Tropomodulin Is Associated with the Free (Pointed) Ends of the Thin Filaments in Rat Skeletal Muscle

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Abstract. The length and spatial organization of thin filaments in skeletal muscle sarcomeres are precisely maintained and are essential for efficient muscle contraction. While the major structural components of skeletal muscle sarcomeres have been well characterized, the mechanisms that regulate thin filament length and spatial organization are not well understood. Tropomodulin is a new, 40.6-kD tropomyosin-binding protein from the human erythrocyte membrane skeleton that binds to one end of erythrocyte tropomyosin and blocks head-to-tail association of tropomyosin molecules along actin filaments. Here we show that rat psoas skeletal muscle contains tropomodulin based on immunoreactivity, identical apparent mobility on SDS gels, and ability to bind muscle tropomyosin. Results from immunofluorescence labeling of isolated myofibrils at resting and stretched lengths using anti-erythrocyte tropomodulin antibodies indicate that tropomodulin is localized at or near the free (pointed) ends of the thin filaments; this localization is not dependent on the presence of myosin thick filaments. Immunoblotting of supernatants and pellets obtained after extraction of myosin from myofibrils also indicates that tropomodulin remains associated with the thin filaments. 1.2–1.6 copies of muscle tropomodulin are present per thin filament in myofibrils, supporting the possibility that one or two tropomodulin molecules may be associated with the two terminal tropomyosin molecules at the pointed end of each thin filament. Although a number of proteins are associated with the barbed ends of the thin filaments at the Z disc, tropomodulin is the first protein to be specifically located at or near the pointed ends of the thin filaments. We propose that tropomodulin may cap the tropomyosin polymers at the pointed end of the thin filament and play a role in regulating thin filament length.

ACTIN filament length and spatial organization in skeletal muscle sarcomeres are precisely regulated and are essential for efficient contraction (Huxley, 1960). The actin filaments (thin filaments) are all ~1.0 μm long and are polarized with their fast growing (barbed) ends located at the Z disc and their slow growing (pointed) ends at the A band, where they interdigitate with myosin thick filaments. At the Z disc, the thin filaments are crosslinked and anchored by α-actinin into regular arrays (I-Z-I brushes) extending with opposite polarity away from each side of the Z disc. Rodlike tropomyosin molecules are associated head-to-tail along each thin filament, forming two polymers, one on each side of the actin filament. Both the barbed and pointed ends of thin filaments in skeletal muscle appear to be capped, based on the inability of exogenous actin to elongate from the filaments at either end when added to isolated myofibril preparations (Sanger et al., 1984; Ishiwata and Funatsu, 1985; Peng and Fischman, 1991). Elongation of actin filaments at the barbed end is thought to be blocked by Cap Z, a high affinity barbed end capping protein from skeletal muscle that is localized at the Z disc (Casella et al., 1987). β-Actinin once appeared to be a good candidate for a pointed end capping protein in skeletal muscle, but its sequence was recently shown to be identical to that of Cap Z (Maruyama et al., 1990). In nonmuscle cells, acumentin was originally thought to be a pointed end capping protein (Southwick and Hartwig, 1982), but was determined later to be identical to human L-plastin (Southwick, F. S., personal communication), a protein homologous to the F-actin bundling protein, fimbrin (de Arruda et al., 1990). Thus, the identity of the protein(s) responsible for preventing actin filament elongation from the pointed end remains unknown.

The length and spatial organization of actin filaments are also precisely regulated in the erythrocyte membrane skeleton, where they are all 33–37 nm long and are crosslinked by long, flexible spectrin molecules into a hexagonal network on the cytoplasmic surface of the plasma membrane (Byers and Branton, 1985; Shen et al., 1986; for a review see Bennett, 1989). Each short actin filament is thought to be associated with two erythrocyte tropomyosin molecules (33–34 nm long), one in each groove of the filament (Fowler and Bennett, 1984; Fowler, 1990). The close similarity in length between erythrocyte tropomyosin and erythrocyte ac-

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tin filaments has led to speculations that tropomyosin both stabilizes the short actin filaments (Fowler and Bennett, 1984) and determines their lengths (Shen et al., 1986). However, purified tropomyosins from both muscle and nonmuscle cells stabilize actin filaments and reduce actin depolymerization, but do not block actin filament elongation (Wegner, 1982; Lal and Korn, 1986; Hitchcock-DeGregori et al., 1988; Broschat et al., 1989; Broschat, 1990).

Tropomodulin is a recently described 40.6-kD tropomyosin-binding protein from the human erythrocyte membrane skeleton that exhibits isoform-specific binding to one of the ends of erythrocyte tropomyosin and blocks tropomyosin head-to-tail association along the actin filament (Fowler, 1987, 1990; Sussman and Fowler, 1992). The cDNA-derived amino acid sequence of tropomodulin has no significant homology to any other proteins, including tropomyosin-binding proteins such as actin, caldesmon, troponin I, and troponin T (Sung et al., 1992). Tropomodulin is present in the erythrocyte membrane skeleton in approximately equimolar amounts with respect to tropomyosin (tropomodulin/tropomyosin = 1.4:1; Fowler, V. M., unpublished data)[1]. Since each short actin filament is only just as long as a tropomyosin molecule, two tropomodulin molecules would be expected to be situated at one end of each actin filament, assuming that tropomodulin binds to the end of tropomyosin in situ as it does in vitro (Fowler, 1990). However, the short length of the erythrocyte actin filaments and their isotropic orientation in the spectrin–actin network makes it difficult to test this prediction. In contrast, the length and parallel orientation of the thin filaments in skeletal muscle provide an ideal system to determine whether tropomodulin is located at one or the other end of a tropomyosin-coated actin filament.

Here we show that tropomodulin is present in rat psoas skeletal muscle and is located at or near the pointed ends of the thin filaments. Between 1.2 and 1.6 copies of tropomodulin are present per thin filament in myofibrils, consistent with the possibility that one or two tropomodulin molecules are associated with the two terminal tropomyosin molecules at the pointed end of each thin filament. We propose that tropomodulin plays a role in limiting thin filament length by blocking tropomyosin head-to-tail association at the pointed end, thus preventing binding of additional tropomyosin molecules. Alternatively, tropomodulin may play a role in cross-linking and organizing the thin filament lattice at the pointed end.

Materials and Methods

Isolation and Extraction of Myofibrils

Fresh psoas major muscles were dissected from adult rats, held at resting length by tying to the plunger of a 5-cm^3 plastic syringe, and incubated overnight at 0°C in an EGTA-Ringer's solution as described by Knight and Trinick (1982), except for the addition of 2.5 mM diisopropylfluorophosphate to inhibit proteases. After mincing and removal of tendons and connective tissue, the muscle was homogenized with a Polytron at medium speed for 1–2 min in 10 vol (vol/wet wt) of a rigor buffer (Knight and Trinick, 1982) containing 1 mM DTT, 100 μg/ml each of tosyl-L-lysyl chloromethyl ketone and PMSF, 5 μg/ml each of leupeptin and pepstatin A, and 1 μg/ml aprotinin. The extent of homogenization was monitored by phase microscopy. Myofibrils were collected by low speed centrifugation at 1,500 *g* (4°C) and then washed four times in 10 vol of ice-cold rigor buffer to remove trapped cytosol and muscle membranes, each time centrifuging at 1,500 *g*. For biochemical experiments, myofibrils were stored frozen at −80°C in small aliquots.

To extract myosin, myofibril pellets were resuspended by sonication with a microprobe sonicator (Heat Systems Incorporated, Farmingdale, NY) in 10 vol of 0.5 M KCl buffer (Ishiwata and Funatsu, 1985) with the protease inhibitors listed above and incubated for 10 min at 0°C. The myosin-extracted myofibrils (I-Z-I brushes) were then collected by centrifugation for 10 min at 15,000 *g* in a microfuge (Eppendorf Inc., Fremont, CA) at 4°C. The 15,000 *g* supernatant was then recentrifuged at 100,000 *g* in a rotor (model TLA100.3; Beckman Instruments, Inc., Fullerton, CA) at 4°C to pellet thin filaments that had broken off and separated from the I-Z-I brushes. Pellets were solubilized directly in SDS-gel sample buffer by sonication and boiling, and supernatants were precipitated with ice-cold, 25% TCA to remove salts before solubilizing in SDS-sample buffer and boiling (Fowler and Bennett, 1984).

