Tropomodulin (Tmod) is a cytoskeletal actin-capping protein that interacts with tropomyosin at the pointed end of actin filaments. E-Tmod is an isoform that expresses predominantly in cardiac cells and slow skeletal muscle fibers. We unexpectedly discovered significant levels of Tmod in nuclei and then defined peptide domains in Tmod responsible for nuclear import and export. These domains resemble, and function as, a nuclear export signal (NES) and a pattern 4 nuclear localization signal (NLS). Both motifs are conserved in other Tmod isoforms and across species. Comparisons of wild-type Tmod and Tmod carrying mutations in these peptide domains revealed that Tmod normally traffics through the nucleus. These observations logically presuppose that Tmod functions may include a nuclear role. Indeed, increasing Tmod in the nucleus severely hampered myogenic differentiation and selectively suppressed muscle-specific gene expression (endogenous p21, myosin heavy chain, myogenin, and Tmod) but did not affect endogenous glyceraldehyde-3-phosphate dehydrogenase or expression from a transfected E-GFP vector. These results suggest that, at least in myogenic cells, nuclear Tmod may be involved in the differentiation process.

Tropomodulin (Tmod) was originally isolated from human erythrocytes and was shown to inhibit the binding of tropomyosin to actin. It was first cloned from a human fetal liver cDNA library and was later shown to be the capping protein for the pointed end of actin filaments. Tmod contains two known functional domains: an N-terminal tropomyosin binding domain and a C-terminal actin binding domain. Comparisons of wild-type Tmod and Tmod carrying mutations in these peptide domains revealed that Tmod normally traffics through the nucleus. These observations logically presuppose that Tmod functions may include a nuclear role. Indeed, increasing Tmod in the nucleus severely hampered myogenic differentiation and selectively suppressed muscle-specific gene expression (endogenous p21, myosin heavy chain, myogenin, and Tmod) but did not affect endogenous glyceraldehyde-3-phosphate dehydrogenase or expression from a transfected E-GFP vector. These results suggest that, at least in myogenic cells, nuclear Tmod may be involved in the differentiation process.

MATERIALS AND METHODS

Site-directed Mutagenesis—Single amino acid mutations on Tmod were generated using custom-designed primers (Invitrogen) that are specific to the site of mutation. The reactions were carried out using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). 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, Foster City, CA). The primers used to generate single mutations are listed below: I134D Forward: GACATCGCAGCT-GACCTGGGCACTGCAC and Reverse: GTGCTAGCCAGGATGCCTG-GAGTGC; L135E Forward: CATCGCAGCTATCGAGGGCATGCAC and Reverse: GTGTGTGCATGCCCTCGATAGCTGCGATG; RAGR Forward: GACCTTGAGGGCGGGAGGCTGTCGAGCAGAC and Reverse: GTCGTCAGGCTCCCGCCCTACAAGGTC. Plasmid Construction—pEGFP-N1 (Clontech, Palo Alto, CA) was used for the construction and expression of GFP fusion proteins. All Tmod constructs (wild type or mutants) and a low molecular weight tropomyosin isoform, TM5, were subcloned into this vector through its EcoR1 site. The forward and reverse primers used to generate the TM5 cDNA insert for subcloning are 5′-GGTGAATTCCTACATCTCGTTCAGGTC-3′ and 5′-TGGGAATTCCTACATCTCGTTCAGGTC-3′, respectively. The TM5 template used in the PCR reactions was a gift from Dr. D. Helfman (16). pCDNA3 (Amersham Biosciences) was also used as a second mammalian expression vector for transfection assays. A FLAG epitope tag, DYKDDDK, was added upstream to all Tmod and TM5 constructs by
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PCR before subcloning into pCDNA3 through its EcoRI site.

pHR-IRES-GFP (provided by Dr. N. Kasahara, UCLA) was used to subclone both wild type and NES Tmod mutants through the EcoRI site for the generation of lentiviruses.

Transient Transfection—C3H10T1/2 cells and C2C12 myoblasts were grown to 50–60% confluency and were transfected with various DNA constructs using calcium phosphate precipitation as previously described (17). Cells were washed with phosphate-buffered saline (PBS) ~16 h after transfection. Fresh medium was added, and cells were allowed to grow for an additional 8–10 h before fixing or harvesting for further analysis.

10T1/2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen). C2C12 muscle cells were grown on ice for growth medium (20% fetal bovine serum in Dulbecco’s modified Eagle’s medium with 1% kanamycin (Invitrogen) containing 0.5% chicken embryo extract (Invitrogen)) before transfection and in differentiation medium (2% horse serum in Dulbecco’s modified Eagle’s medium with 1% kanamycin) after transfection.

Microscopy and Imaging—Cells transfected with GFP constructs were examined with an IMT 2 fluorescence microscope (Olympus, Lake Success, NY). Photomicrographs were taken through the ×20 objective with Fuji 400 ASA film. Cells transfected with pCDNA3 constructs were fixed and immunostained as described under “Immunofluorescence Staining.”

