Identification of a Novel Tropomodulin Isoform, Skeletal Tropomodulin, That Caps Actin Filament Pointed Ends in Fast Skeletal Muscle*

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Tropomodulin (E-Tmod) is an actin filament pointed end capping protein that maintains the length of the sarcomeric actin filaments in striated muscle. Here, we describe the identification and characterization of a novel tropomodulin isoform, skeletal tropomodulin (Sk-Tmod) from chickens. Sk-Tmod is 62% identical in amino acid sequence to the previously described chicken E-Tmod and is the product of a different gene. Sk-Tmod isoform sequences are highly conserved across vertebrates and constitute an independent group in the tropomodulin family. In vitro, chicken Sk-Tmod caps actin and tropomysosin-actin filament pointed ends to the same extent as does chicken E-Tmod. However, E- and Sk-Tmods differ in their tissue distribution; Sk-Tmod predominates in fast skeletal muscle fibers, lens, and erythrocytes, while E-Tmod is found in heart and slow skeletal muscle fibers. Additionally, their expression is developmentally regulated during chicken breast muscle differentiation with Sk-Tmod replacing E-Tmod after hatching. Finally, in skeletal muscle fibers that co-express both Sk- and E-Tmod, they are recruited to different actin filament-containing cytoskeletal structures within the cell: myofibrils and costameres, respectively. All together, these observations support the hypothesis that vertebrates have acquired different tropomodulin isoforms that play distinct roles in vivo.

One of the most spectacular examples of cytoskeletal architecture found in cells is the sarcomere, the contractile unit of striated muscle. Antiparallel polarized actin filaments (thin filaments), bipolar myosin filaments (thick filaments), titin, and nebulin filaments interdigitate and interact with each other to create sarcomeres, which support the contraction of muscle cells (1). Despite the similarity in the basic organization of the filaments in all sarcomeres, a closer examination reveals some important and interesting variations in sarcomeric architecture in different types of muscle. Most strikingly, while thick filament lengths are remarkably constant among different vertebrate muscles (2–4), thin filament lengths exhibit considerable variability among physiologically distinct types of muscles (for review, see Ref. 5). For example, in the rabbit psoas muscle, a fast twitch skeletal muscle, thin filament lengths fall into a narrow range of 1.11 ± 0.03 μm (6). In contrast, in rat cardiac atrial muscle, thin filament lengths vary from 0.6 to 1.1 μm (7). Differences in thin filament length between fast and slow skeletal muscles have also been observed in the fish Perca fluviatilis (8). This variation in thin filament length can be an important determinant of the length-tension relationship during contraction and thus can influence the physiological properties of the muscles (i.e. cardiac, fast skeletal, and slow skeletal) (7, 8). Nevertheless, the mechanisms underlying the regulation of variations in thin filament lengths are not yet understood.

An excellent candidate for regulating the length of the thin filaments is tropomodulin, the capping protein for the thin filament pointed ends (5, 9). When embryonic chick cardiac myocytes are microinjected with an antibody that blocks tropomodulin’s ability to cap actin filament pointed ends in vitro, actin elongates from the thin filament pointed ends (10). Furthermore, the cells are no longer able to beat, demonstrating that maintenance of thin filament length by tropomodulin is critical for normal contraction (10). The levels of tropomodulin also appear to be important for the stabilization and proper organization of thin filaments in myofibrils, based on results from studies employing sense and antisense erythrocyte tropomodulin (E-Tmod) mRNAs in adenovirus vectors in neonatal rat cardiac myocytes (11). In transgenic mice, overexpression of tropomodulin in the heart after birth results in dilated cardiomyopathy, presumably due to alterations in normal myofibril organization (12).