Antibodies

Antibodies to human erythrocyte tropomodulin were generated in rabbits (Fowler, 1990) and affinity-purified over a column of purified erythrocyte tropomodulin coupled to cyanogen-bromide–activated Sepharose (Cl) 4B (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) by standard procedures, eluting with 0.2 M glycine-HCl, pH 2.8. Antibodies specific for two 15 amino acid tropomodulin peptides (amino acids 35–49 and amino acids 297–311) were generated in rabbits and affinity-purified over an erythrocyte tropmodulin-Sepharose column as described (Sussman et al., 1990; Sung et al., 1992). Nonimmune antibody was purified from nonimmune serum by affinity chromatography on protein A–agarose (Pierce Chemical Co., Rockford, IL). A monoclonal antibody to the T12 epitope of titin was kindly provided by Dr. Dieter Fürst, Max-Planck Institute for Biophysical Chemistry, Göttingen, FRG) (Fürst et al., 1988).

Immunoprecipitation and Electrophoresis Procedures

Myofibril pellets were resuspended by sonication in 10 vol of ice-cold immunoprecipitation buffer containing 0.3 M NaCl, 20 mM NaF, 10 mM sodium pyrophosphate, 5 mM EGTA, 2 mM EDTA, 10 mM sodium phosphate, pH 7.5, and the protease inhibitors listed above, and then treated with 2.5 mM diisopropylfluorophosphate for 30 min at 0°C. An equal volume of the same buffer containing 0.8% SDS was added, samples were boiled for 5 min and cooled to room temperature, and Triton X-100 was added to a final concentration of 2%. Insoluble material was removed by centrifugation for 20 min at 100,000 *g* (4°C) and the supernatant was added to protein A-Trisacryl beads (Pierce Chemical Co.) to which affinity-purified anti-erythrocyte tropomodulin antibodies or nonimmune IgG had been preadsorbed. In a titration experiment, we determined that ~50 μg of anti-erythrocyte tropomodulin IgG/mg myofibrils was required to immunoprecipitate all of the tropomodulin. This was determined by sequential immunoprecipitations followed by SDS-gel electrophoresis and silver staining of the immunoprecipitates and by immunoblot analysis of the extracts before and after incubation with antibody (data not shown). After incubation overnight at 4°C on an end-over-end rotator, beads were washed four times in immunoprecipitation buffer containing 0.4% SDS, 2.0% Triton X-100, and 1 mg/ml BSA (Fraction V [ICN Biomedicals, Costa Mesa, CA]), one time in the same solution without the BSA, two times in 50 mM Tris-HCl, pH 7.5, and then solubilized in SDS-sample buffer (Laemmli, 1970).

Samples were electrophoresed on 7.5–15% linear gradient SDS-polyacrylamide gels with a 5% stacking gel (Laemmli, 1970), except that the pH of the gradient gel was 8.6 (Fowler, 1990). After electrophoresis, gels were stained for protein with Coomassie brilliant blue R250 or with silver (Rabilloud et al., 1988), depending on the amount of protein that was immunoprecipitated. Molecular weight markers were from Bio-Rad Laboratories (Richmond, CA), with the addition of actin (M_r 42,000). Electrophoretic transfer of polypeptides from gels to nitrocellulose paper (0.2 μm; Schleicher & Schuell, Inc., Keene, NH) was as previously described (Fowler, 1987). Transfers were labeled with affinity-purified antibodies (2–4 μg/ml) followed by ^{125}I-protein A as described (Fowler, 1990). Labeled polypeptides were visualized by exposure to x-ray film at −80°C (XAR-5; Eastman Kodak Co., Rochester, NY). In some experiments, the relative amount of ^{125}I-protein A bound to the immunoreactive muscle tropomodulin polypeptide was quantitated by cutting out the labeled bands

1. The ratio of tropomodulin to tropomyosin in erythrocyte membranes, prepared by hemolysis in the presence of Mg^{2+} (Fowler and Bennett, 1984), was determined by quantitative immunoblotting using purified erythrocyte tropomyosin and tropomodulin to construct standard curves.

and counting in a gamma counter. This was corrected for nonspecific binding by subtracting the counts associated with a similar-sized piece of nitrocellulose excised from a blank portion of the same lane.

^{125}I-Muscle Tropomyosin Blot-Binding to Muscle Tropomodulin

Skeletal muscle tropomyosin was purified from rabbit back and leg muscles by standard procedures (Bailey, 1948) and labeled with ^{125}I-Bolton and Hunter reagent (New England Nuclear, Boston, MA) to a specific activity of ~1 × 10^6 cpm/μg as previously described (Fowler, 1987). Muscle tropomodulin was isolated by immunoprecipitation from extracts of rat psoas myofibrils, electrophoresed on SDS gels, and transferred to nitrocellulose as described above. ^{125}I-Muscle tropomyosin was incubated overnight at 4°C with nitrocellulose strips in the presence or absence of excess, unlabeled muscle or erythrocyte tropomyosins or erythrocyte tropomodulin, washed extensively, dried, and exposed to x-ray film to detect labeled polypeptides (Fowler, 1987). The relative amount of ^{125}I-tropomyosin bound to the immunoprecipitated muscle tropomodulin polypeptide was determined by cutting out and counting the labeled band in a gamma counter. This was corrected for nonspecific binding as described above. Human erythrocyte tropomyosin and tropomodulin were purified as previously described (Fowler and Bennett, 1984; Fowler, 1990).

Immunofluorescence Staining of Myofibrils

Myofibrils for immunofluorescence staining experiments were isolated from a small piece (~8 mm^3) of fresh rat psoas muscle as described by Knight and Trinick (1982). Myofibrils were adsorbed to glass coverslips and fixed with 4% paraformaldehyde for 15 min at room temperature in rigor buffer (Knight and Trinick, 1982), washed three times with PBS, and incubated 10 min with PBS containing 10% goat serum and 1% BSA. Coverslips were then incubated for 1 h at room temperature with affinity-purified antibodies to human erythrocyte tropomodulin (10 μg/ml) and a monoclonal antibody to the T12 epitope of titin (hybridoma conditioned medium diluted 1:2) in PBS containing 1% BSA, and then washed four times in the same buffer before incubation with a 1:400 dilution of FITC-conjugated goat anti-rabbit IgG (Miles Scientific, Naperville, IL) and a 1:500 dilution of tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG (BCA/Cappel Products, Organon Teknika, Durham, NC) in the same buffer. After washing out unbound antibodies, the coverslips were mounted, viewed, and photographed with epifluorescence and phase contrast optics as described (Daniels, 1990). Myofibrils were stretched according to Huxley and Hanson (1954) with modifications as follows. Myofibrils were suspended 5–10 min in a Ca^{2+}-free relaxing buffer containing ATP (Ishiwata and Funatsu, 1985) and adsorbed to a coverslip, which was then inverted on a slide. Most of the buffer was withdrawn to leave a thin film, and the coverslip was pushed laterally <1 mm and floated off of the slide with fixative, leaving stretched myofibrils attached to either surface. To remove myosin, adsorbed myofibrils were extracted with 0.5 M KCl, 2 mM $MgCl_2$, 10 mM sodium pyrophosphate, 1 mM EGTA, and 10 mM Tris-HCl, pH 6.8 (modified after Ishiwata and Funatsu, 1985), at 4°C for 5 min before fixation.

Results

Identification of Tropomodulin in Rat Psoas Muscle

Affinity-purified antibodies to human erythrocyte tropomodulin immunoprecipitate a 44,000-M_r polypeptide from isolated rat psoas muscle myofibrils that is not immunoprecipitated by nonimmune IgG (Fig. 1, cf. lanes *3* and *4*). Electrophoresis of purified erythrocyte tropomodulin in an adjacent lane demonstrates that the muscle polypeptide comigrates with purified erythrocyte tropomodulin (Fig. 1, cf. lanes *2* and *3*).[2] Other polypeptides visible in the silver-stained gels of the immunoprecipitates are the antibody heavy chains and light chains, as well as various polypeptides nonspecifically immunoprecipitated by both the immune and nonimmune antibodies (Fig. 1, lanes *3* and *4*). In a separate experiment, we determined that only the 44,000-M_r polypeptide is labeled by anti-tropomodulin antibodies on nitrocellulose blots of the immunoprecipitates (Fig. 1, lane *5*).