Nuclear Protein Extraction—24 h after transient transfection, 10T1/2 cells were washed twice in PBS, harvested, and pelleted by centrifugation at 500 × g for 2–3 min. CERI (NE-PER™, Pierce) was added to each sample following the supplier’s guideline (100 μl of CERI per 10-μl cell volume). Cells were resuspended by vortexing for 15 s and then incubated on ice for 10 min. CERI was then added to each sample (5.5 μl of CERI solution for every 10-μl cell volume) followed by repeated vortexing and centrifugation at 14,500 × g for 5 min. The supernatant (cytoplasmic extract) was immediately stored on ice. The insoluble nuclear pellet was resuspended in 50 μl of NER (NE-PER™, Pierce) per 10-μl cell volume and incubated on ice for 40 min with intermittent brief vortexing. The samples were centrifuged at 14,500 × g for 10 min. The supernatant (nuclear extract) was collected.

After measuring the total volumes of each fraction, their protein concentrations were measured spectrophotometrically using the Bio-Rad protein assay reagent. All cytoplasmic and nuclear protein fractions were stored at ~80 °C.

SDS-PAGE and Western Blotting—Aliquots of protein extracts (25% of cytoplasmic or 50% of nuclear extracts) were used for protein gel electrophoresis (4–10% Tris-glycine precast SDS gradient gels; Novex, San Diego, CA). In extracts prepared from non-transfected C2C12 cells (~109), 9% of the cytoplasmic proteins and 90% of the nuclear proteins, respectively, were used for gel assays. Gel-separated proteins were transferred to nitrocellulose membranes (Amersham Biosciences) using a blot transfer chamber (Bio-Rad) at 70 V overnight at 4 °C. Membranes were then washed with PBS and blocked with 5% dry milk/ PBST (0.1% Tween 20 in PBS) for 1 h at room temperature. Membranes were rinsed with PBST several times and then incubated with primary antibodies at room temp for 1 h. After washing with PBST, membranes were incubated with secondary antibodies for 1 h at room temperature. The signal intensities of targeted proteins were detected with ECF chemiluminescence assays (see following).

Primary antibodies used for the assays were anti-GFP polyclonal antibodies (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Tmod polyclonal antibodies (5 μg/ml) (18), anti-HSP90 polyclonal antibody (1:500) (Santa Cruz Biotechnology), and anti-p300 polyclonal antibody (Santa Cruz Biotechnology). Secondary antibodies used were anti-rabbit IgG conjugated with alkaline phosphatase (1: 25,000) (Sigma).

ECF Chemiluminescence Detection—Probed and washed membranes were incubated with Vistra ECF substrate solution (Amersham Biosciences) at a ratio of 1 ml per 24 cm2 for 5 min. Membranes were scanned with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and signals emitted by targeted proteins were measured by the ImageQuant v.1.1 program (Molecular Dynamics).

Primary Neonatal Rat Cardiac Myocyte Culture and Leptomycin B Treatment—Neonatal rat cardiac myocytes from 2–4-day-old Sprague-Dawley rats were prepared by using a previously described protocol (19) and were cultured for 2–3 days at 37 °C in 5% CO2 in modified Eagle’s medium (5% fetal calf serum, 2 mM glutamine, 1% penicillin/streptomycin, and 1% 5-bromodeoxyuridine). Leptomycin B (LMB) was then added to the medium at a final concentration of 10 ng/ml, and the incubation of the cells continued at 37 °C for 24 h before fixation. Control cells were supplemented with the same volume of 0% methanol, the LMB solution. LMB was a gift from Dr. M. Yoshida (20) and was used to block protein export from the nucleus (21–23).

Immunofluorescence Staining—Untreated (control) and LMB-treated cardiac cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min. Fixed cells were first rinsed with PBS, then with 0.1 M glycine and then with PBS again before incubation with 0.1% Triton X-100 for 10 min. After rinsing extensively with PBS, cells were incubated with anti-Tmod polyclonal antibodies (10 μg/ml) in 10% horse serum in PBS at 1 h at ambient temperature. Cells were then washed several times with PBS and incubated with anti-rabbit IgG fluorescein isothiocyanate-conjugated secondary antibodies (1:80) (Sigma) for 1 h in the dark. After the final washing with PBS, cells grown on cover slips were mounted with slide mounting medium (American Type Culture Laboratories, Burlingame, CA), sealed and secured with nail polish (Revinail™, European Touch). 10T1/2 cells transiently transfected with pCDNA3 constructs bearing FLAG-tagged Tmod or TM5 were fixed after 24 h and treated similarly except the primary antibody used for immunostaining was anti-FLAG M2 monoclonal antibody (10 μg/ml) (Sigma), and the secondary antibody was rhodamine-conjugated anti-mouse IgG (1:100) (Sigma).

