The N-terminal End of Nebulin Interacts with Tropomodulin at the Pointed Ends of the Thin Filaments

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Strict regulation of actin thin filament length is critical for the proper functioning of sarcomeres, the basic contractile units of myofibrils. It has been hypothesized that a molecular template works with actin filament capping proteins to regulate thin filament lengths. Nebulin is a giant protein (~800 kDa) in skeletal muscle that has been proposed to act as a molecular ruler to specify the thin filament lengths characteristic of different muscles. Tropomodulin (Tmod), a pointed end thin filament capping protein, has been shown to maintain the final length of the thin filaments. Immunofluorescence microscopy revealed that the N-terminal end of nebulin colocalizes with Tmod at the pointed ends of thin filaments. The three extreme N-terminal modules (M1-M2-M3) of nebulin bind specifically to Tmod as demonstrated by blot overlay, bead binding, and solid phase binding assays. These data demonstrate that the N terminus of the nebulin molecule extends to the extreme end of the thin filament and also establish a novel biochemical function for this end. Two Tmod isoforms, erythrocyte Tmod (E-Tmod), expressed in embryonic and slow skeletal muscle, and skeletal Tmod (Sk-Tmod), expressed late in fast skeletal muscle differentiation, bind on overlapping sites to recombinant N-terminal nebulin fragments. Sk-Tmod binds nebulin with higher affinity than E-Tmod does, suggesting that the Tmod/nebulin interaction exhibits isoform specificity. These data provide evidence that Tmod and nebulin may work together as a linked mechanism to control thin filament lengths in skeletal muscle.

Sarcomeres, the basic contractile units of myofibrils, are complex structures composed of numerous structural and regulatory proteins organized in an exquisitely precise manner. These structures are composed of alternating parallel arrays of thin and thick filaments. Helical, polar actin polymers are the principal component of the thin filaments. In vitro, actin monomers polymerize at both the fast growing (barbed) and the slow growing (pointed) ends of the actin filaments until the critical concentration is reached. Once this is attained, the length distribution of the filaments becomes exponential (for further discussion, see Refs. 1 and 2). In sharp contrast to this, skeletal muscle cells exhibit a strikingly narrow thin filament length distribution (e.g. 1.1 ± 0.03 μm in rabbit psoas muscle) (3). Additionally, although cardiac muscle has a wider range of thin filament lengths compared with skeletal muscle (e.g. 0.6–1.1 μm in rat heart) (4), the distribution is much more uniform than that of pure actin filaments in vitro. Thus, regulatory mechanism(s) must exist in striated muscle to tightly control thin filament length. Although these mechanisms remain unknown, one highly favored hypothesis that has existed for years is that a “molecular ruler” protein works together with actin filament capping proteins to regulate thin filament length (reviewed in Refs. 1 and 5).

The giant, actin-binding protein, nebulin, is an excellent candidate for a molecular ruler that functions to specify thin filament length in skeletal muscle (reviewed in Refs. 1 and 6–9). Strikingly, the molecular mass of nebulin (~600–800 kDa) correlates with variations in thin filament lengths observed in different types of skeletal muscle (10). Additionally, a great deal of evidence indicates that single molecules of nebulin associate with the thin filaments along their entire length. The extreme C-terminal region of nebulin is inserted in the Z disc (11–14). This region contains a unique 20-kDa domain and an Src homology 3 domain (15). More is known about this region of nebulin in comparison with other parts of the molecule, since a significant amount of recent work has focused on deciphering the molecular interactions and targeting domains responsible for anchoring nebulin in the Z disc (e.g. 15–17). The vast majority of nebulin, however, is composed of ~185 modular repeats that are each ~35 amino acids in size. Groups of seven of these modules are classified as “super repeats” (13, 18, 19). Biochemical, biophysical, and structural studies suggest that a single nebulin module interacts with a single actin monomer and that each super repeat interacts with each regulatory unit of the thin filament (comprised of a troponin complex and a tropomyosin molecule for every seven actin monomers) (13, 18, 19). Last, the N-terminal region of nebulin is predicted to be located at or near the pointed ends of the thin filaments (12, 13, 20). This region contains eight unique “linker” modules (M1–M8) as well as a unique, acidic 8-kDa domain (13). The function of these N-terminal domains is unknown, but it is intriguing to speculate that they are specialized for interacting with other sarcomeric proteins and have distinct functions.

Nebulin’s periodic, modular structure probably enables it to dictate the number of actin monomers to be polymerized into the thin filaments in skeletal muscle. What is the mechanism,
then, that prevents the thin filaments from elongating once their specified length is attained? In this regard, actin filament capping proteins that bind to the ends of actin filaments prevent actin monomer addition and filament growth (for a review, see Ref. 21). In striated muscle, CapZ has been shown to cap the barbed ends of the thin filaments at the Z line, while the pointed ends of the filaments are capped by tropomodulin (Tmod)1 (22–24). Investigations of CapZ and Tmod in cultured myocytes suggest that CapZ plays a role in nucleating the initial assembly of actin filaments into sarcomeres, while Tmod functions later to maintain thin filament lengths (25–27). For example, microinjection of an antibody to Tmod that disrupts its capping activity in in vitro assays results in abnormal elongation of the thin filaments from their pointed ends in chick cardiac myocytes (26). Additionally, cells in which Tmod function is inhibited no longer beat, and overexpression of Tmod in the myocardium of transgenic mice causes myofibrillar disarray and dilated cardiomyopathy (26–28). These studies indicate that Tmod regulation of thin filament length is critically important for muscle function.

Tmod, first identified as a tropomyosin-binding protein in the erythrocyte membrane cytoskeleton (E-Tmod; Ref. 29), is present at thin filament pointed ends in chicken slow skeletal muscle fibers, in skeletal muscle nuclei, and in heart (30, 31). A second isoform, skeletal Tmod (Sk-Tmod), is present at thin filament pointed ends late in chicken fast skeletal muscle differentiation (32). In vitro assays indicate that both E-Tmod and Sk-Tmod bind to tropomyosin (KJ ~150–200 nM) (29, 32). In the presence of tropomyosin, Tmod completely blocks the polymerization and depolymerization of actin filaments at their pointed ends (KJ ~50 μM), while in the absence of tropomyosin, Tmod has a lower affinity for the pointed ends (KJ ~0.3–0.4 μM). This suggests that the tight capping of tropomyosin-coated actin filaments is due to Tmod’s ability to bind both tropomyosin and actin (reviewed in Ref. 34). To date, actin and tropomyosin are the only identified binding partners for Tmod.

