in both assays. Notably, the rank order of the interactions was the same for the four mutants in both assays.
Subcellular Location of Exogenous Wild Type and Mutant Tmod Fusion Proteins in C3H10T1/2 Fibroblast Cells—The identification of single residues on Tmod-1 that are important for its interaction with TM5 allowed us to test the effects of these point mutations on the cellular expression and localization of Tmod-1. Tmod-1 GFP wild type or mutant constructs encoding the fusion proteins were transfected into C3H10T1/2 cells and examined by fluorescence confocal microscopy (Fig. 4). Wild type Tmod accumulates in the cytoplasm as expected, as does the missense mutant with minimally perturbed TM5-binding ability, G136A (Fig. 4). Surprisingly, both the I134D and the L135E mutants accumulate in the nucleus of the transfected cells. These results correlate well with the identification of a novel nuclear export signal defined by residues 127–136 on Tmod-1 we recently described (15).

I134D, I135E, and L135V Tmod Mutants Cause Accelerated Disruption of Sarcomeric Structures of Well Differentiated Cardiomyocytes—We used primary cardiomyocytes to examine the interaction between these four missense mutation Tmods and the endogenous TM in situ. Endogenous wild type Tmod-1 is well expressed in cardiac muscle cells and forms highly organized striated patterns within the center of the A bands of the sarcomeres (28).

Following transduction, protein expression of all FLAG-tagged Tmod-IRE5-GFP lentiviral constructs were similar, as confirmed by Western immunoblotting (data not shown). Transduced cardiomyocytes were fixed and immunostained at ~24 h after the viral infection. The well-organized pattern of striated actin filaments of cells transfected with empty lentiviral vectors as a control was normal (Fig. 5, A and B). Cells transfected with either the wild type Tmod vector or the vector bearing the TG136A mutant continued to have a reasonably well-organized striated immunostaining pattern within the actin filament structures (Fig. 5, C, D, I, and J, white arrows), and neither showed a predominant nuclear localization signal. Thus, the interaction between exogenous wild type or G136A mutant Tmod-1 and endogenous TM appears to remain unperturbed. In contrast, however, cells that were infected with either the TI134D, the TL135E, or the TL135V mutant, appeared to have two pools of exogenous Tmod proteins within different subcellular compartments. A large fraction of the exogenous Tmod protein of these three mutants was present in the nuclei as expected (Fig. 5, E–H, K, and L). The cells shown in these panels also have cytosolic pools of exogenous Tmod proteins scattered apparently randomly and with severely disrupted striated filament actins. Some of these cells (not shown) had a more organized, striated plating patterns but still evinced severe fragmentation of sarcomeric structures. Overall, cells infected with either the I134D, L135E, or L135V mutants show severe defects in incorporation of mutant Tmod proteins into sarcomeric structures and a severe disruption of sarcomeric structures as early as ~24 h after the viral infection (Fig. 5, E–H, K, and L).

Mutations of Residue 135 Do Not Affect Tmod-1 Binding to Muscle Tropomyosin Isoforms—While we were examining protein-protein interactions between wild type Tmod-1 and wild type TM5 (nonmuscle TM isoform) by GST affinity chromatography, we were also able to determine the interactions between wild type Tmod and wild type α- or β-TM (muscle TM isoforms) (Fig. 6A). T-mod-1 interacts very weakly with both muscle TM isoforms compared with its ability to interact with the nonmuscle TM isoforms TM5 (Fig. 6A). We then examined the interactions between Tmod and two other nonmuscle TM isoforms, TM5a and TM5b (Fig. 6B). Both of these other nonmuscle TM isoforms (Fig. 6B) also strongly inter-

![Figure 3](image3.png)

**FIGURE 3.** Co-immunoprecipitation of HA-tagged TMS with FLAG-tagged Tmod and single residue mutants. A, Western blot analysis of immunoprecipitated proteins. The top and bottom blots were probed with anti-FLAG M2 monoclonal antibody and anti-HA polyclonal antibodies, respectively. B, Western blot analysis of total protein extracts. The top and bottom blots were probed with anti-FLAG M2 monoclonal antibody and anti-HA polyclonal antibodies, respectively.