Photomicrographs of immunostained cardiac myocytes were taken with a ×40 objective on a Zeiss LSM 5 Pascal confocal system, and images were processed by LSM 5 Pascal software V 2.5.10.134. Cells from separate experiments were examined to calculate the percentage of cells expressing the representative phenotype. Cells from each experiment were further analyzed to determine the percentage of Tmod located in the nuclei of untreated cardiomyocytes and in the nuclei of LMB-treated cardiomyocytes. Intensities of the fluorescence signal within the whole cell and within the nuclear area of the same cell were quantified separately using the ImageJ software (NIH Image). % of nuclear Tmod = (pixel intensity of nuclear area/pixel intensity of whole cell area) × 100%. The p value of the difference between nuclear Tmod in untreated cardiomyocytes versus LMB-treated cardiomyocytes was calculated by Student’s t test. Photomicrographs of immunostained 10T1/2 fibroblasts were taken using a ×40 objective on an Olympus camera with Fuji 400 ASA film.

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 (24). All viral stocks used in the transduction of C2 myoblasts were from un fractionated culture media. Titer 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 Research Laboratory.

Viral Transduction, Cell Sorting, and Cell Enrichment of C2 Myoblasts—C2C12 myoblasts were cultured in 6-cm tissue culture plates until reaching ~30–40% confluency. An MOI of five copies of virus per cell was used for all lentiviral transductions. After 8 h of incubation, fresh medium (20% fetal bovine serum, 1% kanamycin, and 0.05% CEE) was added to each plate, and cells were cultured for three more days. Cells were then trypsinized and collected into 5-ml round bottom polystyrene tubes for cell sorting by a MoFlo Fluorescence-activated cell sorter (Cytometry, Fort Collins, CO). Cells expressing moderate to high GFP signals were selected and deposited into a premounted 35-mm culture plate containing fresh culture medium. Approximately 5,000 to 10,000 GFP-positive cells were recovered from each viral transduction preparation. After another week of culturing, cells were trypsinized again and subjected to a second round of cell sorting to enrich the GFP-positive population.

Approximately 5,000 GFP-positive C2 cells were cultured in parallel with uninfected C2 myoblasts as controls for time course studies on the change of cell morphology and the expression of myogenic markers before and after the induction of differentiation.

Northern (RNA) Blot Analyses—Total RNA was extracted with TRIzol (Invitrogen). Aliquots (10 μg) of total RNA were fractionated by agarose gel electrophoresis, transferred to nytran membranes (Molecular Probes, Eugene, OR), then hybridized with radiolabeled probe and examined by autoradiography.

Probes used for Northern blots were all generated by RT-PCR reactions using the RETROscript™ first strand synthesis kit (Ambion, Austin, Texas) except for EGFP and G3PDH. Briefly, 1 μg of total RNA was reverse transcribed (RT) reagent and incubated. Each RT reaction from a total volume of 20 μl was used for each PCR reaction afterward. Annealing temperature for the PCR reactions were from 55 to 58 °C, depending on the predicted melting temperature of the primers. Random sequence oligonucleotides from the RT-PCR kit were used
Subcellular Location of Exogenous Tmod, Tmod Mutants, and Tropomyosin Fusion Proteins—We have used biochemical and cellular protein-protein interaction studies to define residues on Tmod that interact with tropomyosin and actin. During the course of those studies we examined the effect on cellular phenotype of mutations that affected the protein interactions and others that did not. Several of the Tmod mutant proteins that we engineered with single amino acid substitutions showed surprising differences in intracellular localization from that of wild-type Tmod. When wild-type Tmod-GFP fusion protein was overexpressed in cells, it accumulated in the cytoplasm as expected and any suggestion of nuclear immunofluorescence was not noticeably different from that of cells expressing a GFP control vector (Fig. 2A). However, two Tmod mutants that we had been studying, I134D and I135E (see Fig. 1A), showed a strong and dominant nuclear localization following transient transfection of their expression vectors in 10T1/2 fibroblasts (Fig. 2A). The same results were also observed when these vectors were transfected into C2C12 myoblasts (data not shown).

Although GFP has been reported to diffuse into the nucleus even though it has no NLS, we are unaware of GFP causing nuclear localization as a fusion protein. To eliminate this possibility we included a low molecular weight (LMW) tropomyosin domains of the WT Tmod protein and the locations of single substitution mutations introduced at amino acid residues 134 and 135. B, residues surrounding these two mutation sites (denoted by *) fit established criteria for a leucine-rich NES motif, and the leucine amino acids (underlined) within this class of NES can sometimes be replaced by Val, Phe, or Ile. C, locations of putative NES and pattern 4 NLS of wild-type Tmod (NLS WT). Two amino acid substitutions were introduced into the pattern 4 NLS (Pat 4 NLS) sequence to change the RKRR motif into RAGR (NLS mutant).