Many myofibrillar components exist as families of multiple isoforms, which are differentially expressed in distinct types of muscle (for review, see Ref. 13). The variable expression of protein isoforms among muscles of different types is a major determinant of the contractile properties of each muscle. Our previous studies and those of others had identified so far only one tropomodulin isoform, E-Tmod, which is expressed in both skeletal and cardiac muscle (14–17). In this study, we have identified and cloned a novel tropomodulin isoform from vertebrates, Sk-Tmod, which is highly expressed in fast skeletal muscle and is not present in slow skeletal or cardiac muscle. In vitro, chicken Sk-Tmod caps actin and tropomysosin-actin filament pointed ends to the same extent as does chicken E-Tmod. However, E- and Sk-Tmods differ in their tissue distribution; Sk-Tmod predominates in fast skeletal muscle fibers, lens, and erythrocytes, while E-Tmod is found in heart and slow skeletal muscle fibers. Additionally, their expression is developmentally regulated during chicken breast muscle differentiation with Sk-Tmod replacing E-Tmod after hatching. Finally, in skeletal muscle fibers that co-express both Sk- and E-Tmod, they are recruited to different actin filament-containing cytoskeletal structures within the cell: myofibrils and costameres, respectively. All together, these observations support the hypothesis that vertebrates have acquired different tropomodulin isoforms that play distinct roles in vivo.

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The high conservation of Sk-Tmod sequences among vertebrates and its different tissue and subcellular distribution as compared with E-Tmod suggest that the Sk-Tmod isoform may be functionally distinct from the E-Tmod isoform.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—A panel of 19 monoclonal anti-tropomodulin antibodies were generated using recombinant chicken E-Tmod as an antigen (16) (Chris W. Grant, Custom Monoclonals, West Sacramento, CA). The antibodies were purified from the hybridoma culture supernatants over a Protein G-Sepharose fast performance liquid chromatography column (Amersham Pharmacia Biotech, Uppsala, Sweden) and eluted with 0.2 m glycine-HCl, pH 2.8. The specificity of the antibodies was characterized by two-dimensional gel electrophoresis, followed by Western blotting (see below) and by immunofluorescence. Monoclonal antibodies (mAbs) 17, 23, and 47 recognize a single tropomodulin spot by Western blots in both heart and breast muscle (pectoralis major, PM) (Fig. 1, left panel). In contrast, mAbs 9 and 95 only recognize a tropomodulin spot in heart and none in breast muscle (PM) (Fig. 1, right panel). Mixing experiments demonstrate that tropomodulin spots from heart and breast muscle (PM) do not comigrate (Fig. 1, bottom left panel). By immunofluorescence, mAb 95 specifically recognizes E-Tmod, as demonstrated by staining of embryonic breast muscle myofibrils, which contain Sk-Tmod (data not shown and see below). In contrast, although mAb 17 recognizes both E- and Sk-Tmod isoforms on Western blots, it preferentially recognizes Sk-Tmod by immunofluorescence since it does not stain E-Tmod in the myofibrils of embryonic breast muscle but stains the pointed ends of thin filaments in isolated myofibrils from adult chicken breast muscle, which contain Sk-Tmod (data not shown and see below).

Polyclonal antibodies to recombinant chicken Sk-Tmod (see below) were generated in rabbits (R3577) and affinity-purified over a column of recombinant chicken Sk-Tmod coupled to cyanogen bromide-activated Sepharose 4B (Sigma) by standard procedures. The monoclonal anti-sarcocereic α-actinin antibody (clone 9A2B2S) (18) was generously provided by Drs. S. and H. Holtzer (University of Pennsylvania, Philadelphia, PA). The monoclonal anti-chicken fast C-protein antibody (clone MF1) (19) developed by Dr. A. Fischman was obtained from Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Polyclonal rabbit antibodies generated against bovine brain α-spectrin were prepared as described previously (20). Anti-rabbit antibodies were labeled with fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG, and rhodamine-conjugated goat anti-rabbit antibodies were purchased from Molecular Probes, Inc. (Eugene, OR), Accurate Chemical and Scientific Corp. (Westbury, NY), and Roche Molecular Biochemicals, respectively.

**Comparison of Tropomodulin Isoforms by Two-dimensional Electrophoresis**—Tropomodulin (21) from a variety of species, including mammalian erythrocytes and lens, could be compared with that of the other muscle tissues.