![Figure 4](image4.png)

**FIGURE 4.** Subcellular location of exogenous Tmod fusion proteins in transfected C3H10T1/2 fibroblasts. Confocal microscopy images of 10T1/2 fibroblasts that had been transfected by either the GFP vector, the wild type TmodGFP (TWT), the TI134DGFP (TI134D), the TL135EGFP (TL135E), or the TG136AGFP (TG136A) Tmod-1 mutants. All nuclei were counterstained with ToPro3 iodide fluorescence. Scale bar, 20 μm. The total brightness and contrast of the images has been digitally adjusted. 300 cells were examined from separate experiments of each transfection, and the mean percentages of cells ± S.D. with the representative phenotypes (shown in this figure) were as follows: GFP vector, 98 ± 3%; TWT, 96 ± 3%; TI134D, 85 ± 7%; TL135E, 92 ± 4%; and TG36A, 89 ± 9%. 
FIGURE 5. Expression of wild type and single mutation Tmod mutants in lentiviral transduced cardiomyocytes. Shown are confocal microscopy images of immunostained neonatal rat cardiomyocytes following infection with various lentiviral vectors as described under “Experimental Procedures.” In all micrographs, white bars represent 5 μm, and red bars represent 10 μm. A and B, immunostaining of endogenous Tmod (Tmod-FITC) (left) and endogenous actin filaments (Phall-TRITC) (middle) in control cardiomyocytes infected with parental lentiviral construct. B, an enlarged area selected from A (box) to show the details of each immunostaining. C and D, immunostaining of exogenous wild type Tmod (FLAG-Tmod-FITC) (left) and endogenous actin filaments (Phall-TRITC) (middle) in cardiomyocytes infected with FLAG-tagged wild type Tmod lentivirus. D, an enlarged area selected from C (box) to show the details of each immunostaining. E and F, immunostaining of exogenous I134D Tmod mutant (FLAG-TI134D-FITC) (left) and endogenous actin filaments (Phall-TRITC) (middle) in cardiomyocytes infected with FLAG-tagged I134D Tmod mutant lentivirus. F, an enlarged area selected from E (box) to show the details of each immunostaining. G and H, immunostaining of 12 exogenous L135E Tmod mutant (Flag-TL135E-FITC) (left) and endogenous actin filaments in cardiomyocytes infected with FLAG-tagged TL135E Tmod mutant lentivirus. H, an enlarged area selected from G (box) to show the details of each immunostaining. I and J, immunostaining of exogenous G136A Tmod mutant (FLAG-TG136A-FITC) (left) and endogenous actin filaments (Phall-TRITC) (middle) in cardiomyocytes infected with FLAG-tagged TG136A Tmod mutant lentivirus. J, an enlarged area selected from I (box) to show the details of each immunostaining. K and L, immunostaining of exogenous L135V Tmod mutant (FLAG-TL135V-FITC) (left) and endogenous actin filaments (Phall-TRITC) (middle) in cardiomyocytes infected with FLAG-tagged TL135V Tmod mutant lentivirus. L, an enlarged area selected from K (box) to show the details of each immunostaining.
FIGURE 5—continued

Novel Functional Domain of Tropomodulin-1
break down this region into smaller segments using deletions of amino acids 39 –138 did completely abolish the interaction between Tmod and TM5, and the truncated Tmod fragment 39 –138 retains a strong affinity for TM5 (Table 2 and Fig. 1). However, when we tried to break down this region into smaller segments using deletions of amino acids 39 –138 did completely abolish the interaction between Tmod-1 and TM5, and the truncated Tmod fragment 39 –138 retains a strong affinity for TM5 (Table 2 and Fig. 1). Therefore, when we tried to break down this region into smaller segments using deletions of amino acids 39 –138 did completely abolish the interaction between Tmod-1 and TM5.

FIGURE 6. GST affinity chromatography assays between TMs (muscle or nonmuscle isoforms) and L135E single mutation Tmod-1 mutant. A–C, lane 1, 10% input from in vitro translated lysate; lane 2, GST protein plus 100% in vitro translated lysate; lane 3, GST fusion protein plus 100% in vitro translated lysate. TM5, TM5a, and TM5b are low molecular weight/nonmuscle TM isoforms. α- and β-TM are high molecular weight/muscle TM isoforms.