Identification of Nuclear Transport Signals on Tmod—The amino acids encompassing residues 134 and 135 do not resemble known nuclear localization import motifs making it unlikely that our mutations had artificially created an NLS. However, these mutations did fail in the midst of a previously unsuspected motif resembling a class III nuclear export signal (NES) motif (26–28), which is usually composed of short leucine-rich amino acid sequences (underlined) within this class of NES can sometimes be replaced by Val, Phe, or Ile. C, locations of putative NES and pattern 4 NLS of wild-type Tmod (NLS WT). Two amino acid substitutions were introduced into the pattern 4 NLS (Pat 4 NLS) sequence to change the RKRR motif into RAGR (NLS mutant).

FIG. 1. Schematic diagrams of tropomodulin and various introduced mutations. A, tropomodulin (Tmod) binding and actin binding domains of the WT Tmod protein and the locations of single substitution mutations introduced at amino acid residues 134 and 135. B, residues surrounding these two mutation sites (denoted by *) fit established criteria for a leucine-rich NES motif, and the leucine amino acids (underlined) within this class of NES can sometimes be replaced by Val, Phe, or Ile. C, locations of putative NES and pattern 4 NLS of wild-type Tmod (NLS WT). Two amino acid substitutions were introduced into the pattern 4 NLS (Pat 4 NLS) sequence to change the RKRR motif into RAGR (NLS mutant).

Mutations at NLS Affect Nuclear Accumulation of Tmod—To determine whether this motif indeed plays a role in Tmod nuclear entry we created a Tmod expression vector that carries R341A and R342G mutations. Tmod proteins carrying this RAGR mutation (Fig. 1C) at the site of the putative NLS RKKR (Fig. 1C) indeed have been excluded from nuclei compared with wild-type Tmod in either 10T1/2 cells or C2C12 myoblasts (Fig. 2C). Thus, this mutation appears to have disrupted a functional NLS in Tmod that is required for its previously unsuspected nuclear import.

* K. Y. Kong and L. Kedes, manuscript in preparation.
If the accumulation of Tmod in nuclei we observed with the mutations at residues 134 and 135 is caused by disruption of a normal NES, then double mutants also carrying the RAGR substitution would be expected to reduce the nuclear accumulation of Tmod. As predicted, the presence of Tmod in nuclei appears to be greatly diminished in either 10T1/2 cells or the C2 myoblasts expressing the double mutants (Fig. 3B).

Measurement of Tmod in Nuclear Extracts—To measure the levels of Tmod in nuclei and confirm the effects of the various mutations, we extracted nuclear proteins from the transfected cells and measured the relative Tmod abundance by semiquantitative Western blotting using fluorescent ECF signals detected by a phosphorimager. The degree of cytoplasmic contamination in the nuclear extracts was monitored using a probe for HSP90, a purely cytoplasmic protein. Conversely the degree of nuclear contamination in the cytoplasmic extracts was monitored by measuring the presence of the nuclear transcription co-factor, p300. Following separation of nuclear and cytoplasmic components, minimal amounts of HSP90 were detected in the nuclear extracts, and p300 was almost undetectable in cytoplasmic extracts (Fig. 3A). Thus, we subsequently used the nuclear/cytoplasmic ratio of HSP90 and p300 to correct the fraction of various Tmods in the nuclear compartment.

The RAGR mutation of the NLS reduced the fraction of Tmod detected in nuclei to between 45% to 80% of the levels of wild type or the I134D and L135E mutations (Fig. 3B). Further, all three constructs carrying the RAGR mutation (wild type, I134D, and L135E) had reduced levels of nuclear Tmod accumulation compared with the fully wild-type construct. Cells expressing the L135E RAGR double mutant consistently accumulated in nuclei less than half the ECF detectable signal emitted by cells expressing the wild-type construct.

Subcellular Localization of Endogenous Tmod in Myogenic C2 Cells—We analyzed the subcellular location of Tmod in proliferating C2 myoblasts and differentiated myotubes both by immunofluorescence microscopy and by Western blotting of nuclear and cytoplasmic extracts. Tmod could not be detected in myoblasts by immunostaining but in myotubes we observed strong Tmod staining exclusively in the cytoplasm (data not shown). However, Tmod expression was detectable by Western blot in proliferating C2 myoblasts (Fig. 4A), and its levels rise strongly in myotubes (Fig 4B; also see Fig. 7, A and B) in both nuclei and cytoplasm. In addition, the less abundant E-Tmod transcript (1.6–1.8 kb) (3) can also be detected by Northern blot with longer autoradiographic exposure (Fig. 7B, day 4 culture in differentiation medium). Myotube nuclei appear to contain ~13 ± 2% of the total cellular Tmod.