**Isolation of Chicken Sk-Tmod cDNAs**—Tropomodulin from adult chicken breast muscle was immunoprecipitated as described (24) using anti-tropomodulin mAb 17. Samples were electrophoresed on 7.5–15% acrylamide linear gradient SDS-polyacrylamide gels at pH 9.1 (20, 25), and polypeptides were transferred to polyvinylidene difluoride membrane (0.45 μm; Millipore Corp., Bedford, MA) in CAPS buffer (10 mCAPS, pH 11.0, 10% methanol). The membrane was stained briefly in 0.1% Ponceau S in 1% acetic acid and the region of the membrane containing the tropomodulin band was excised and subjected to tryptic digestion. Microsequencing and peptide sequence frequencies were obtained (Dr. John Leszyk, University of Massachusetts Medical School, Shrewsbury, MA) (underlined sequences in Fig. 2).

Degenerate primers to amplify Sk-Tmod cDNA were designed based on the peptides TLQSLSNIFSSAGMMSVIK and YKVPDPFPNNTVVEETL (see below, Fig. 2). These primers were used to amplify chicken cDNA fragments by polymerase chain reaction reactions, which were obtained (Dr. John Leszyk, University of Massachusetts Medical School, Shrewsbury, MA) (underlined sequences in Fig. 2).

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**Identification of Additional Sk-Tmod Sequences**—The available tropomodulin sequences at GenBank together with the new chicken Sk-Tmod cDNA sequence reported in this study, were used to screen the National Center for Biotechnology Information (NCBI) expressed sequence tag (dbEST). Several ESTs from the IMAGE Consortium clone collection (Genome Systems, St. Louis, MO) and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Polyclonal rabbit antibodies generated against bovine brain α-spectrin were prepared as described previously (20). Anti-rabbit antibodies were labeled with fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG, and rhodamine-conjugated goat anti-rabbit antibodies were purchased from Molecular Probes, Inc. (Eugene, OR), Accurate Chemical and Scientific Corp. (Westbury, NY), and Roche Molecular Biochemicals, respectively.

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A Novel Tropomodulin Isoform in Skeletal Muscle

Indirect Immunofluorescence Microscopy—Isolated myofibrils from adult chicken breast muscle myofibrils were prepared as described previously for rat psoas myofibrils (24). The immunostaining was carried out as described previously (33). Briefly, coverslips were washed with phosphate-buffered saline to remove excess fixative, incubated with 3% bovine serum albumin in phosphate-buffered saline for 30 min, and then incubated for 1 h with rabbit serum (R3577) generated against recombinant Sk-Tmod (1:100 dilution) or mAb anti-a-actinin antibody (1:400 dilution) followed by rhodamine-conjugated goat anti-rabbit or rhodamine conjugated-donkey anti-mouse secondary antibodies (1:200 dilution each) together with bodipy-phallacidin (1:200 dilution).

To obtain muscle cryosections, small pieces of adult chicken biceps femoris (BF) muscle were dissected, embedded in Tissue-Tek O.C.T. (Sakura Finetek, Torrance, CA) in a cryomold (Miles, Elkhart, IN) and immersed in liquid N2. Serial cross-sections of approximately 10 µm thickness were obtained at −16 °C on a Cryocut 1800 (Leica, Heidelberg, West Germany) and collected on a glass coverslip. Sections were processed for immunofluorescence as described above. Myofibrils and muscle sections were observed on a Zeiss Axioskop using a Zeiss 63× Plan-Apochromat objective lens (1.4 numeric aperture) and a Zeiss 25× multi-immersion objective (0.8 numeric aperture). Images were acquired with a cooled CCD-1230Y camera equipped with a Sony interline chip (Roper Scientific, Trenton, NJ) using IP laboratory software and then processed using Adobe Photoshop (Adobe Systems Inc. San Jose, CA). In Fig. 9, images were recorded using TrIx (400 ASA) film.