While investigations into the properties of Tmod described above demonstrated that Tmod is a critical component for maintaining thin filament length by blocking actin polymerization at the pointed end, there is no evidence that Tmod is involved in specifying the lengths (for a review, see Ref. 35). In fact, it is difficult to envision how a capping protein, which is present only at the end of the filament, could alone determine the characteristic thin filament lengths that differ among various types of skeletal muscle. Thus, we hypothesized that the N-terminal region of nebulin, the part of the molecule predicted to be at or near the pointed ends of the thin filaments, interacts directly with Tmod as part of a linked regulatory mechanism in skeletal muscle to determine and maintain the lengths of the thin filaments. To test this idea, we first established by immunofluorescence microscopy that Tmod and the N-terminal end of nebulin colocalize in skeletal tissue. Next, we determined that Tmod specifically binds to a 13.5-kDa recombinant fragment from the extreme N-terminal modules of nebulin, as demonstrated by blot overlay, bead binding, and solid phase binding assays. Interestingly, the Sk-Tmod/nebulin interaction is of higher affinity than the E-Tmod/nebulin interaction. This isoform specificity of nebulin binding suggests that thin filament length regulation by nebulin and Tmod may differ in some respects in fast and slow skeletal muscle fibers and/or during skeletal muscle differentiation. The data presented here demonstrate that the N terminus of the nebulin molecule extends to the extreme end of the thin filament and also establish a novel biochemical function for this end. This study provides direct evidence to support the proposed model that the interaction of the capping protein, Tmod, with the molecular ruler, nebulin, may be pivotal for regulating actin filament assembly and length in skeletal muscle.

**EXPERIMENTAL PROCEDURES**

**Purified Proteins and Antibodies—** Antibodies against human E-Tmod were generated in rabbits and affinity-purified as described (36). Antibodies to recombinant fragments from the N-terminal region of embryonic chicken skeletal myotubes were prepared as described (37) and indirect immunofluorescent staining to localize Tmod and the N-terminal region of nebulin was performed (40). Briefly, paraformaldehyde-fixed cultures were permeabilized in 0.2% Triton X-100/phosphate-buffered saline for 15 min and immediately blocked in 2% bovine serum albumin/phosphate-buffered saline for 1 h. The myotubes were stained with monoclonal antibodies to Tmod (monoclonal antibody 95) at 2 μg/ml, followed by rhodamine-conjugated sheep anti-mouse IgG (1:200) (Roche Molecular Biochemicals). Next, the cells were incubated with affinity-purified polyclonal rabbit anti-neb N-term + M1 (see below for nomenclature of nebulin fragments) antibodies (10 μg/ml), followed by fluorescein-conjugated goat anti-rabbit IgG (1:200) (Roche Molecular Biochemicals). The myotubes were observed using a confocal microscope (Bio-Rad MRC600) and digital images were processed and merged using Adobe Photoshop software (San Jose, CA).

**Blot Overlays—** Nebulin was solubilized from rat psoas muscle fibers and resolved from other myofibrillar proteins using a large pore, 3.3–12% linear gradient SDS-gel electrophoresis system, under conditions optimized to resolve giant myofibrillar proteins (41, 42). After transfer to nitrocellulose (0.2 μm) (Schleicher and Schuell), blocking, and incubation with 1 μg/ml purified human E-Tmod in binding buffer (80 mM KCl, 2 mM MgCl2, 10 mM Hepes, pH 7.3, 2% Triton X-100, 20 mg/ml bovine serum albumin, 1 mM dithiothreitol), Tmod binding to nebulin was detected by incubation with affinity-purified rabbit anti-human E-Tmod antibodies followed by 125I-protein A and autoradiography. Nebulin was detected on a parallel nitrocellulose strip by immunoblotting with goat anti-nebulin antibodies (generously provided by Dr. Kuan Wang, University of Texas at Austin) followed by rabbit anti-goat antibodies and 125I-protein A.

**Indirect Immunofluorescence Microscopy—** Primary cultures of embryonic chicken skeletal myotubes were prepared as described (37) and indirect immunofluorescent staining to localize Tmod and the N-terminal region of nebulin was performed (40). Briefly, paraformaldehyde-fixed cultures were permeabilized in 0.2% Triton X-100/phosphate-buffered saline for 15 min and immediately blocked in 2% bovine serum albumin/phosphate-buffered saline for 1 h. The myotubes were stained with monoclonal antibodies to Tmod (monoclonal antibody 95) at 2 μg/ml, followed by rhodamine-conjugated sheep anti-mouse IgG (1:200) (Roche Molecular Biochemicals). The myotubes were observed using a confocal microscope (Bio-Rad MRC600) and digital images were processed and merged using Adobe Photoshop software (San Jose, CA).