The trafficking of endogenous Tmod through the nucleus was further confirmed by treating cells with the nuclear export inhibitor leptomycin B (LMB). Tmod-rich cardiac myocytes appear to retain most Tmod in the cytoplasmic compartment (Fig. 5A). However, following exposure of the cells to LMB, more than 90% of the cardiacmyocytes clearly show a moderate accumulation of nuclear Tmod detected by immunofluorescence staining with anti-Tmod antiserum (Fig. 5B).

Accumulation of Tmod in the Nucleus Inhibits Myogenic Differentiation of C2 Cells—To begin to understand the possible nuclear function of Tmod, we examined the effects of expression of wild type and of NES Tmod mutants on the phenotypes of proliferating C2 myoblasts induced to undergo myogenic differentiation. In order to ensure transduction of a majority of cells we delivered the genes with lentiviral vectors followed by selection of GFP-positive cells using a MoFlo high speed fluorescence-activated cell sorter. The cell preparations were highly enriched for GFP-positive cells: lentiGFP control ~90% +ve, lentiTmodWT ~85% +ve, lentiI134D mutant ~72% +ve, and lentiL135E mutant ~82% +ve. The enriched cells were plated in growth medium and, once they reached high confluency (established as day 0) they were switched to differentiation medium and followed for 8 days of continuous culturing (Fig. 6). By day 2, a few cells have begun to form some short myotubes in all cell types. However, cells expressing the
Western blots of cytoplasmic (C) and nuclear (N) extracts from both TmodGFP transfected and control non-transfected 101/2 fibroblasts comparing the amounts of HSP90 and p300, a cytoplasmic and a nuclear marker, respectively. B, fraction of Tmod and Tmod mutant proteins found in nuclear versus cytoplasmic compartments from three separate experiments (mean ± S.D.). TmodWT, TmodGFP construct with no mutation within the NES region; TT134D, TmodGFP with an Ile to Asp mutation at amino acid 134; TL135E, TmodGFP with a Leu to Glu mutation at amino acid 135; RKRR, wild-type NLS; RAGR, mutated NLS.

FIG. 5. Subcellular localization of endogenous Tmod in well differentiated cardiomyocytes. Representative fluorescence photomicrographs of immunostained neonatal rat cardiomyocytes either untreated (control) (A) or treated with LMB (B). Fixed cells were stained with Tmod polyclonal antiserum and fluorescein isothiocyanate-conjugated secondary antibodies. Scale bar, 10 μm. Control and treated cells typically had phenotypes of Tmod localization as seen in these examples. The accumulation of Tmod in nuclei of LMB-treated cells was evaluated by confocal microscopy through the nuclear plane. Pixel intensities of fluorescence in the cytoplasm versus the nucleus of 50 representative cells in each group were measured as described under “Materials and Methods.” A mean (± S.D.) of 3 ± 1% of total Tmod fluorescence was located in the nuclei of untreated cardiomyocytes and 7 ± 2% of Tmod fluorescence was located in nuclei of the LMB-treated cardiomyocytes (p < 0.0001). whereas the cells transduced with NES mutants have formed only a few large myotubes and continue to persist as morphologically undifferentiated myoblasts.

We also compared the time courses of appearance of mRNA markers of myogenic differentiation in C2C12 cells and in the several lines of lentiviral transduced cells. We followed the expression of the early marker, myogenin; an intermediate marker, p21; and a late marker, myosin heavy chain (Fig. 7A). The expression patterns of all these myogenic markers from the lentiGFP-infected cells and the lentiTmodWT-infected cells were very similar to the uninfected C2 control cells during the time course study. However, the onset of expression of the same markers was significantly delayed and less robust in both cell lines that had been transduced with the NES mutations (Figs. 7A and 8). We examined the expression of endogenous Tmod in these cells because Tmod is itself a late myogenic marker although lower levels of Tmod mRNA are present in proliferating myoblasts (Fig. 7B). The expression levels of endogenous Tmod were also reduced in the two cell lines expressing the NES mutations (Figs. 7 and 8). All lentiviral-transduced cells continued to express the GFP transgene even at day 8 as determined by probing with a full-length EGFP cDNA (Figs. 7 and 8). Because both Tmod (wild type or mutants) and GFP used the same cytomegalovirus promoter in the lentiviral construct for expression, the continued expression of EGFP mRNA throughout the 8-day differentiation period likely reflects the
continued expression of Tmod transgenes. Overexpression of Tmod in proliferating C2 cells does not appear to be as cytotoxic as in well differentiated muscle cells as previously described (10, 11, 31). The delay of myogenic markers expressed in cells transduced with NES mutants does not appear to be caused by a loss of transgene-expressing cells (Figs. 7 and 8). Thus the morphological changes and the gene expression markers normally observed in differentiating C2 cells are both delayed and less robust when the cells accumulate Tmod in the nucleus.