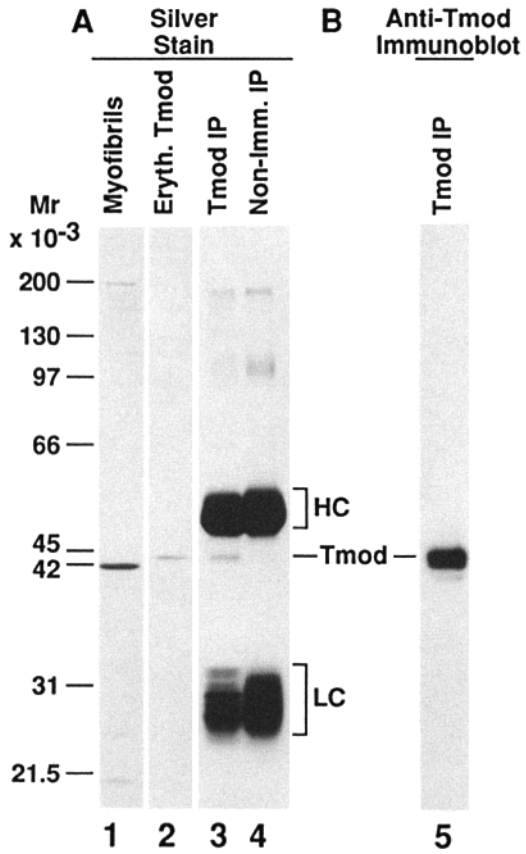

Figure 1. Identification of tropomodulin (*Tmod*) in myofibrils from rat psoas skeletal muscle by immunoprecipitation with anti-human erythrocyte tropomodulin antibodies. (*A*) Silver-stained gel of rat psoas myofibrils (~5 μg; lane *1*), purified human erythrocyte tropomodulin (100 ng; lane *2*), anti-erythrocyte tropomodulin immunoprecipitate from rat psoas myofibrils (lane *3*), and nonimmune immunoprecipitate from rat psoas myofibrils (lane *4*). Note that α-helical proteins like myosin and tropomyosin do not stain well with silver. (*B*) Anti-tropomodulin-labeled nitrocellulose blot of anti-tropomodulin immunoprecipitate as in lane *3*. In addition to the 44,000-M_r polypeptide, a minor, faster-migrating polypeptide is also labeled on the blot. This minor component was not always present in the immunoprecipitates and is probably a proteolytic fragment of the major 44,000-M_r polypeptide (e.g., compare lanes *3* and *5*). The migration positions of tropomodulin (*Tmod*) and the antibody heavy and light chains (*HC* and *LC*, respectively) are indicated. Positions of molecular weight markers are indicated on the left.

Both erythrocyte tropomodulin and the 44,000-M_r immunoreactive muscle polypeptide are also recognized by anti-peptide antibodies prepared against synthetic peptides derived from either the NH_2-terminal portion (residues 35–49) (Fig. 2, lanes *2* and *5*) or the COOH-terminal portion (residues 297–311) (Fig. 2, lanes *3* and *6*) of the erythrocyte tropomodulin sequence (Sung et al., 1992). Competition

2. Like erythrocyte tropomodulin, this immunoprecipitated muscle polypeptide exhibits anomalous mobility on SDS gels, migrating slower than actin when electrophoresed at pH 8.6 (M_r 44,000) (Fig. 1), comigrating with actin when electrophoresed at pH 8.8 (M_r 42,000), and migrating faster than actin when electrophoresed at pH 9.1 (M_r 39,000) (data not shown; see Fowler, 1987, 1990). The basis for this pH-dependent mobility shift is not known.

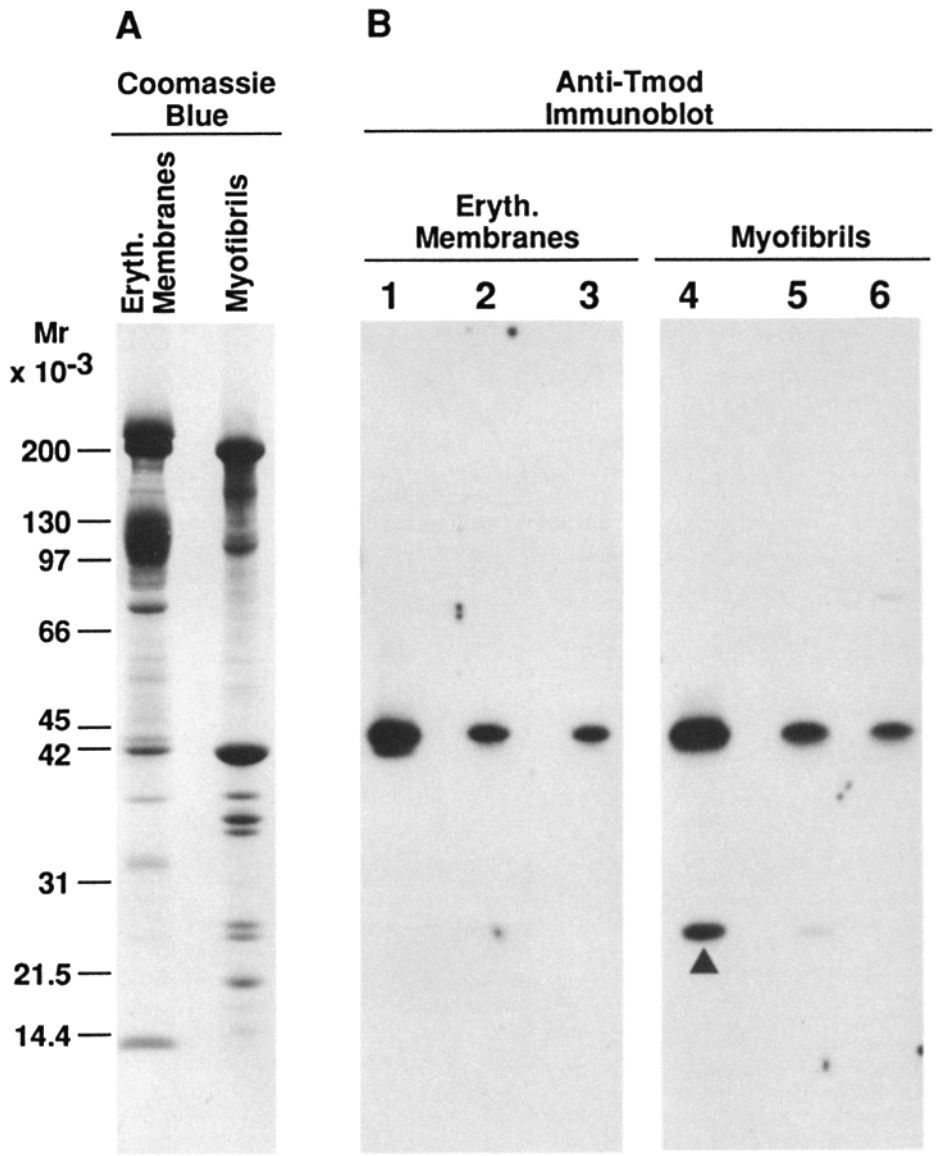

Figure 2. Identification of tropomodulin (*Tmod*) on immunoblots of human erythrocyte membranes and of myofibrils from rat psoas skeletal muscle, using affinity-purified antibodies to human erythrocyte tropomodulin (*Tmod*) (lanes *1* and *4*) or anti-peptide antibodies specific for tropomodulin residues 35–49 (lanes *2* and *5*) or tropomodulin residues 297–311 (lanes *3* and *6*). (*A*) Coomassie blue–stained gel of erythrocyte membranes (80 μg) and myofibrils (50 μg). (*B*) Anti-tropomodulin-labeled nitrocellulose blots of erythrocyte membranes (8 μg) (lanes *1–3*) and myofibrils (50 μg) (lanes *4–6*). Tropomodulin is the major labeled polypeptide at M_r 44,000. The crossreactive M_r 22,000 polypeptide marked with an arrowhead is troponin I (see text).

with the appropriate peptides demonstrates that the anti-peptide antibodies are specific for the correct sequences (data not shown; see Sussman et al., 1990). Taken together, these experiments indicate that the immunoreactive 44,000-M_r polypeptide in rat psoas muscle is skeletal muscle tropomodulin.

As shown previously for rat diaphragm and rabbit skeletal muscle (Fowler, 1990), anti-tropomodulin antibodies also crossreact with troponin I on nitrocellulose blots of rat psoas muscle myofibrils (Fig. 2, lane *4*, *arrowhead*). However, we are confident that crossreaction with troponin I is not a factor in our results because anti-erythrocyte tropomodulin antibodies appear to recognize only an epitope of troponin I that is exposed on nitrocellulose blots. This conclusion is supported by the following experiments. (*1*) Troponin I is not immunoprecipitated from myofibril extracts by anti-tropomodulin antibodies, as demonstrated by the absence of a 22,000-M_r band in the silver-stained gels (Fig. 1, lane *3*) or in the anti-tropomodulin-labeled blots of the immunoprecipitates (Fig. 1, lane *5*). Anti-troponin I labeling of blots of the tropomodulin immunoprecipitates is also negative (data not shown). (*2*) Immunofluorescence staining of myofibrils with anti-tropomodulin antibodies does not stain the I band as would be expected for antibodies to troponin I (see below, Fig. 5). (*3*) Preadsorption of erythrocyte tropomodulin antisera by passage through a troponin I affinity column has no effect on anti-tropomodulin staining of myofibrils or on labeling of the 44,000-M_r muscle tropomodulin polypeptide on blots, while completely eliminating labeling of troponin I on blots (data not shown).