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**Bacterial Expression and Purification of Cloned Nebulin Fragments—** Nebulin cDNA fragments encoding various domains were amplified from total human skeletal muscle cDNA by polymerase chain reaction, using a matchmaker cDNA library (CLONTECH, Palo Alto, CA). The primer sequences used are listed in the Appendix. The obtained fragments were subcloned into the T7 promoter-regulated pET vectors, which express their inserts C-terminally fused to 6x histidine tags (Novagen, Madison, WI). The expressed nebulin fragments correponded to neb N-term (11.3 kDa); neb N-term + M1 (13.6 kDa); neb N-term + M1-M2-M3 (21.5 kDa); M1-M2-M3 (13.5 kDa); M1-M8 (33.7 kDa); and M9-M15 (32.8 kDa). Additionally, a fragment corresponding to the unique acidic N-terminal domain plus the first three N-terminal modular repeats (nette + R1-R2-R3) of the cardiac nebulin-like protein, nebulrete, was also expressed as a control (24.3 kDa). To exclude potential artifacts from the histidine tags, the M1-M2-M3 nebulin fragment also was expressed in the pETM-11 vector, which allows histidine tag removal after cleavage by the TEV protease (Life Technologies, Inc, San Francisco, CA).
Inc.). The inserts were confirmed by DNA sequencing, and the expression vectors were transformed into Epicurian Coli BL21-Codon Plus (DE3)-RIL competent cells (Stratagene, La Jolla, CA). Large scale preparations of the nebulin fragments and the nebuette fragment were purified from the cell lysates according to instructions from Qiagen (Valencia, CA). Briefly, a 100-ml overnight culture of the transformed bacterial cells was used to inoculate 1 liter of Luria broth containing 35 μg/ml kanamycin. The cells were grown to an A600 of 0.6–0.8, induced with 0.2–0.4 mM isopropyl β-D-thiogalactopyranoside (Sigma), and 3–4 h later were collected by centrifugation. The bacterial pellets were resuspended in 15 ml of ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0, 10 mM imidazole, pH 7.4, 200 mM NaCl, 0.1% Nonidet P-40, and 2 mM β-mercaptoethanol plus 100 μg/ml of tosyl-L-lysyl chloromethyl ketone, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 μg/ml aprotinin, and 0.5 mM Pefabloc) and sonicated to lyse cells and reduce viscosity of the lysate. The lysate was ultracentrifuged for 30 min at 50,000 × g, and the supernatant was loaded onto a column containing 300 μl of Ni2+-nitrilotriacetic acid-agarose (Qiagen). The column was washed sequentially with 30 ml of lysis buffer, 30 ml of lysis buffer without Nonidet P-40, 1 ml of 1 M NaCl, and 50 ml of lysis buffer without Nonidet P-40. The His-tagged protein was eluted from the column with 10 ml of 300 mM imidazole, pH 7.5, and peak fractions were pooled. Some nebulin fragment preparations (containing contaminating bacterial proteins) were further purified using a fast protein liquid chromatography system on a Mono Q anion exchange Superose 6 gel filtration column (Amersham Pharmacia Biotech). The pooled peak fractions were concentrated using Centriplus centrifugal filter devices (molecular weight cut-off of 3,000) (Millipore Corp.), and dialyzed against binding buffer for the solid phase binding assays (20 mM Hepes, pH 7.4, 80 mM KCl, 2 mM MgCl2, 0.002% NaN3, 0.05% Tween 20, 0.2% bovine serum albumin) and then blocked in the same buffer for 1 h at 4°C. The wells were then incubated with 100 nM skeletal muscle tropomyosin (as a positive control), diluted with binding buffer, and bound proteins were eluted by incubating the slurry with 20 μl of 0.1 M glycine, pH 2.8, for 1 h at room temperature. The eluted proteins were neutralized with 1 μl of 1 M Tris-HCl, pH 9.0, and boiled in SDS sample buffer. The same volume of unbound and eluted proteins was separated on 18% SDS-polyacrylamide gel electrophoresis (Merck), boiled in SDS sample buffer, and then run for 2 h. The proteins were transferred to nitrocellulose membranes, and the strips were then incubated with blocking buffer containing 10% nonfat dried milk, 0.02% NaN3, and 0.05% Tween 20, at room temperature with gentle rocking. 20 μl of a 1:1 slurry of streptavidin-conjugated Sepharose 4B beads (Amersham Pharmacia Biotech) in binding buffer were added to the protein solution and incubated for 1 h at room temperature with gentle rocking. 20 μl of the supernatant, containing unbound proteins, were recovered and boiled in SDS sample buffer. The Sepharose slurry was washed five times with 400 μl of binding buffer, and bound proteins were eluted by incubating the slurry with 20 μl of 0.1 M glycine, pH 2.8, for 1 h at room temperature. The eluted proteins were neutralized with 1 μl of 1 M Tris-HCl, pH 9.0, and boiled in SDS sample buffer. The same volume of unbound and eluted proteins was separated on 18% SDS-polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining.

**RESULTS**

**Nebulin Colocalizes with Tmod at the Pointed Ends of the Thin Filament**—Previous immunolocalization studies have revealed that the N-terminal region of nebulin is located at or in close proximity to the pointed ends of the thin filaments (10, 12, 20), which is where Tmod is located. To determine if the N-terminal region of nebulin colocalizes with Tmod at the pointed ends of the thin filaments, indirect immunofluorescence microscopy studies were performed. Polyclonal antibodies were generated against a recombinant fragment comprising nebulin’s extreme N-terminal unique 8-kDa domain (which we refer to as neb N-term) plus the first unique module, M1. These anti-nebulin (neb N-term + M1) antibodies were shown to be specific by Western blotting, since they detected a single band corresponding to the molecular weight of nebulin in muscle lysates (data not shown). Double staining of primary cultures of embryonic chicken skeletal myotubes with the anti-nebulin (neb N-term + M1) (Fig. 1A) and Tmod antibodies (Fig. 1B) revealed that these two proteins colocalize (Fig. 1C). This demonstrated that the extreme N-terminal region of nebulin is in the correct location in the sarcomere to interact with Tmod.