The conclusion that there are multiple sites critical for interaction with TM5 is strongly supported by the results we obtained using random mutagenesis. We identified residues at both N- and C-terminal ends of the regions from 108 to 138 that are important for maintaining the integrity of the Tmod-1 interaction with TM5 (Table 3). The initial goal of our study was to more narrowly define the TM-binding domain of Tmod-1 previously delineated to lie between amino acids 39 and 138 (2, 21). Our domain deletion experiments failed to pinpoint a smaller region that would be solely responsible for the interaction between the two proteins (Table 2 and Fig. 1). Indeed, deletion of amino acids 39 –138 did completely abolish the interaction between Tmod-1 and TM5, and the truncated Tmod fragment 39 –138 retains a strong affinity for TM5 (Table 2 and Fig. 1).

The conclusion that there are multiple sites critical for interaction with TM5 is strongly supported by the results we obtained using random mutagenesis. We identified residues at both N- and C-terminal ends of the regions from 108 to 138 that are important for maintaining the integrity of the Tmod-1 interaction with TM5 (Table 3). Closer examination of the effects of mutating residue 134 or 135 revealed that both are important for Tmod-1 to maintain its relatively strong interaction with TM5 (Fig. 2 and 3). Indeed, deletion of amino acids 39 –138 did completely abolish the interaction between Tmod-1 and TM5, and the truncated Tmod fragment 39 –138 retains a strong affinity for TM5 (Table 2 and Fig. 1). However, when we tried to break down this region into smaller segments using deletions of amino acids 39 –138 did completely abolish the interaction between Tmod-1 and TM5.

A recent study elegantly showed that a Tmod-1 N-terminal fragment (amino acids 1–130) binds with a $K_d$ value of 0.23 ± 0.15 µM with a low molecular weight/nonmuscle isoform (LMW) truncated TM fragment (TMZip) (29). However, in this binding study, full-length Tmod-1 and TM were not tested, because interactions between full-length Tmod-1 and TMZip complexes were not reversible and thus could not be used for measuring changes of the thermodynamics of folding. As a result, it remains unclear whether the Tmod-1 fragment (amino acids 1–130) has similar binding affinity to the LMW TM construct as does the full-length Tmod-1, since there was no direct comparison (28). Furthermore, the LMW TM construct, TM1bZip, is composed of only the first 19 residues (AGSSSLLEAVRKKIRSLQEQ) of rat α-TM encoded by exon 1b plus the 18 C-terminal residues of the leucine zipper domain of the yeast transcription factor, GCN4, whereas the LMW TM we used in our study was full-length TM5, a product of the α-TM gene that is homologous to the human hTMmn gene (17). Although TM isoforms usually have rather high similarity among their coding sequences, the first 19 residues of TM5 (MAGSTTTIEAVKRIKLQEQ) are quite different from those encoded by the rat α-TM gene exon 1b. Accordingly, the results of the studies by Greenfield...
and Fowler (29) and others analyzing TM1hZip-Tmod 1 binding (15–17) are not directly comparable with those we report here.

There is yet another interesting cellular effect caused by mutation of residue 134 or 135 of Tmod-1. In a previous study, it was shown that myofibril structures in well differentiated cardiomyocytes were disrupted by overexpressed wild type Tmod-1 at 48 h after infection (9). Consistent with this observation, at 24 h, cardiocytes infected with either wild type or the minimally perturbing G136A mutant showed intact actin filament structures that were comparable at 24 h with those of cells infected with empty viral vectors (Fig. 5, A–D, I, and J). Some myofibril disruption did occur 36 h after infection, and more severe disruption was observed at 48 and 72 h (data not shown). In stark contrast, there is already severe actin filament fragmentation at 24 h in cardiocytes infected with either L135E or L135V (Fig. 5, G, H, K, and L). We originally proposed that overexpression of wild type Tmod in well differentiated cardiomyocytes disrupted the myofibrillar structures by excessive binding of Tmod to its target tropomyosin and consequent production of truncated actin filaments (9). Although L135E and L135V mutants do not bind TM5 very effectively, the mutations do not appear to affect the residual interaction between Tmod-1 and muscle TM isoforms (Fig. 6). One might expect that both wild type and mutants of Tmod-1 would have similar effects on the sarcomeric structures in cells that overexpressed these proteins, yet the occurrence of myofibrillar disruption was much faster in cells infected by the Tl135E and TL135V mutants than in cells infected with wild type Tmod or with the G136A mutant (24 versus 48 h). Thus, it is likely that the actin filament disruption involves an alternative pathway, perhaps one involving the accumulation of Tmod-1 in the nuclei of well differentiated cardiomyocytes.

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