Muscle Tropomodulin Is Associated Exclusively with Myofibrils

To determine whether the tropomodulin in rat psoas muscle is associated exclusively with myofibrils, we used immunoblotting to compare the amount of tropomodulin in whole muscle homogenates with the amount of tropomodulin in the initial supernatant obtained after sedimentation of myofibrils at 1,500 *g*, and in the isolated, washed myofibrils obtained after several cycles of resuspension and sedimentation

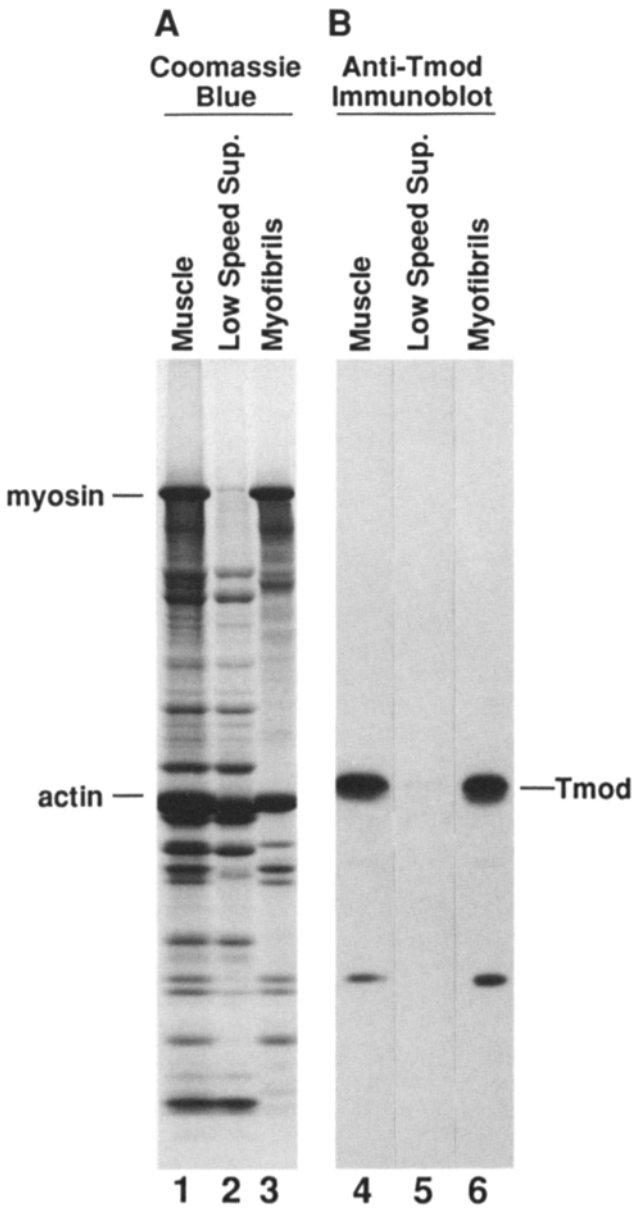

Figure 3. Comparison of the amount of tropomodulin (*Tmod*) present in equivalent amounts of whole muscle homogenate (*Muscle*), initial low speed supernatant obtained after collection of myofibrils (*Low Speed Sup.*) and isolated, washed myofibrils (*Myofibrils*). (*A*) Coomassie blue–stained gel. (*B*) Anti-tropomodulin-labeled nitrocellulose blot of a duplicate gel.

(Knight and Trinick, 1982; see Materials and Methods). All of the myofibrils in the muscle homogenate are recovered by this procedure, as demonstrated by SDS-gel electrophoresis and Coomassie blue staining, which shows that all of the actin and myosin is in the low speed pellet and none is in the low speed supernatant (Fig. 3 *A*). (Note that the polypeptide remaining in the low speed supernatant [Fig. 3, lane *2*] migrates slightly faster than actin [M_r 42,000]; this polypeptide can be well resolved from actin after longer electrophoresis times [not shown].) Immunoblots of these samples show that >90% of the muscle tropomodulin is associated with the isolated, washed myofibrils, while <10% remains in the low speed supernatant (Fig. 3 *B*). Since the low speed

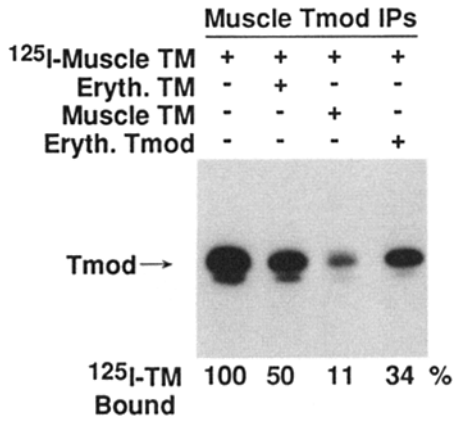

Figure 4. Binding of ^{125}I-skeletal muscle tropomyosin to rat psoas muscle tropomodulin. Tropomodulin (*Tmod*) was immunoprecipitated from extracts of rat psoas muscle, electrophoresed on an SDS gel, transferred to nitrocellulose, and incubated with 2.3 μg/ml ^{125}I-Bolton and Hunter labeled skeletal muscle tropomyosin (*TM*) in the absence or presence of a 20-fold molar excess of cold, unlabeled muscle tropomyosin, erythrocyte tropomyosin, or purified erythrocyte tropomodulin. The number at the bottom of each lane indicates the relative amount of ^{125}I-muscle tropomyosin bound to each immunoprecipitated band, and was determined as described (see Materials and Methods). Approximately 63 ng of immunoprecipitated muscle tropomodulin was loaded in each lane (determined as described in Table I).

supernatant contains muscle membrane vesicles in addition to cytosol, muscle tropomodulin appears not to be associated with membranes. This experiment also shows that the 44,000-M_r polypeptide associated with myofibrils is the only immunoreactive tropomodulin polypeptide detected in rat muscle (other than troponin I; see above).

^{125}I-Tropomyosin Binding to Muscle Tropomodulin

To determine whether rat skeletal muscle tropomodulin binds skeletal muscle tropomyosin, we performed a ^{125}I-tropomyosin blot-overlay experiment on the immunoprecipitated material. This method was used previously to characterize erythrocyte tropomyosin binding to erythrocyte tropomodulin and is both sensitive and specific (Fowler, 1987). Fig. 4 shows that ^{125}I-skeletal muscle tropomyosin binds to muscle tropomodulin on a nitrocellulose blot. No other polypeptides in the immune or nonimmune immunoprecipitates are labeled by ^{125}I-muscle tropomyosin (data not shown). Binding is specific since it is competitively inhibited by a 20-fold molar excess of cold, unlabeled muscle tropomyosin. However, erythrocyte tropomyosin is considerably less effective at competing for ^{125}I-muscle tropomyosin binding to muscle tropomodulin. Cutting out the bands and counting in a gamma counter indicates that competition with muscle tropomyosin reduces binding by 90%, while competition with erythrocyte tropomyosin reduces binding by only 50%. Binding of ^{125}I-muscle tropomyosin to muscle tropomodulin is also inhibited by a 20-fold molar excess of purified erythrocyte tropomodulin, presumably due to binding of erythrocyte tropomodulin to the ^{125}I-muscle tropomyosin. (^{125}I-Erythrocyte tropomodulin does not bind to itself on blot overlays [data not shown].) These experiments demonstrate qualitatively that muscle tropomodulin is a