**Tmod Binds to Nebulin in Blot Overlay Studies**—As an initial approach to determine whether Tmod binds to nebulin, we performed a blot overlay assay with isolated rat myocardial thin filaments. A large pore, SDS gradient polyacrylamide gel system was used to resolve giant proteins (41, 42). Coomassie Blue staining of the proteins demonstrated that a band corresponding to the molecular mass of nebulin (~800 kDa) was resolved using this system (Fig. 2, lane 1). The proteins were transferred onto nitrocellulose membranes, and the strips were then incubated with a series of linearized plots to accurately quantify association constants. Briefly, in a series of ELISA experiments, the association constant, k, was calculated according to a constant, unknown calibration factor (c) to the amount of immobilized “receptor” (i.e., the M1-M2-M3 nebulin fragment) bound to its soluble “ligand” (i.e., the biotinylated Tmod). This calibration factor can be determined assuming that a defined amount of ligand in solution becomes completely bound to an immobilized receptor upon repeated transfer from one receptor-coated well to another in a series of “transfer assays.” For the deELISA, three sets of experiments were required, using the solid phase binding assay conditions described above. First, the incubation time of the receptor with the ligand was varied in a time course to determine the rate constant, k,. Second, a series of “transfer assays” (where unbound ligand from one receptor-coated well is transferred to the next, identified with serial well numbers) was performed at various times of incubation (10, 15, 20, and 30 min) in order to determine the transfer factor, F,, as well as c. Finally, a saturation curve under equilibrium conditions was generated to determine the amount of immobilized receptor available to bind to the ligand. The obtained results allowed for the calculation of the association constant, k,, the inverse of which is equal to the K value.
Tmod Binds to the M1-M2-M3 Modules in the N-terminal Region of Nebulin—If the binding of nebulin to Tmod is physiologically relevant, the Tmod-binding site on nebulin must be present in nebulin’s N-terminal region, which is located at the pointed ends of the thin filaments. The extreme N-terminal region of nebulin, consisting of an 8-kDa domain (neb N-term), is adjacent to eight unique modules (M1 through M8) (13). These modules are each approximately 35 residues in size and are similar to one another but only distantly related to the other classes of nebulin modules (13). To determine the location of the Tmod-binding site on nebulin, we generated recombinant fragments, including various combinations of these N-terminal modules with and without the neb N-term domain, for use in solid phase Tmod-binding assays (Fig. 3).

The recombinant nebulin fragments, as well as control fragments and proteins (see below), were adsorbed onto microtiter plates at equal molar concentrations to obtain equal numbers of putative Tmod-binding sites per well. Biotinylated recombinant E- and Sk-Tmods were incubated with the immobilized fragments or with the known Tmod binding partner protein, skeletal troponymosin, as a positive control (Fig. 4A). As expected, Sk-Tmod bound to skeletal troponymosin, verifying the utility of this binding assay. Comparison of Tmod binding to the N-terminal nebulin fragments showed that no binding of Sk-Tmod to neb N-term (the unique 8-kDa acidic domain at the extreme N-terminal end of nebulin) was detected. Occasionally, Sk-Tmod binding to neb N-term + M1 (the 8-kDa domain linked to the first unique module) was detected, albeit weakly, suggesting that the M1 module may contain a partial binding site for Sk-Tmod. In contrast, Sk-Tmod was observed to bind strongly and consistently to neb N-term + M1-M2-M3 (the 8-kDa domain fused to the first three N-terminal unique modules), to M1-M2-M3 alone, and to the modules M2-M8. To exclude potential artifacts that may have resulted from the presence of the histidine tag fused to the recombinant nebulin fragments, the tag was cleaved from the M1-M2-M3 fragment, and no difference in binding to Tmod was detectable (data not shown). Further mapping of the Tmod binding site using smaller recombinant fragments was hindered by the insolubility of these fragments (data not shown).

Interestingly, in comparing the binding of Sk-Tmod to neb N-term + M1-M2-M3 with the binding of Sk-Tmod to M1-M2-M3, it was found that Sk-Tmod bound more strongly to M1-M2-M3 alone. Why the larger neb N-term + M1-M2-M3 nebulin fragment, which also contains the Tmod binding site, was not able to bind to Tmod as strongly as M1-M2-M3 alone is not known, although one possibility is that the neb N-term domain sterically hinders the complete Sk-Tmod binding site. The results from these studies indicate that the first three unique N-terminal modules of nebulin, the M1-M2-M3 region (representing 13.5 kDa of the ~800-kDa molecule), are sufficient for binding to Tmod.

In order for binding of Sk-Tmod to the N-terminal region of nebulin to be specific, Tmod should bind only to this region and not elsewhere along the length of the nebulin molecule. In fact, binding of Sk-Tmod to a series of modules comprising the first nebulin super repeat (SR1), M9–M15, which is located adjacent to (toward the C-terminal end of) the unique M1–M8 modules, was not detected (Fig. 4A). As an additional control, a recombinant fragment comprising the N-terminal domain and first three modular repeats of nebullette, nette + R1-R2-R3, was used. Nebulette is a small (107 kDa), Z-line-associated protein found in cardiac muscle that shares sequence homology and a similar domain layout with nebulin (14, 45, 46). No binding of Sk-Tmod to this nebullette fragment was detected. This was expected, since the location of the nebullette N terminus is predicted to be located only ~0.2 μm from the Z line and not at the pointed ends of the filaments where Tmod is found (45). We conclude from these data that the binding of Tmod to nebulin is specific for the M1-M2-M3 modules at the N-terminal end of nebulin. Furthermore, this binding of Tmod to the unique M1-M2-M3 modules strongly indicates that the N-terminal end of nebulin does, in fact, extend to the extreme end of the thin filaments.

Using this assay, we also found that Sk-Tmod appeared to bind more strongly to nebulin compared with E-Tmod binding to nebulin (Fig. 4A). E-Tmod was observed to bind consistently only to M1-M2-M3, although it sometimes bound weakly to neb N-term + M1-M2-M3 and to M1-M8. Since both Sk-Tmod and E-Tmod bound to the recombinant nebulin fragment M1-M2-M3 to a greater extent than to other nebulin fragments (Fig. 4A), M1-M2-M3 was used in studies designed to further characterize the Tmod/nebulin interaction. First, the interaction between Sk-Tmod and M1-M2-M3 was compared with the interaction of E-Tmod with M1-M2-M3. We performed competition assays using our solid phase binding system, where biotinylated Tmods were incubated with excess unlabelled Tmods (Fig. 4, B and C). The results show that the binding of both biotinylated Tmod isoforms to M1-M2-M3 was inhibited by the addition of excess amounts of unlabelled Tmods. Thus, the binding of the biotinylated Tmods to M1-M2-M3 in the assay is not due to the biotin label (on Tmod) binding nonspecifically to nebulin. Additionally, unlabelled Sk-Tmod was more effective than unlabelled E-Tmod at inhibiting the binding of biotinylated Sk-Tmod or E-Tmods to the immobilized M1-M2-M3 (Fig. 4, B and C). These results indicate that, first, both Tmod isoforms probably bind to similar or overlapping sites on nebulin and, second, that Sk-Tmod binds to nebulin with a higher affinity than E-Tmod.
affinity than does E-Tmod, which is consistent with the results described above (Fig. 4A).