**DISCUSSION**

In this study, we have identified novel nuclear transport signals on the actin-capping protein Tmod and established the presence of Tmod in nuclei of several mammalian cell types. Nuclear Tmod proved difficult to detect by immunostaining, which may explain why the nuclear entry of the protein had previously been overlooked. The presence of Tmod in nuclei presupposes previously unsuspected cellular functions there. The previously unsuspected presence of endogenous Tmod in nuclei is not a complete surprise because compelling evidence has accumulated recently (23, 32, 33) for the presence and accrual in nuclei of other proteins traditionally associated with the cellular cytoskeleton. In addition, actin and α-actinin were found within intranuclear rods in muscle biopsies from patients with Nemaline myopathy (34, 35) although their intranuclear presence in this instance may be related more to the pathological state.

In the process of engineering Tmod mutant proteins to map Tmod functional domains, we discovered that certain point mutations could cause the exogenously introduced Tmod to be trapped in the nucleus of 10T1/2 fibroblasts and C2C12 muscle cells. Because there is no detectable endogenous Tmod expression in 10T1/2 fibroblasts, this NES seems not to be cell type-specific. However, the nuclear localization of wild type and NES Tmod mutants is likely to be caused by a more specific transport mechanism than by random diffusion because even the smaller actin-binding protein TM5 (29 kDa) seems to be unable to diffuse freely into the nuclei of the transfected cells (Fig. 2, A and B). Also, nuclear extraction assays from transiently transfected 10T1/2 cells and from non-transfected differentiated C2 cells, which express the endogenous gene, provided strong support for the notion that a significant fraction of wild-type Tmod protein normally is present in the nucleus.

**FIG. 6.** Phase contrast photomicrographs of differentiating C2 myoblasts compared with C2 cells transduced with lentiviral constructs. C2C12 ctr, uninfected C2 cells; GFP ctr, C2 cells transduced with a lentiviral construct containing just the GFP transgene; TWT, C2 cells transduced with lentiviral construct containing both wild-type Tmod plus GFP transgene; TI134D, C2 cells transduced with lentiviral construct containing both the NES Tmod mutant TI134D plus GFP transgene; TL135E, C2 cells transduced with lentiviral construct containing NES Tmod mutant TL135E plus GFP transgene. Photomicrographs taken at day 0 were of cells cultured in growth medium. Photomicrographs taken at day 2 to day 8 were of cells cultured in differentiation medium. Scale bar, 50 μm.
FIG. 7. Northern blot analyses comparing expression of different myogenic markers at different times after induction of myogenesis. A, aliquots of RNA extracted from cells treated as described in the legend to Fig. 6 were used for Northern blot analysis. Total cellular RNA was collected from cells cultured in growth medium (day 0) and then switched to differentiation medium (days 2–8). MHC, myosin heavy chain; G3PDH, glyceraldehyde-3-phosphate dehydrogenase. B, Northern blot analyses of endogenous Tmod expression in C2C12 myoblast and myotubes. Aliquots of RNA extracted from either mock-transfected (C2 ctr) or wild-type Tmod-transfected (TWT) C2 cells were used for Northern blot analysis. Total cellular RNA was collected from cells cultured in growth medium (day 0) and then switched to differentiation medium (day 1 and day 4). An E-Tmod 3′-UTR probe was used for the hybridization. Autoradiograph after (I) 24-h exposure and (II) 72-h exposure. III, methylene blue staining on original blot to detect levels of 28 S and 18 S rRNA from each RNA sample.
discovery that Tmod can be found in the nucleus and carries a clear nuclear export motif led us to search for a functional nuclear localization signal. Indeed, a likely NLS, RKRR, was detected. When this NLS motif was mutated, the presence of Tmod in nuclei was significantly reduced. However, the RKRR → RAGR modifications did not entirely block nuclear import of the mutated proteins (Fig. 3B). At least two possibilities could explain the residual nuclear presence of these RAGR mutants. First, only two out of four amino acids of the putative NLS were mutated which might only partially cripple the import mechanism. Alternatively, there may be a second, cryptic, NLS elsewhere in the molecule. Although endogenous Tmod might be small enough (40.6 kDa) to diffuse into nuclei with low efficiency, we are not considering it as one of the possible cause for the residual nuclear presence of these RAGR mutants because the proteins expressed from the various TmodGFP constructs used in our nuclear extraction experiments were much larger (~70 kDa) than the maximum size limit for energy-free diffusion into nuclei (26, 27, 36).

In untreated control cardiomyocytes, the subcellular localization of Tmod is predominantly in the cytosol when detected by immunostaining. However, treatment with a nuclear export blocking agent, LMB, led to easily detectable nuclear Tmod in more than 90% of the cells (Fig. 5). Of interest is that not all Tmod ends up in nuclei following treatment with LMB. One reason for this limited relocalization may be because most Tmod is already incorporated in stable cytoskeletal structures in these well differentiated cells and the soluble pools of Tmod in cardiomyocytes and skeletal muscle are actually quite small (13). This explanation may account for the similar restrained level of nuclear actin following LMB treatment as reported by Wada et al. (23). After 24 h of treatment with LMB, these authors reported that about 70% of 3Y1 cells showed actin staining intensity levels in the nucleus similar to those in the cytoplasm but only 4.5% of the LMB-treated cells showed a stronger actin signal in the nucleus.