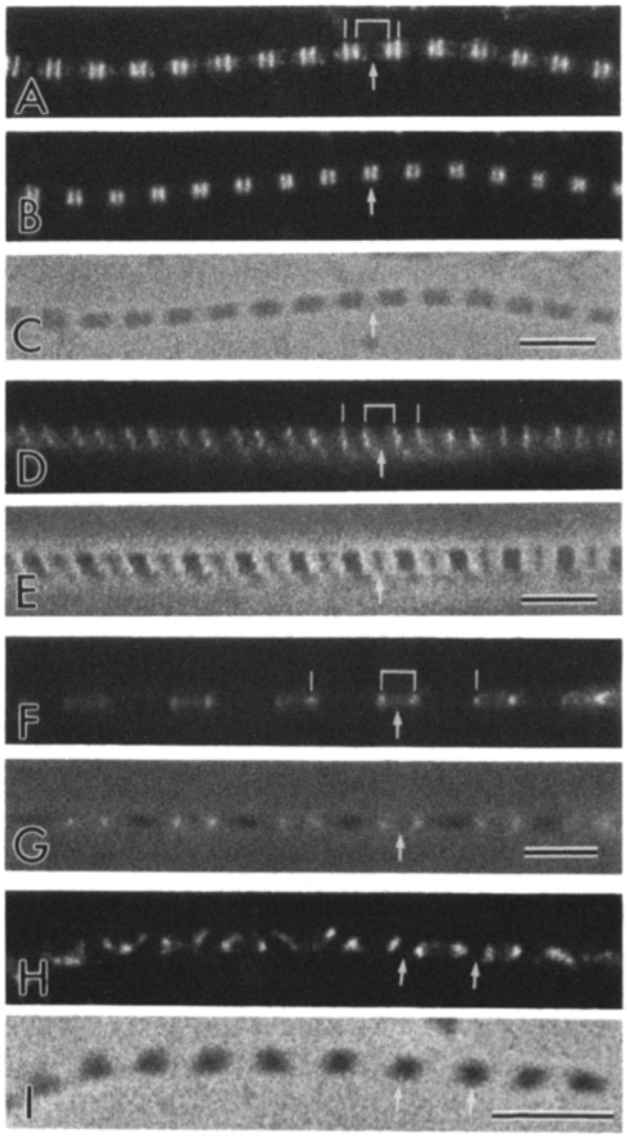

Figure 5. Immunofluorescence localization of tropomodulin in rat psoas myofibrils. Double staining of resting length myofibrils for tropomodulin (*A*) and for the T-12 epitope of titin (*B*). *C* is the corresponding phase micrograph. The vertical white lines indicate the locations of four adjacent tropomodulin-stained bands; the horizontal line connects two of the tropomodulin bands across the Z line, which is indicated by the arrow. Tropomodulin staining of myofibrils stretched to 125% resting length (*D* and *E*) and 230% of resting length (*F* and *G*). In *E* and *G* the phase contrast and fluorescence images were photographed simultaneously (by balancing the illumination), allowing direct visualization of tropomodulin's position in the sarcomere. Tropomodulin staining of a myofibril extracted with 0.5 M KCl to remove myosin is shown in *H*; *I* is the corresponding phase micrograph. An additional minor component of tropomodulin labeling is visible in some of the unextracted myofibrils at the A-I junction (*A*). This labeling is much fainter than the major pair of tropomodulin bands and is more evident in resting length (*A*) or contracted myofibrils (not shown) than in stretched myofibrils (*D* and *F*). The significance of this variable, minor staining component is uncertain. Bars, 5 μm.

tropomyosin-binding protein which interacts preferentially with muscle as compared with erythrocyte tropomyosin.

Immunofluorescence Localization of Tropomodulin in Isolated Myofibrils

Immunofluorescence labeling of resting length, isolated myofibrils from rat psoas muscle with anti-erythrocyte tropomodulin antibodies localizes tropomodulin to a pair of narrow bands within the A band of each sarcomere (Fig. 5 *A*; compare with phase contrast micrograph in Fig. 5 *C*). This is also demonstrated by comparison of tropomodulin staining (Fig. 5 *A*) with staining for the titin T12 epitope which is localized in a pair of narrow bands, one on each side of the Z line (Fig. 5 *B*) (Fürst et al., 1988). Double labeling of myofibrils with rhodamine-phalloidin and anti-tropomodulin antibodies shows that the tropomodulin staining is located at the edges of the phalloidin-stained I bands (data not shown). Immunofluorescence staining of myofibrils with the anti-peptide antibodies used in Fig. 1 and with anti-tropomodulin antibodies preadsorbed against troponin I also stains the same pair of narrow bands within the A band (data not shown).

When the myofibrils are stretched to 125% of their resting length, the distance between tropomodulin bands measured across the A band increases, while the distance between tropomodulin bands measured across the Z line remains constant (~2.2 μm) (compare Fig. 5 *D* with Fig. 5 *A*). In hyperstretched myofibrils that have been stretched to 230% of their resting length so that the thin and thick filaments no longer overlap, the tropomodulin-stained bands are now completely outside the A band but still remain a constant distance from the Z line (Fig. 5, *F* and *G*). The distance of each tropomodulin band from the Z line fits well with the reported length of sarcomeric actin filaments in psoas muscle (1.1 μm; Page and Huxley, 1963; Traeger and Goldstein, 1983; Kruger et al., 1991) and indicates that the tropomodulin-stained bands are located at or near the free (pointed) ends of the thin filaments. A diagram depicting the position of the tropomodulin-stained bands with respect to the thin and thick filaments in resting and stretched myofibrils is shown in Fig. 6.

Association of Tropomodulin with Myofibrils Is Independent of Myosin

To determine whether the association of tropomodulin with myofibrils depends on the presence of myosin thick filaments, we localized tropomodulin in myofibrils that had been treated with 0.5 M KCl to solubilize myosin (Huxley and Hanson, 1954; Ishiwata and Funatsu, 1985). Although the linear organization of these myosin-extracted myofibrils (I-Z-I brushes) is somewhat distorted, immunofluorescence staining shows clearly that tropomodulin is localized at the edges of the phase-dense I band (Fig. 5, *H* and *I*). Staining with rhodamine-phalloidin confirms that the phase-dense bands correspond to the actin filament-containing I bands (data not shown; see Ishiwata and Funatsu, 1985).

In independent experiments, we determined by SDS-gel electrophoresis and immunoblotting that ~50% of the tropomodulin remains associated with the extracted myofibrils after sedimentation at 15,000 *g* (Fig. 7 *B*; compare lanes *a–c*). However, when the 15,000-*g* supernatant is recentrifuged at 100,000 *g* (to pellet thin filaments that had broken

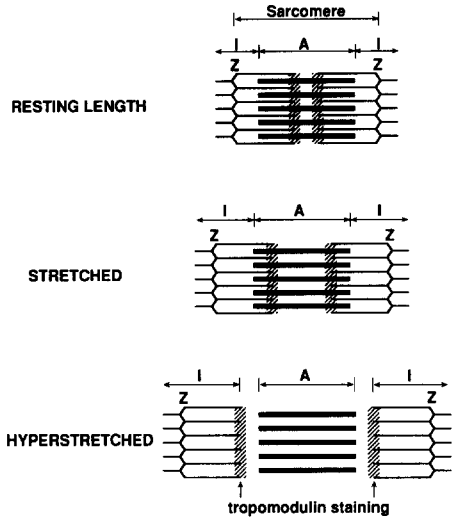

Figure 6. Diagram illustrating the location of tropomodulin staining with respect to the thick and thin filaments in muscle sarcomeres at resting and stretched lengths. Tropomodulin staining is indicated by the hatched area at the free (pointed) ends of the thin filaments.

off and separated from the I-Z-I brushes), essentially all of the remaining tropomodulin cosediments with the actin (Fig. 7 *B*; compare lanes *d* and *e*). Under these conditions, >90% of the myosin is solubilized and remains in the 100,000-*g* supernatant (Fig. 7 *A*). These experiments demonstrate that tropomodulin is associated with thin filaments and that this association is not dependent on intact myosin thick filaments.

Stoichiometry of Tropomodulin/Thin Filament

If tropomodulin is associated with the pointed ends of thin filaments, we might expect that the amount of tropomodulin in myofibrils would be stoichiometric with respect to the number of thin filaments. To test this prediction, we determined the ratio of tropomodulin to actin in isolated myofibrils by quantitatively immunoprecipitating tropomodulin from myofibrils and determining the micrograms of tropomodulin immunoprecipitated per milligram of actin present in the myofibril preparations. In three separate preparations of myofibrils, we found that the molar ratio of actin to tropomodulin varied between 236 and 323 (Table I). Since there are 13 actin monomers per 37 nm of filament and the thin filaments in psoas muscle are ~1.1 μm long (Page and Huxley, 1963; Traeger and Goldstein, 1983; Kruger et al., 1991), there are ~386 actin monomers per thin filament. Therefore, we calculate that there are between 1.2 and 1.6 tropomodulin molecules per thin filament (Table I). This low number further indicates that tropomodulin is likely to be associated with the end rather than along the length of the thin filaments.