**Tmod Binds to the N-terminal End of Nebulin in Solution**—The solid phase binding assays described above require that the nebulin fragments be adsorbed onto the wells of a microtiter plate, which can result in partial denaturation of the peptides. In order to determine whether Sk-Tmod binds to nebulin under native conditions, we performed bead binding studies in which the proteins were incubated together in a soluble state. The recombinant nebulin fragment M1-M2-M3 was incubated with biotinylated Sk-Tmod, and the protein mixture was then incubated with streptavidin-conjugated Sepharose beads. The bound (lanes B) and unbound (lanes U) fractions were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue (Fig. 5). When M1-M2-M3 was incubated with the streptavidin beads alone, all of the fragment was recovered in the unbound fraction; i.e. M1-M2-M3 did not bind alone to the beads (Fig. 5, lanes 1 and 2). Consistently, however, when we added biotinylated Sk-Tmod to the M1-M2-M3 fragment, a portion of M1-M2-M3 (ranging from 20 to 50% of the total) was recovered in the bound fraction (Fig. 5, lane 4).

The presence of unbound M1-M2-M3 in the supernatant may be due to the saturation of all of the potential binding sites (Fig. 5, lane 3). Similar results were obtained when the M1-M2-M3 fragment was incubated with biotinylated E-Tmod (data not shown). The results of the bead binding assays indicate that Tmod also binds to nebulin when both proteins are present under native conditions.

**Binding of Tmod to the Nebulin M1-M2-M3 Modules Is Saturable and of High Affinity**—The next series of experiments was designed to determine whether Tmod binding to the nebulin fragment, M1-M2-M3, was saturable and to obtain an approximate value for the dissociation constant ($K_d$) for the binding of Tmod to M1-M2-M3. Immobilized M1-M2-M3 was incubated with increasing amounts of either biotinylated Sk-Tmod (Fig. 6A) or E-Tmod (Fig. 6B) for 1 h in saturation binding studies. Determination of the concentration of Tmod corresponding to the half-maximal $A_{405}$ value yields a semi-quantitative estimate of the $K_d$ value for Tmod binding to M1-M2-M3. These experiments showed that the binding of Tmod to M1-M2-M3 is saturable. Sk-Tmod bound to M1-M2-M3 with an apparent $K_d$ of approximately 15 nM, while E-Tmod bound to M1-M2-M3 with an apparent $K_d$ of approximately 30 nM. The results from these saturation studies are consistent with the previous finding (Fig. 4) that more Sk-Tmod binding to M1-M2-M3 was detected compared with E-Tmod. We also analyzed the interaction of E-Tmod with immobilized skeletal muscle tropomyosin (Fig. 6B). In these assays, E-Tmod bound to tropomyosin with a much weaker apparent affinity than it bound to M1-M2-M3, with a $K_d$ in the range of 100–150 nM (Fig. 6B); this $K_d$ value is comparable with that obtained from previous binding studies (−200 nM) (38).

To more accurately determine $K_d$ values for the Tmod/M1-M2-M3 interaction, a series of equilibrium and kinetic binding experiments in a dcELISA was performed (43). The dcELISA utilizes a series of linearized plots to accurately quantify association constants. Three sets of experiments are required. First, the incubation time of the receptor with the ligand is varied in a time course to determine the binding complex formation rate constant, $k_c$. Second, a series of transfer assays at various binding times is performed to determine the transfer factor, $F_t$, and the calibration factor, $c$. Finally, a saturation curve is used to determine the amount of immobilized M1-M2-M3, $R_c$. The results obtained allow for the calculation of the association constant, $K_a$, the inverse of which is equal to the $K_d$ value.

Fig. 7 shows results obtained from the dcELISA study. In Fig. 7A, we determined the M1-M2-M3-Tmod complex formation rate constant, $k_c$, by varying the time that biotinylated Tmod was incubated with the immobilized recombinant M1-M2-M3 fragment. Following linear regression analysis on the data plotted as $\ln(A_c - A_t)$ versus time (Fig. 7A), we determined that the $k_c$ for Sk-Tmod binding to M1-M2-M3 is $4.3 \times 10^{-4}$ s$^{-1}$ and the $k_c$ for E-Tmod binding to M1-M2-M3 is $2.5 \times 10^{-3}$ s$^{-1}$. In the next experiments, transfer assays were performed where immobilized M1-M2-M3 was incubated with biotinylated Tmod for various amounts of time (Fig. 7B). Unbound Tmod remaining in solution was then removed from the well and transferred to the next well, also containing immobilized M1-M2-M3. The results from one of a total of four transfer assays are shown, where Tmod was incubated with M1-M2-M3 for 30 min/well (Fig. 7B). Before unbound Tmod was transferred to the next, identically coated well. After generating semilogarithmic plots of $\ln$ versus the total number of transfers performed at each incubation time, linear regression was performed to obtain the $F_t$ values (data not shown). These experiments also were used to calculate the calibration constant, $c$, according to the equation $c = L/A_t \times (1 - F)$ (data not shown).

Finally, to determine the $K_a$ values, saturation curves were generated under equilibrium conditions, where Tmod was in-
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Fig. 4. Tmod specifically binds to recombinant fragments from the N-terminal end of nebulin in solid phase binding assays. A, 100 nM of purified rabbit skeletal muscle tropomyosin, recombinant nebulin fragments nebulin plus the unique 8-kDa domain from the extreme N terminus, nebulin plus the first unique module, nebulin plus the first three unique modules, M1-M2-M3, M1-M2-M3 (the first three unique modules), M2-M8 (seven of the eight unique N-terminal modules), M9-M15 (internal modules comprising the first super repeat), as well as a recombinant nebulin fragment, nettel-R1-R2-R3 (the nebulin N-terminal domain plus the first three unique modules), were adsorbed onto microtiter plates. After washing and blocking, the wells were incubated with 2.5 nM biotinylated E-Tmod (open bars) or biotinylated Sk-Tmod (solid bars). Following washes and incubation with alkaline phosphatase-conjugated streptavidin, bound Tmod was determined by a colorimetric reaction at 405 nm. Background values of wells coated with buffer alone were subtracted from the values obtained from all other wells. The graph is representative of three experiments. Values are the mean of duplicates ± S.D.