When C2 cells expressing a wild-type Tmod transgene were induced to differentiate, there was a modest delay of myogenesis when compared with regular C2 cells or cells that carried only the GFP transgene (Fig. 6). However, when C2 cells expressing NES Tmod mutant transgenese were induced to differentiate, the process was severely hampered (Figs. 6 and 7).

A small fraction of C2 cells among the enriched population of cells expressing the NES mutant Tmod did manage to differentiate into myotubes. It is possible that such cells were derived from the non-transduced contaminants (Fig. 6) and that no cells expressing NES mutant Tmod differentiate. This same cell group could also fully account for the delayed and less robust expression of myogenic markers during the differentiation induction process (Figs. 7 and 8).

Nuclear Trafficking Is Likely Conserved Among Tmod Isoforms and Across Species—All four Tmod isoforms (E-, N-, Sk-, and U-), and the Drosophila melanogaster homologue sanpodo, are highly likely to be nuclear proteins as predicted by the PSORT program. E-Tmod, N-Tmod, and U-Tmod contain a pattern 4 NLS. Although there is no consensus NLS in either Sk- or sanpodo, they each contain the arginine-lysine (RK)-rich region at their C termini that is well conserved in all four Tmods examined (Table I). Evidence has accumulated to suggest that such RK-rich motifs favor nuclear importation (29, 37–39).

What Is the Nuclear Role of Tmod?—An important clue may be found in the role of sanpodo, a Drosophila homologue of Tmod that interacts with numb and notch to determine cell fates in neural and muscle development (40–42). Other cytoskeletal proteins have also been found in nuclei (32, 33, 43, 44). Zhao et al. (45) recently showed that β-actin is a subunit of the SWI/SNF-like BAF (Brg-associated factor) complex in the nucleus and is involved in chromatin remodeling in mammalian cells. Because Tmod is an actin filament capping protein, it is highly possible that Tmod might also interact with the same complex in the nucleus. Also, our lentiviral transduction experiments show that accumulation of Tmod protein in nuclei can delay or inhibit the normal myogenic process (Figs. 6–8).

E-Tmod levels do rise dramatically during myocyte differentiation but our Northern and Western analyses do demonstrate the presence of E-Tmod in myoblasts albeit at a low level. Clearly the rise of E-Tmod mRNA and protein levels in myo-

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**Table I**

| Protein | Species          | Amino acid sequence |
|---------|------------------|---------------------|
| E-Tmod  | *Mus musculus*   | RKRRVLADLTPIMPKPCKSG |
| N-Tmod  | *Rattus norvegicus* | RKRRVLADLTPIMPKPCKSG |
| Sk-Tmod | *Gallus gallus*  | RKRRVLADLTPIMPKPCKSG |
| U-Tmod  | *Homo sapiens*   | RKRRVLADLTPIMPKPCKSG |
| Sanpodo | *Drosophila melanogaster* | RKRRVLADLTPIMPKPCKSG |

* The C termini of known Tmod isoforms, beginning at the indicated residues, were compared and aligned. RK-rich motifs are underlined in boldface.
tubes is predominantly related to the assembly requirements of cytoplasmic structures. However the presence of low levels of E-Tmod mRNA and protein in myoblasts does allow for the possibility of a role in the early myogenic process. Indeed, overexpression of wild-type Tmod causes a moderate delay in myogenesis (Fig. 6). We speculate that nuclear Tmod may be involved in the regulation of proliferation and differentiation processes in muscle cells. Ongoing studies are attempting to identify possible interacting partners of Tmod in nuclei.