Discussion

We report here the identification of tropomodulin in rat skeletal muscle, and show by immunofluorescence staining of isolated myofibrils at resting and stretched lengths that it is located at or near the pointed ends of the thin filaments. Tropomodulin localization is not dependent on the presence

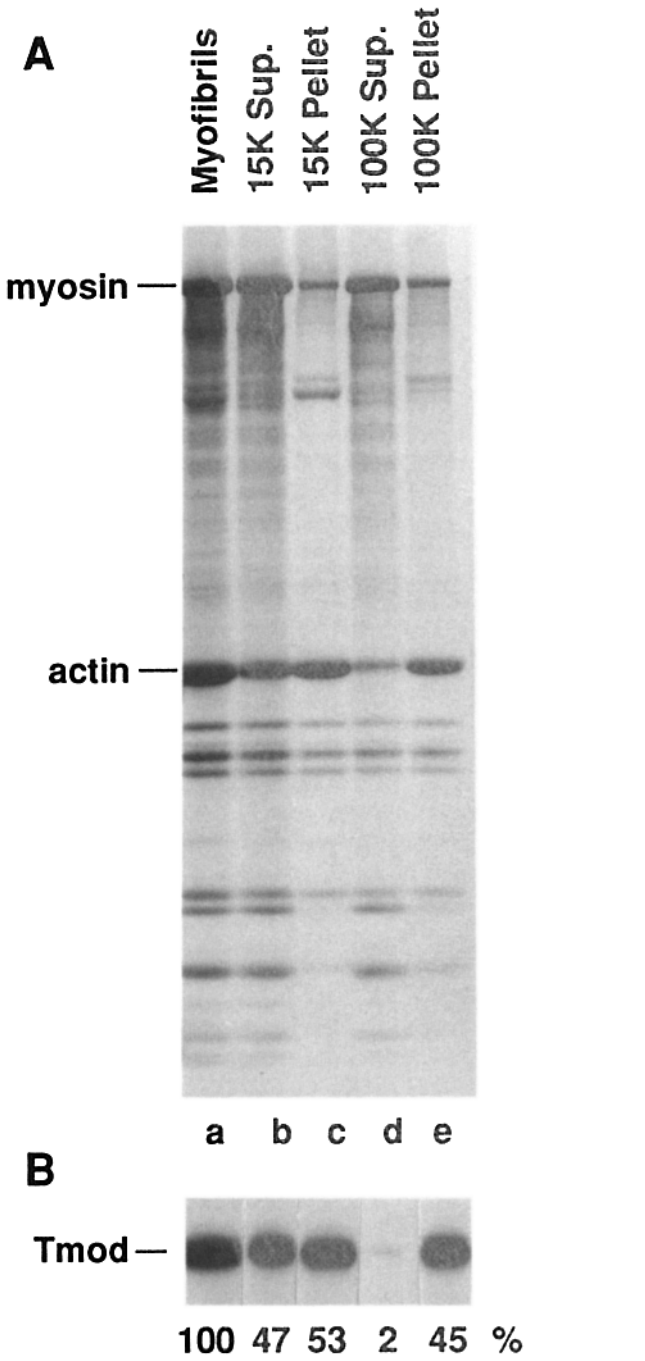

Figure 7. Effect of 0.5 M KCl extraction on association of tropomodulin with rat psoas myofibrils. (*A*) Coomassie blue-stained gel of unextracted myofibrils (*Myofibrils*, lane *a*), supernatant (*15K Sup.*, lane *b*), and pellet (*15K Pellet*, lane *c*) obtained after 0.5 M KCl extraction and centrifugation at 15,000 *g*. Supernatant (*100K Sup.*, lane *d*) and pellet (*100K Pellet*, lane *d*) were obtained by recentrifugation of the 15,000-*g* supernatant at 100,000 *g*. (*B*) Anti-tropomodulin (*Tmod*) labeled nitrocellulose blot of a duplicate gel. The numbers below the blot represent the relative amount of tropomodulin in each sample, determined as described in Materials and Methods. The sum of the counts in the 15,000-*g* supernatant and pellet combined was used as the 100% value for these calculations.

Table I. Number of Tropomodulin Molecules per Thin Filament in Rat Psoas Myofibrils

	μg Tmod* / mg protein	μg actin‡ / mg protein	mol actin§ / mol Tmod	Tmod‖ / Thin filament
Expt. 1	0.536	160	289	1.33
Expt. 2	0.437	146	323	1.20
Expt. 3	0.512	125	236	1.62

* 0.5–1.5 μg of tropomodulin (Tmod) was quantitatively immunoprecipitated from SDS-solubilized rat psoas myofibrils under conditions of antibody excess, and electrophoresed on SDS gels as described in Materials and Methods. The amount (micrograms) of tropomodulin in the immunoprecipitates was determined by laser scanning densitometry of Coomassie blue–stained gels with reference to a standard curve constructed with purified erythrocyte tropomodulin. The μg tropomodulin/mg of total protein in the myofibril extract was corrected for the percentage of tropomodulin immunoprecipitated, which was determined by immunoblotting of extracts before and after immunoprecipitation. In all cases, 80–90% of the total tropomodulin was immunoprecipitated. The milligrams of myofibrillar protein in the extract was determined by the BCA assay (Pierce Chemical Co.). The μg tropomodulin/mg protein listed for each experiment are the averages of duplicate immunoprecipitations; duplicates were within 10% of one another.

‡ The μg actin/mg myofibrillar protein was calculated from the percentage of protein that was actin, determined by scanning densitometry of Coomassie blue–stained gels of myofibrils.

§ The molar ratio of actin to tropomodulin was calculated using molecular weights of 42,000 for actin and 40,600 for tropomodulin (calculated from the predicted amino acid sequence; see Sung et al., 1992).

‖ The number of tropomodulin molecules per thin filament was calculated assuming each thin filament contains 386 actin monomers (see text).

of myosin thick filaments, and immunoblotting analysis shows that all of the tropomodulin remains associated with the thin filaments after extraction of myosin. These data, together with the low number of tropomodulin molecules we determined per thin filament (1.2–1.6), strongly supports the possibility that tropomodulin is associated with the pointed end of each thin filament.

We further propose that tropomodulin is likely to be associated directly with the NH_2-terminal ends of the two terminal tropomyosin molecules at the pointed end of the thin filament (Fig. 8). This model is based on the following observations: (*1*) tropomyosin molecules are polarized with their NH_2-terminal ends oriented toward the pointed end of the thin filament (Ohtsuki, 1979), (2) the NH_2-terminal ends of the terminal tropomyosin molecules are expected to be available for binding since they are not associated with the COOH terminus of a neighboring tropomyosin molecule or with the T1 domain of troponin T (Zot and Potter, 1987), (*3*) erythrocyte tropomodulin binds to only one of the ends of tropomyosin (Fowler, 1990), and (*4*) holo-troponin (which interacts principally with the COOH-terminal one-third of tropomyosin [Zot and Potter, 1987]) does not inhibit erythrocyte tropomyosin–tropomodulin interactions (Fowler, 1987).

Head-to-tail associations of tropomyosin molecules along the length of the thin filament might serve to restrict muscle tropomodulin binding to the free tropomyosin NH_2-termini at the pointed end of the thin filament. If so, head-to-tail interactions of muscle tropomyosin along the thin filament might be expected to be stronger than the interaction of muscle tropomodulin with the end of muscle tropomyosin. In contrast, the ability of erythrocyte tropomodulin to abolish cooperative binding of erythrocyte tropomyosin to F-actin suggests that erythrocyte tropomodulin–tropomyosin interactions are stronger than head-to-tail interactions of erythrocyte tropomyosin (Fowler, 1990). This could partly explain the absence of long, tropomyosin-coated actin filaments in

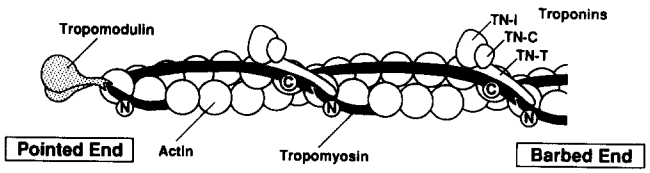

Figure 8. Molecular model for the association of tropomodulin with tropomyosin at the pointed end of a muscle thin filament. Attachment of tropomodulin to tropomyosin via a short tail reflects the actual appearance of erythrocyte tropomodulin–tropomyosin complexes as visualized by rotary shadowing electron microscopy (Fowler, 1990). This tail also leaves room for additional molecule(s) which could interact directly with actin at the pointed end of the thin filament. Troponin I (*TN-I*), troponin T (*TN-T*), and troponin C (*TN-C*) molecules are drawn as in Flicker et al. (1982).

the erythrocyte membrane skeleton (Fowler and Bennett, 1984; Shen et al., 1986). Sequence differences at the NH_2-terminal ends of skeletal muscle vs. nonmuscle tropomyosins (Lees-Miller and Helfman, 1991) may partly account for differences in head-to-tail association between tropomyosins (Cote, 1983; Mak et al., 1987). Conversely, sequence differences between erythrocyte and skeletal muscle tropomodulins would be expected to determine the isoform specificity and affinity of these tropomodulins for tropomyosins (Fig. 4; see Sussman and Fowler, 1992). Recently, we have isolated a chicken skeletal muscle cDNA clone for tropomodulin that codes for a 40.4-kD polypeptide whose amino acid sequence is 86% identical to that of erythrocyte tropomodulin.[3] We are currently mapping the tropomyosin-binding domains of muscle and erythrocyte tropomodulins and investigating their effects on tropomyosins' self-association in the presence and absence of actin filaments.