Fig. 5. Sk-Tmod binds to M1-M2-M3 under native conditions. 1 µg of recombinant M1-M2-M3 was incubated in 20 µl of binding buffer for 1 h at room temperature in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 1 µg of biotinylated Sk-Tmod. Streptavidin-conjugated Sepharose beads were then added (1:1) to the protein solution, and the unbound (lanes U) and bound (lanes B) fractions were separated by 18% SDS-polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining. Note that under elution conditions using 0.1 M glycine, pH 2.8, the biotinylated Tmod remained bound to the streptavidin beads and therefore was not recovered in either bound or unbound fractions. The gel is representative of 10 experiments.

Fig. 6. The binding of Tmod to M1-M2-M3 is saturable in solid phase binding assays. 100 nM M1-M2-M3 (A and B, squares) or 100 nM purified rabbit skeletal tropomyosin (B, diamonds) was adsorbed onto microtiter plates and incubated with increasing concentrations of biotinylated Sk-Tmod (A) or biotinylated E-Tmod (B) for 1 h at 4 °C. Following washes and incubation with alkaline phosphatase-conjugated streptavidin, wells were incubated with pNPP substrate. Binding of Tmod was determined by a colorimetric reaction at 405 nm. Background values of wells coated with buffer alone were subtracted from the values obtained from all other wells. A<sub>405</sub> values were plotted versus the concentration of biotinylated Tmod and fitted to a moving average trendline with a period of 2, using Microsoft Excel. The graphs are representative of three experiments. Values are the mean of duplicates ± S.D. The approximate K<sub>j</sub> is estimated by determining the concentration of Tmod at half-maximal A<sub>405</sub> for the fitted curve. Note that the saturation curves for the three proteins, Sk-Tmod, E-Tmod, and tropomyosin, were performed in various combinations, and similar results were obtained.

Incubated with nebulin for 4 h at 4 °C (Fig. 7, C for Sk-Tmod and D for E-Tmod). Using the saturation absorbance obtained by nonlinear regression analysis (data not shown), as well as the values obtained for c and F in the previous series of experiments, we calculated the K<sub>c</sub> values to be 0.227 × 10<sup>9</sup> liters/mol for Sk-Tmod binding to M1-M2-M3 and 0.067 × 10<sup>9</sup> liters/mol for E-Tmod binding to M1-M2-M3. The inverse of these values
The initial concentration of M1-M2-M3 (μM) was adsorbed onto microtiter plates and incubated with 2.5 nM biotinylated Sk-Tmod (squares) or E-Tmod (diamonds) for various times, washed, and incubated with alkaline phosphatase-conjugated streptavidin. Following additional washes and incubation with pNPP substrate, binding of Tmod was determined by a colorimetric reaction at A405. The A405 values were plotted versus time, and nonlinear regression analysis was performed to determine A∞ (data not shown). The data points were then plotted as ln(A∞ – A) versus time and represent the mean of duplicates (y = –0.2718 + 0.03889 and R² = 0.9715 for Sk-Tmod; y = –0.0145x – 0.2738 and R² = 0.99 for E-Tmod). A linear regression analysis leads to a kₜ = 4.3 × 10⁻⁴ s⁻¹ for Sk-Tmod binding to M1-M2-M3, and a kᵦ value of 2.5 × 10⁻⁴ s⁻¹ for E-Tmod binding to M1-M2-M3.

B. 100 nM M1-M2-M3 was adsorbed onto microtiter plates and incubated with 2.5 nM biotinylated Sk-Tmod (squares) or E-Tmod (diamonds) for various times. Next, the Tmod solution was transferred to an adjacent well for the same amount of time. Between four and seven transfers were performed for each experiment, for either 10, 15, or 20 min (data not shown) or 30 min (B) of incubation for each well. Each well was washed and then incubated with alkaline phosphatase-conjugated streptavidin, followed by incubation with pNPP substrate. The data were plotted as lnA405 versus the transfer number (y = –0.337x + 0.6929 and R² = 0.981 for Sk-Tmod; y = –0.2718x – 0.7459 and R² = 0.9331 for E-Tmod). Using the absorbances of the first wells as A₁ and the initial concentration of M1-M2-M3 (L₀), calibration constant (c) values were calculated for each transfer plot and averaged to c = 0.32 nm for Sk-Tmod and 0.30 nm for E-Tmod. C and D, to determine Kᵦ values, saturation curves for biotinylated Sk-Tmod and E-Tmod were calculated under equilibrium conditions. 100 nM M1-M2-M3 was adsorbed onto microtiter plates and incubated with various concentrations of biotinylated Sk-Tmod (C) or E-Tmod (D) for 4 h. After washes and incubation with alkaline phosphatase-conjugated streptavidin, followed by incubation with pNPP substrate, binding of Tmod was determined by a colorimetric reaction at A₄⁰⁵. The saturation absorbance was used along with the obtained c and F values, and the association equilibrium constant was calculated as Kᵦ = 0.227 × 10⁴ liters/mole for Sk-Tmod binding to M1-M2-M3 and 0.067 × 10⁴ liters/mole for E-Tmod binding to M1-M2-M3. The inverse of the Kᵦ values yields the Kᵦ values of 4.4 nm for Sk-Tmod and 15.0 nm for E-Tmod binding to M1-M2-M3.

Strikingly, the interaction of Tmod with the N-terminal region of nebulin (Kᵦ ~4–16 nm) is considerably tighter compared with previously reported interactions of nebulin with other proteins. Although these previous investigations were performed using different in vitro binding assays, it is nevertheless of interest to compare these interactions with the Tmod/nebulin interaction. For example, nebulin has been reported to bind F-actin with a Kᵦ ranging from ~0.1 to ~400 μM (depending on the nebulin fragment utilized), troponin with a Kᵦ of ~100–200 nm, tropomyosin with a Kᵦ of ~500 nm, calmodulin with a Kᵦ of ~100 nm, and myosin with a Kᵦ of ~160 nm (19, 47–51). The affinity of the Tmod/M1-M2-M3 interaction is more similar, however, to the interaction of Tmod with the pointed ends of tropomyosin-coated actin filaments (Kᵦ < 1 nm) (24, 33). The tight binding of Tmod to both the N-terminal end of nebulin and to the pointed ends of the thin filaments suggests that together these interactions have a critical role in the sarcomere.