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REFERENCES
1. Fowler, V. M. (1987) J. Biol. Chem. 262, 12792–12800
2. Fowler, V. M. (1990) J. Cell Biol. 111, 471–481
3. Sung, L. A., Fowler, V. M., Lambert, K., Sussman, M. A., Karr, D., and Chien, S. (1992) J. Biol. Chem. 267, 2616–2621
4. Weber, A., Pennise, C. R., Babcock, G. G., and Fowler, V. M. (1994) J. Cell Biol. 127, 1627–1635
5. Gregorio, C. C., and Fowler, V. M. (1995) J. Cell Biol. 129, 683–695
6. Almenar-Queralt, A., Lee, A., Conley, C. A., Ribas de Pouplana, L., and Fowler, V. M. (1999) J. Biol. Chem. 274, 28466–28475
7. Conley, C. A., Fritz-Six, K. L., Almenar-Queralt, A., and Fowler, V. M. (2001) Genomics 73, 127–139
8. Cox, P. R., and Zoghbi, H. Y. (2000) Genomics 63, 97–107
9. Watakabe, A., Kobayashi, R., and Helfman, D. M. (1996) J. Cell Sci. 109, 2299–2310
10. Sussman, M. A., Baque, S., Uhm, C. S., Daniels, M. P., Price, R. L., Simpson, D., Terracio, L., and Kedes, L. (1996) Circ. Res. 82, 94–105
11. Gregorio, C. C., Weber, A., Bondad, M., Pennise, C. R., and Fowler, V. M. (1995) Nature 377, 83–86
12. Sussman, M. A., and Fowler, V. M. (1992) Eur. J. Biochem. 205, 355–362
13. Fowler, V. M., Sussman, M. A., Miller, P. G., Fischer, B. E., and Daniels, M. P. (1993) J. Cell Biol. 120, 411–420
14. Ito, M., Swanson, B., Sussman, M. A., Kedes, L., and Lyons, G. (1995) Dev. Biol. 177, 317–328
15. Sussman, M. A., Sakhi, S., Barrientos, P., Ito, M., and Kedes, L. (1994) Circ. Res. 75, 221–232
16. Temm-Grove, C. J., Guo, W., and Helfman, D. M. (1996) Cell Motil. Cytoskeleton 33, 223–240
17. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
18. Sussman, M. A., Sakhi, S., Tocco, G., Najm, I., Baudry, M., Kedes, L., and Schreiber, S. S. (1994) Brain Res. Dev. Brain Res. 80, 45–53
19. Bishepic, N. H., and Kedes, L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2132–2136
20. Yoshida, M., Nishikawa, N., Nishi, K., Abe, K., Horinouchi, S., and Beppu, T. (1990) Exp. Cell Res. 187, 150–156
21. Ossareh-Nazari, B., Bachelier, F., and Dargent, C. (1997) Science 278, 143–144
22. Welf, B., Sanglier, J. J., and Wang, Y. (1997) Chem. Biol. 4, 139–147
23. Wada, A., Fukuda, M., Mishima, M., and Nishida, E. (1988) EMBO J. 17, 1635–1641
24. Sakoda, T., Kanahebi, N., Hamamori, Y., and Kedes, L. (1999) J. Mol. Cell Cardiol. 31, 2037–2047
25. Sung, L. A., and Lin, J. J. (1994) Biochem. Biophys. Res. Commun. 201, 627–634
26. Nigg, E. A. (1997) Nature 386, 779–787
27. Turpin, P., Ossareh-Nazari, B., and Dargent, C. (1999) FEBS Lett. 452, 82–86
28. Wen, W., Meinkoth, J. L., Tsien, R. Y., and Taylor, S. S. (1995) Nature 372, 267–270
29. Burglin, T. R., and De Robertis, E. M. (1987) EMBO J. 6, 2617–2625
30. Nakai, N., Kedes, L., and Horton, P. (1999) Trends Biochem. Sci. 24, 34–36
31. Sussman, M. A., Welch, S., Cambron, N., Klevitsky, R., Hewett, T. E., Price, R., Witt, S. A., and Kimball, T. R. (1998) J. Clin. Investig. 101, 51–61
32. Correas, I. (1991) Biochem. J. 270, 581–585
33. Rando, O. J., Zhao, K., and Crabtree, G. R. (2000) Trends Cell Biol. 10, 92–97
34. Goebel, H. H., and Warlo, I. (1997) Neuronmus. Disord. 7, 13–19
35. Weeks, D. A., Nixon, R. R., Kaimaktchiev, V., and Mierau, G. W. (2003) Ultrastuct. Pathol. 27, 151–154
36. Golicht, D., and Mattaj, I. W. (1996) Science 271, 1513–1518
37. Cochrane, A. W., Perkins, A., and Rosen, C. A. (1990) J. Virol. 64, 881–885
38. Hauber, J., Malin, M. H., and Cullen, B. R. (1989) Trends Biochem. Sci. 24, 34–36
39. Taagepera, S., McDonald, D., Loeb, J. E., Whitaker, L. L., McElroy, A. K., and Crabtree, G. R. (1998) J. Mol. Cell Cardiol. 31, 2037–2047
40. Dye, C. A., Lee, J. K., Atkinson, R. C., Brewster, R., Han, P. L., and Bellen, H. J. (1998) Development 125, 1845–1856
41. Park, M., Yaich, L. E., and Bodmer, R. (1998) Mech. Dev. 75, 117–126
42. Skeath, J. B., and Doe, C. Q. (1998) Development 125, 1857–1865
43. Taagepera, S., McDonald, D., Loeb, J. E., Whimaker, L. L., McElroy, A. K., Wang, J. Y., and Hope, T. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7457–7462
44. Zeng, X. L., Duan, M. D., Xing, M., Wang, X. G., and Hao, S. (1999) Cell Res. 9, 61–69
45. Zhao, K., Wang, W., Zandi, O. J., Xue, Y., Swiderek, K., Kuo, A., and Crabtree, G. R. (1998) Cell 95, 625–636