The number of tropomodulin molecules that we determined per thin filament (1.2–1.6) is consistent with one *or* two tropomodulin molecules associated with the pointed end of each thin filament. Two tropomodulin molecules are depicted in our model, one bound to each of the two terminal tropomyosin molecules at the pointed end of a thin filament (Fig. 8). We favor this possibility by analogy with the erythrocyte membrane, where the ratio of tropomodulin to tropomyosin is 1.4:1,[1] and there are two tropomyosin molecules per short actin filament (Fowler and Bennett, 1984). In this study, determination of the percentage of actin in myofibrils by scanning of Coomassie blue–stained gels could have led to an overestimation of the percentage of actin and thus an underestimation of the number of tropomodulin molecules per thin filament.

In vitro, the ratio of erythrocyte tropomodulin to tropomyosin at a saturation determined in a solid phase binding assay is two tropomodulins to one tropomyosin (Fowler, 1987; Sussman and Fowler, 1992). Therefore, if a tropomodulin dimer is required for tropomyosin binding, only one of the two tropomyosin molecules at the pointed end of the thin filament would be occupied by a tropomodulin dimer. Alternatively, a tropomodulin monomer may be sufficient to bind tropomyosin in situ at the end of the thin filament and half of the tropomodulin molecules may be inaccessible or

3. Babcock, G. A., and V. M. Fowler, unpublished observations; and Sussman, M. A., M. Ito, B. E. Flucher, M. P. Daniels, V. M. Fowler, and L. Kedes, manuscript submitted for publication.

inactivated in our solid phase binding assay in vitro. Further experiments to characterize the interaction of tropomodulin with tropomyosin in solution and with reconstituted thin filaments will be required to resolve this discrepancy. In addition, immunogold labeling of isolated thin filaments will be required to determine whether tropomodulin is indeed situated at their extreme pointed ends.

Tropomodulin Function in Skeletal Muscle

Although a number of proteins are associated with the barbed ends of the thin filaments in skeletal muscle (Chowrashi and Pepe, 1982; Maher et al., 1985; Casella et al., 1987; Saide et al., 1989), tropomodulin is the only protein that is likely to be specifically associated with the pointed ends of the thin filaments. There are a number of functions that such a protein could perform. For example, tropomodulin could regulate thin filament length by blocking head-to-tail association of tropomyosin at the pointed end of the actin filament, thus capping the tropomyosin polymers and preventing additional tropomyosin molecules from binding to the distal portion of the actin filament (Wegner, 1979; Fowler, 1990). This would be expected to indirectly reduce the extent of actin filament elongation from the pointed end because naked actin filaments are more susceptible to spontaneous fragmentation (Wegner, 1982), depolymerization (Broschat et al., 1989; Broschat, 1990; Weigt et al., 1990), as well as disassembly and severing by actin-binding proteins (Bonder and Mooseker, 1983; Nishida et al., 1985; Prulière et al., 1986; Ishikawa et al., 1989). On its own, tropomyosin is not sufficient to terminate filament elongation because purified muscle tropomyosin blocks depolymerization but has no effect on elongation (Broschat, 1990; Hitchcock-DeGregori et al., 1988). Since we have not detected any direct interaction of erythrocyte tropomodulin with actin (Fowler, 1990), it is probable that another protein (a tropomodulin-associated component?) binds to actin and directly caps the pointed end, preventing elongation of the actin filaments (Ishiwata and Funatsu, 1985; Sanger et al., 1984).

Specification and stabilization of thin filament length in skeletal muscle is likely to require multiple interacting components. For example, nebulin is a giant protein found in skeletal muscle that is coextensive with the thin filaments and is proposed to function as a length-regulating template for the thin filaments (Jin and Wang, 1991; Kruger et al., 1991; Labeit et al., 1991). Perhaps nebulin functions to specify the minimum length of the thin filaments and tropomyosin stabilizes the filaments, while tropomodulin and associated proteins at the pointed end (and Cap Z at the barbed end) terminate growth and restrict the length of the thin filaments. Alternatively, tropomodulin may be a component of a structural lattice that links thin filaments to one another at the pointed end. At least in the erythrocyte, additional tropomodulin binding components are expected to exist since tropomodulin remains tightly associated with the membrane skeleton even after all of the tropomyosin has been extracted (Fowler, 1987).

Tropomodulin Function in Nonmuscle Cells

The pointed ends of certain actin filaments found in nonmuscle cells have been reported to be capped and unavailable for elongation, including the actin filaments in the mature hair cells of the cochlea of birds (discussed in Tilney et al., 1992), the actin filaments in the comet-like tail of *Listeria monocytogenes* in macrophages (Tilney et al., 1992), and under some conditions, the short actin filaments in the erythrocyte membrane skeleton (Matsuzaki et al., 1985; Pinder et al., 1986). Results presented in this paper, together with the ratio of tropomodulin/tropomyosin/short actin filament (2.8:2:1) (Fowler and Bennett, 1984),[1] indicates that tropomodulin is likely to be situated at the pointed end of the short, tropomyosin-coated actin filaments in the erythrocyte membrane skeleton. Tropomyosin is also associated with the actin filaments in the cuticular plate and circumferential band in the cochlear hair cells (Drenckhahn et al., 1991) and with the actin filaments in the *Listeria* tail (Dabiri et al., 1990). Although the predicted amino acid sequence of erythrocyte or muscle tropomodulin is not homologous to any other proteins (Sung et al., 1992),[3] immunoreactive tropomodulin polypeptides are present in a variety of other cells and tissues (Fowler, 1990). We speculate that tropomodulin could be a representative of a new family of tropomyosin-binding proteins that are associated with the pointed ends of tropomyosin-coated actin filaments in nonmuscle as well as muscle cells. These proteins could function to regulate actin filament length and/or play a role in actin filament organization.

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References

Bailey, K. 1948. Tropomyosin: a new asymmetric protein component of the muscle fibril. *Biochem. J.* 43:271–279.

Bennett, V. 1989. The spectrin-actin junction of erythrocyte membrane skeletons. *Biochim. Biophys. Acta.* 988:107–121.

Bonder, E. M., and M. S. Mooseker. 1983. Direct electron microscopic visualization of barbed end capping and filament cutting by intestinal microvillar 95-kdalton protein (Villin). A new actin assembly assay using the *Limulus* acrosomal process. *J. Cell Biol.* 96:1097–1107.

Broschat, K. O. 1990. Tropomyosin prevents depolymerization of actin filaments from the pointed end. *J. Biol. Chem.* 265:21323–21329.

Broschat, K. O., A. Weber, and D. R. Burgess. 1989. Tropomyosin stabilizes the pointed end of actin filaments by slowing depolymerization. *Biochemistry.* 28:8501–8506.

Byers, T. J., and D. Branton. 1985. Visualization of the protein associations in the erythrocyte membrane skeleton. *Proc. Natl. Acad. Sci. USA.* 82: 6153–6157.

Casella, J. F., S. W. Craig, D. J. Maack, and A. E. Brown. 1987. Cap $Z_{(36/32)}$, a barbed end actin-capping protein, is a component of the Z-line of skeletal muscle. *J. Cell Biol.* 105:371–379.

Chowrashi, P. K., and F. A. Pepe. 1982. The Z-band: 85,000-dalton amorphin and alpha-actinin and their relation to structure. *J. Cell Biol.* 94:565–573.

Cote, G. P. 1983. Structural and functional properties of the nonmuscle tropomyosins. *Mol. Cell. Biochem.* 57:127–146.

Dabiri, G. A., J. M. Sanger, D. A. Portnoy, and F. S. Southwick. 1990. *Listeria monocytogenes* moves rapidly through the host-cell cytoplasm by inducing directional actin assembly. *Proc. Natl. Acad. Sci. USA.* 87:6068–6072.

Daniels, M. P. 1990. Localization of actin, β-spectrin, 43 × 10^3 M_r and 58 × 10^3 M_r proteins to receptor-enriched domains of newly formed acetylcholine receptor aggregates in isolated myotube membranes. *J. Cell Sci.* 97:615–627.

de Arruda, M. V., S. Watson, C.-S. Lin, J. Leavitt, and P. Matsudaira. 1990.