**Tropomyosin Does Not Affect Binding of Tmod to M1-M2-M3**—It has previously been established that the interaction of tropomyosin with Tmod is important for modulating the interaction of Tmod with actin and for Tmod’s actin filament capping activity (24, 36). Thus, we next determined if tropomyosin affects the interaction of Tmod with nebulin. Biotinylated Tmod was incubated with immobilized M1-M2-M3 in the presence of increasing amounts of purified rabbit skeletal muscle tropomyosin (Fig. 8). Since the affinity of Tmod for tropomyosin is 2 orders of magnitude weaker than the affinity of Tmod for M1-M2-M3 (150–200 nm versus 4–16 nm; see above and Ref. 38), we included a 200-fold excess of tropomyosin over Tmod in these assays. Tropomyosin had no detectable effect on the interaction of either Sk-Tmod or E-Tmod with M1-M2-M3. This suggests that skeletal tropomyosin and M1-M2-M3 have distinct, or nonoverlapping, binding sites on Tmod. Since tropomyosin binds to Tmod within the Tmod residues 1–130 (38, 52), it is expected that the primary M1-M2-M3 binding site is located outside of these Tmod residues.

**DISCUSSION**

The length distributions of the thin filaments in skeletal muscle are strikingly narrow and uniform, indicating that an intricate regulatory mechanism exists for precisely controlling...
their lengths. It has been proposed that this mechanism must involve at least two types of proteins, namely template molecules to specify thin filament length (e.g. nebulin) and capping proteins to maintain the specified length (e.g. CapZ at the barbed ends and Tmod at the pointed ends) (1, 5). In this study, we report that the actin-capping and tropomyosin-binding protein, Tmod, has a third binding partner, nebulin. Tmod binds specifically to the extreme N-terminal modules, M1-M2-M3 (13.5 kDa), of the giant nebulin molecule (~600–800 kDa) as demonstrated by blot overlay studies, solid phase binding assays, and bead binding assays. To our knowledge, this is the first report of a biochemical function for the N-terminal region of nebulin. The establishment of the Tmod/nebulin interaction demonstrates that nebulin’s final N-terminal modules indeed extend out to, and interact with, the extreme ends of the thin filaments, a requirement necessary to function as a molecular ruler for thin filament length specification. These data provide evidence that nebulin’s N-terminal end, together with Tmod, probably function as part of a linked regulatory mechanism for thin filament length regulation in skeletal muscle.

Based on our results and the investigations of others, we propose a molecular model that demonstrates how the interaction of nebulin with Tmod could regulate thin filament length at the pointed ends in various types of skeletal tissues (Fig. 9). First, the association of each actin subunit in the thin filament with an individual nebulin module specifies the length of the thin filament (10, 13, 19). Specific thin filament lengths are precisely defined by different nebulin isoforms containing varying numbers of modules. It should be noted that the exact mode of association of nebulin along the length of the actin/thin filament has not yet been resolved. Structural studies and microscopic investigations indicate that nebulin is likely to bind in the central cleft of the actin filament, i.e. in the phalloidin binding site (53–55). However, in vitro binding studies have led to the alternative proposal that nebulin associates with the outer edges of the actin filament, forming a composite regulatory complex with tropomyosin and the troponins (19, 47, 48, 56). Interestingly, recombinant nebulin fragments have also been reported to inhibit actomyosin ATPase activity (49). Therefore, nebulin may serve additional regulatory roles in the sarcosome. Second, a tropomyosin polymer is associated with each of the strands in the actin filament in a head-to-tail manner, stabilizing the filament and helping to prevent actin disassembly from the pointed ends (e.g. see Ref. 57). Third, once the filaments attain their mature lengths, nebulin’s extreme N-terminal modules target Tmod to the pointed ends of the thin filaments that have polymerized to the length of the nebulin ruler. Fourth, Tmod caps the actin filament pointed ends and perhaps the tropomyosin polymers, thus maintaining the correct lengths of the filaments (26). Finally, targeting of Tmod to the pointed ends of thin filaments of correct lengths, via binding to nebulin, is likely to be a dynamic process. This hypothesis is based on recent studies indicating that the thin filaments are not irreversibly capped by Tmod and can still exchange actin subunits with the monomer pool (2). Therefore, a nebulin-mediated mechanism may regulate and/or stabilize Tmod’s capping activity at the pointed ends. Future studies will be directed at investigating the effect of the N-terminal region of nebulin on the capping activity of Tmod in actin filament polymerization assays.

We found that the N-terminal region of nebulin specifically binds to both Sk-Tmod and E-Tmod. However, Sk-Tmod binds to nebulin with a higher affinity compared with the binding of E-Tmod to nebulin. These two Tmod isoforms are products of different genes but are 62% identical and 75% similar to each other at the amino acid level in chicken (32, 58). Previous in vitro studies have not detected differences in their ability to cap pure actin filaments or tropomyosin-coated actin filaments (24, 32). However, our studies now reveal that the two Tmod isoforms differ in their interactions with the N-terminal region of nebulin. Although it is not yet known if this ~4-fold difference in affinity is physiologically significant, it is tempting to speculate that it may explain the previously reported differences in the cellular properties of Sk-Tmod and E-Tmod.

First, the distinct interactions of Sk-Tmod and E-Tmod with nebulin may explain the previously reported differences in the subcellular localization patterns of these isoforms in skeletal tissue. In chicken slow muscle fibers, the predominant Tmod isoform expressed is E-Tmod, which is associated with the thin filament pointed ends (32). In chicken fast skeletal muscle fibers, which co-express both Tmod isoforms, there is distinct targeting of E- and Sk-Tmods to different actin filament-containing structures (32). In these fibers, E-Tmod predominantly colocalizes with α-spectrin in costameric subsarcolemmal domains, while Sk-Tmod is present at the pointed ends of the thin filaments in sarcomeres of those same cells (32). Therefore, the
higher affinity interaction of nebulin with Sk-Tmod compared with E-Tmod may cause Sk-Tmod to outcompete E-Tmod for the nebulin-binding site at the pointed ends of the thin filaments in fast muscle.

It is also possible that the isoform-specific interaction of Tmod with nebulin may provide the molecular basis for the observed variations in thin filament lengths between slow and fast skeletal muscle fibers as well as during muscle differentiation (e.g. see Refs. 59 and 60). For instance, E-Tmod is located at the pointed ends of thin filaments in embryonic chicken pectoralis muscle. This embryonic tissue contains longer thin filaments with a wider length distribution (~0.95–1.1 μm) compared with adult pectoralis muscle, which has Sk-Tmod at the pointed ends and shorter, more uniform thin filaments (all of the thin filaments are ~0.9 μm long) (60). We speculate that this phenomenon is a result of the differential nebulin-Tmod isoform interaction that functions to regulate thin filament lengths. Specifically, the tighter association of Sk-Tmod with nebulin, compared with the association of E-Tmod with nebulin, may cause tighter capping at the pointed ends, resulting in a reduced actin monomer exchange at the pointed ends and, hence, more precise thin filament lengths in adult fast skeletal muscle fibers.

Finally, it is striking that cardiac muscle, which expresses E-Tmod (32) but does not appear to contain nebulin (11) has a significantly broader range of thin filament lengths (e.g. 0.6–1.1 μm in rat) as compared with skeletal muscle (e.g. 1.1 ± 0.03 μm in rabbit) (3, 4). Small proteins with homology to nebulin have recently been identified in heart, but these proteins do not localize to the pointed ends of the thin filaments. These include nebulette, which associates with the Z-disc and is likely to extend only about 25% along the length of the thin filament, and N-RAP, which is found at intercalated discs (14, 45, 46, 61). Thus, in cardiac muscle, E-Tmod may work with proteins other than nebulin to regulate thin filament lengths at the pointed ends, although the particular regulatory mechanism appears to be less precise than that for skeletal muscle, which contains full-length nebulin.

Investigations into the physiological significance of the Tmod/nebulin interaction are needed and are expected to be pivotal for deciphering the mechanisms of thin filament regulation in skeletal muscle. Since the precise organization of the thin filaments is pivotal for deciphering the mechanisms of thin filament regulation, investigations into the function of the Tmod/nebulin interaction will aid in understanding actin filament dynamics in a variety of cell types.

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APPENDIX

Expression of Recombinant Nebulin Fragments—The expression constructs encoding fragments from the N-terminal region of nebulin, as well as the expression construct encoding a fragment from the N-terminal region of the cardiac protein, nebulette, were expressed in Escherichia coli using the pET system (65) and are as follows (to simplify protein purification, histidine hexamer tags were included either as N-terminal or C-terminal fusions): 1) Neb N-terminal unique acidic domain, in pET9d + C-terminal His$_{6}$ (11.3 kDa); 2) Neb N-Term + M1, in pET8c + N-terminal His$_{6}$ (13.6 kDa); 3) Neb N-Term + M1-M2-M3, in pET9d + C-terminal His$_{6}$ (21.8 kDa); 4) M1-M2-M3, in pET9d C-terminal His$_{6}$ and in pET-M11 (13.5 kDa); 5) M1-M8, in pET9d C-terminal His$_{6}$ (33.7 kDa); 6) M9-M15, in pETd C-terminal His$_{6}$ (32.8 kDa); 7) nebulette N-term + R1-R2-R3 in pET9d C-terminal His$_{6}$ (24.5 kDa).

Nebulin cDNA fragments were amplified by polymerase chain reaction from total human skeletal muscle cDNA (66) using combinations of the primers listed below. Skeletal muscle cDNA was used as a template (CLONTECH, Matchmaker HL4047AH). For polymerase chain reaction, about 50 ng of the amplified cDNA was used as template, and 25 cycles were performed with the following profile: 10 s, 95 °C; 2 min, 68 °C; and 5 min, 74 °C final incubation. After polymerase chain reaction amplification, the products were agarose gel-purified and subcloned into pET vectors essentially as described (67).

 Primer Sequences—All primer sequences were derived from EMBL data library accession number X83957 (20.9-kilobase pair full-length human skeletal nebulin cDNA). The following primer pairs were used for amplification of the nebulin N-terminal unique acidic domain (beginning with the amino acid sequence MADD) to the M15 module of nebulin. Small capital letters denote primer mismatch portions harboring cloning sites, whereas capital letters correspond to nebulin codons/reverse codons: neb N-term sense (MADD-sense), ttctcagc GCA GAT GAC GAA GAC TATG; X83957 nebulin 444S; neb 731R (MADD-reverse), ttacgct-ta-GCT AAA AAG ATC CTG CATT; nebulin 663S (M1-sense), ttctcagggc-AAA GTG GAT CCT TCA AAG TTC ATG ACC CCC TAC; neb 797R (M1-reverse), ttgacagt-ta-AGT ATC TGT TGT GCT GGC; neb 774S (M2-sense), tttcagc-CCA TAC GCC AGC ACA ACA GA; neb 890R (M2-reverse), ttgacacct-ta-TAC GTG ATC CCT ATC AAC ACC ATC ACC; neb 996S (M4-sense), tttcagct-CCT GAC GCC CCT TCA TTC GTC CAG; neb 1,533S (M9-sense), ttctcctag-gcc-GCT TCA GAG AAC CCA CAG CTT AGG CAG; neb 1,007R (M3-reverse), ttgacacct-ta-GGC ATC AGG AAG CAG CTA TTC ATC; neb 1,532R (M8-reverse), ttgacagt ta-AAG CAC ATT ATC ATC GTC; neb 2,385R (M15-reverse), tttgacacct-ta-ATT CAT ACT CTT TGC CTT TTC CTA AAG.

As discussed above, to compare the properties of the expressed nebulin N-terminal + M1-M2-M3 with those of a corresponding nebulette fragment, nebulette N-terminal + R1-R2-R3 (14) was amplified with the following primer pair: ttctcagc-ggg AGG GTC CCT GTA TTT GAG GAT (human nebulin 398S) and ttgacacct-tta-GGC CTT CTT ATC GTG GCC ATA (human nebulin 830R).

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