Fimbrin is a homologue of the cytoplasmic phosphoprotein plastin and has domains homologous with calmodulin and actin gelation proteins. *J. Cell Biol.* 111:1069–1079.

Drenckhahn, D., K. Engel, D. Höfer, C. Merte, L. Tilney, and M. Tilney. 1991. Three different actin filament assemblies occur in every hair cell. Each contains a specific actin crosslinking protein. *J. Cell Biol.* 112:641–651.

Flicker, P. F., G. N. Phillips, Jr., and C. Cohen. 1982. Troponin and its interactions with tropomyosin: an electron microscope study. *J. Mol. Biol.* 162:495–501.

Fowler, V. M. 1987. Identification and purification of a novel M_r 43,000 tropomyosin-binding protein from human erythrocyte membranes. *J. Biol. Chem.* 262:12792–12800.

Fowler, V. M. 1990. Tropomodulin. A cytoskeletal protein that binds to the end of erythrocyte tropomyosin and inhibits tropomyosin binding to actin. *J. Cell Biol.* 111:471–482.

Fowler, V. M., and V. Bennett. 1984. Erythrocyte membrane tropomyosin: purification and properties. *J. Biol. Chem.* 259:5978–5989.

Fürst, D. O., M. Osborn, R. Nave, and K. Weber. 1988. The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunoelectron microscopy. A map of ten nonrepetitive epitopes starting at the Z line extends close to the M line. *J. Cell Biol.* 106:1563–1572.

Hitchcock-DeGregori, S. E., P. Sampath, and T. D. Pollard. 1988. Tropomyosin inhibits the rate of actin polymerization by stabilizing actin filaments. *Biochemistry.* 27:9182–9185.

Huxley, H. E. 1960. Muscle cells. *In* The Cell: Biochemistry, Physiology, Morphology. Vol. 4. J. Brachet and A. E. Mirsky, editors. Academic Press, New York. 365–481.

Huxley, H., and J. Hanson. 1954. Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation. *Nature (Lond.).* 173:973–976.

Ishikawa, R., S. Yamashiro, and F. Matsumura. 1989. Differential modulation of actin-severing activity of gelsolin by multiple isoforms of cultured rat cell tropomyosin. Potentiation of protective ability of tropomyosins by 83-kDa nonmuscle caldesmon. *J. Biol. Chem.* 264:7490–7497.

Ishiwata, S., and T. Funatsu. 1985. Does actin bind to the ends of thin filaments in skeletal muscle? *J. Cell Biol.* 100:282–291.

Jin, J.-P., and K. Wang. 1991. Cloning, expression, and protein interaction of human nebulin fragments composed of varying numbers of sequence modules. *J. Biol. Chem.* 266:21215–21223.

Knight, P. J., and J. A. Trinick. 1982. Preparation of myofibrils. *Methods Enzymol.* 85:9–12.

Kruger, M., J. Wright, and K. Wang. 1991. Nebulin as a length regulator of thin filaments of vertebrate skeletal muscles. Correlation of thin filament length, nebulin size, and epitope profile. *J. Cell Biol.* 115:97–107.

Labeit, S., T. Gibson, A. Lakey, K. Leonard, M. Zeviani, P. Knight, J. Wardale, and J. Trinick. 1991. Evidence that nebulin is a protein-ruler in muscle thin filaments. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 282:313–316.

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

Lal, A. A., and E. D. Korn. 1986. Effect of muscle tropomyosin on the kinetics of polymerization of muscle actin. *Biochemistry.* 25:1154–1158.

Lees-Miller, J. P., and D. M. Helfman. 1991. The molecular basis for tropomyosin isoform diversity. *Bioessays.* 13:429–437.

Maher, P. A., G. F. Cox, and S. J. Singer. 1985. Zeugmatin. A new high molecular weight protein associated with Z lines in adult and early embryonic striated muscle. *J. Cell Biol.* 101:1871–1883.

Mak, A. S., G. Rosborough, and H. Baker. 1987. Tropomyosin from human erythrocyte membrane polymerizes poorly but binds F-actin effectively in the presence and absence of spectrin. *Biochim. Biophys. Acta.* 912:157–166.

Maruyama, K., H. Kurokawa, M. Oosawa, S. Shimaoka, H. Yamamoto, M. Ito, and K. Maruyama. 1990. β-Actinin is equivalent to Cap Z protein. *J. Biol. Chem.* 265:8712–8715.

Matsuzaki, F., K. Sutoh, and A. Ikai. 1985. Structural unit of the erythrocyte cytoskeleton: isolation and electron microscopic examination. *Eur. J. Cell Biol.* 39:153–160.

Nishida, E., E. Muneyuki, S. Maekawa, Y. Ohta, and H. Sakai. 1985. An actin-depolymerizing protein (Destrin) from porcine kidney: its action on F-actin containing or lacking tropomyosin. *Biochemistry.* 24:6624–6630.

Ohtsuki, I. 1979. Molecular arrangement of troponin-T in the thin filament. *J. Biochem. (Tokyo).* 89:491–497.

Page, S. G., and H. E. Huxley. 1963. Filament lengths in striated muscle. *J. Cell Biol.* 19:369–390.

Peng, I., and D. A. Fischman. 1991. Post-translational incorporation of actin into myofibrils in vitro: evidence for isoform specificity. *Cell Motil. Cytoskeleton.* 20:158–168.

Pinder, J. C., A. G. Weeds, and W. B. Gratzer. 1986. Study of actin filament ends in the human red cell membrane. *J. Mol. Biol.* 191:461–468.

Prulière, G., A. d'Albis, and E. Der Terrosian. 1986. Effects of tropomyosin on the interactions of actin with actin-binding proteins isolated from pig platelets. *Eur. J. Biochem.* 159:535–547.

Rabilloud, T., G. Carpentier, and P. Tarroux. 1988. Improvement and simplification of low-background silver staining of proteins by using sodium dithionite. *Electrophoresis.* 9:288–291.

Saide, J. D., S. Chin-Bow, J. Hogan-Sheldon, L. Busquets-Turner, J. O. Vigoreaux, K. Valgeirsdottir, and M. L. Pardue. 1989. Characterization of components of Z-bands in the fibrillar flight muscle of *Drosophila melanogaster*. *J. Cell Biol.* 109:2157–2167.

Sanger, J. W., B. Mittal, and J. M. Sanger. 1984. Analysis of myofibrillar structure and assembly using fluorescently labeled contractile proteins. *J. Cell Biol.* 98:825–833.

Shen, B. W., R. Josephs, and T. L. Steck. 1986. Ultrastructure of the intact skeleton of the human erythrocyte membrane. *J. Cell Biol.* 102:997–1006.

Southwick, F. S., and J. H. Hartwig. 1982. Acumentin, a protein in macrophages which caps the 'pointed' end of actin filaments. *Nature (Lond.).* 297:303–307.

Sung, L. A., V. M. Fowler, K. Lambert, M. A. Sussman, D. Karr, and S. Chien. 1992. Molecular cloning and characterization of human fetal liver tropomodulin: a tropomyosin-binding protein. *J. Biol. Chem.* 267:2616–2621.

Sussman, M. A., and V. M. Fowler. 1992. Tropomodulin binding to tropomyosins: isoform-specific differences in affinity and stoichiometry. *Eur. J. Biochem.* 205:355–362.

Sussman, M. A., E. Battenberg, F. E. Bloom, and V. M. Fowler. 1990. Identification of two nerve growth factor-induced polypeptides in PC12 cells. *J. Mol. Neurosci.* 2:163–174.

Tilney, L. G., D. J. DeRosier, A. Weber, and M. S. Tilney. 1992. How *Listeria* exploits host cell actin to form its own cytoskeleton. II. Nucleation, actin filament polarity, filament assembly, and evidence for a pointed end capper. *J. Cell Biol.* 118:83–93.

Traeger, L., and M. A. Goldstein. 1983. Thin filaments are not of uniform length in rat skeletal muscle. *J. Cell Biol.* 96:100–103.

Wegner, A. 1979. Equilibrium of the actin-tropomyosin interaction. *J. Mol. Biol.* 131:839–853.

Wegner, A. 1982. Kinetic analysis of actin assembly suggests that tropomyosin inhibits spontaneous fragmentation of actin filaments. *J. Mol. Biol.* 161: 217–227.

Weigt, C., B. Schoepper, and A. Wegner. 1990. Tropomyosin-troponin complex stabilizes the pointed ends of actin filaments against polymerization and depolymerization. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 260:266–268.

Zot, A. S., and J. D. Potter. 1987. Structural aspects of troponin-tropomyosin regulation of skeletal muscle contraction. *Annu. Rev. Biophys. Biophys. Chem.* 16:535–559.