Expression and Purification of Recombinant Sk-Tmod—The full-length Sk-Tmod cDNA was excised from the pBluescript plasmid at EcoRI sites and subcloned in frame into the plasmid pGEX-KG (16) to express a glutathione S-transferase (GST) fusion protein in E. coli. Proper orientation of all insert was confirmed by restriction mapping. For high yields, cells were grown to an A600 of approximately 1 before inducing with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h, and the GST fusion protein was purified as described for E-Tmod (16) with some modifications. After affinity isolation of the GST fusion protein on a glutathione column and release from the GST by thrombin cleavage, Sk-Tmod was further purified by sequential anion exchange chromatography on a Resource Q column (Amer sham Pharmacia Biotech), followed by a Mono Q column (Amersham Pharmacia Biotech). The 20 µM HEPS, pH 7.3, 80 mM KC1, 1 mM dithiothreitol, 0.02% sodium azide, 20 µg/ml phenylmethylsulfonyl fluoride and stored frozen at −80 °C. The concentration of Sk-Tmod was determined by light absorption, using ε290 = 10.24 mM−1 cm−1. The extinction coefficient was calculated from the amino acid composition as described (34) using the ProtParam tool available at the EXPASY web site. The amino acid sequence of the GST linker that remains at the N-terminal end of Sk-Tmod is GSPGISGGGGGG, which is directly followed by the Sk-Tmod sequence.

Actin Elongation Assays—Measurements of actin elongation were carried out as described previously (35, 36), using pyrenyl-labeled rabbit skeletal muscle actin (10% pyrylabeled) and short gelsolin-capped actin filaments as nuclei for polymerization. Actin polymerization was followed continuously for 5–7 h and measured again the next morning. The fluorescence changes (excitation 366.5 and emission 407 nm) were standardized against a Raman excitation peak and measured in a photon counting fluorimeter (Photon Technology International, Princeton, NJ). All experiments were carried out at 20 °C with Mg2+–actin (converted from Ca2+–actin as described previously (36) in a medium containing 10 mM imidazole buffer, pH 7.0, 0.1 mM KC1, 2 mM MgCl2, 1 mM azide, 1 mM dithiothreitol, 0.5 mM ATP, and 0.1 mM CaCl2 (polyribonuclease medium)). Gelsolin-capped actin filaments used as nuclei for polymerization were obtained by copolymerizing actin (usually 10 µM) with gelsolin in the presence of calcium. Average sizes for filaments are given in the figure legend as the ratios of actin:gelsolin. The K0 of Sk-Tmod for capping the pointed ends of pure actin filaments

RESULTS

Isolation of Sk-Tmod, a New Tropomodulin Isoform—While screening different muscles with a panel of mAbs generated against E-Tmod, we observed a spot in chicken breast muscle that migrated differently from E-Tmod on two-dimensional gels (Fig. 1). To determine whether this new tropomodulin spot was a novel isoform of tropomodulin, sequences of four peptides were obtained from tropomodulin immunoprecipitated from adult breast muscle. Comparison of the peptide sequences to the chicken E-Tmod amino acid sequence (16) demonstrated that all of the peptides were homologous but not identical to sequences in the C-terminal half of E-Tmod (Figs. 2 and 3). Using degenerate primers from these peptides, we isolated a polymerase chain reaction product, which was then used to isolate a full-length Sk-Tmod cDNA from an adult chicken skeletal muscle cDNA library. The complete nucleotide sequence of two of the longest clones (−1.3 kb) was determined and is shown in Fig. 2. The sequence contains a single open reading frame of 1044 bp starting at nucleotide 56 (ATG) with a stop codon at position 1098 (TGA), followed by 131 bp of a 3′-untranslated region (3′-UTR), containing a polyadenylation signal (Fig. 2, bold) (38) and a short poly(A) tail. All four peptides obtained from the immunoprecipitated protein are present in the predicted amino acid sequence of the Sk-Tmod cDNA (Fig. 2, underlined).

We have designated this new isoform Sk-Tmod since, as we describe later, adult skeletal muscle is a major site of expression for this isoform. Comparison of the cDNA sequences of chicken Sk- and E-Tmods demonstrates that they are 64% identical along their entire length, which indicates that these sequences are unlikely to be alternatively spliced products from the same gene. In agreement with this conclusion, the chicken E-Tmod and Sk-Tmod genes are located on different chromosomes: E-Tmod is located on the chicken Z (sex) chro-
A striking difference between the Sk-Tmod and E-Tmod cDNA sequences is the difference in size of their 3'-UTRs. The chicken E-Tmod cDNA has a long 3'-UTR of 2.5 kb (16), whereas the chicken Sk-Tmod cDNA has a short 3'-UTR of 131 bp (Fig. 2). Sk-Tmod Sequences Are Highly Conserved among Vertebrates—The chicken Sk-Tmod cDNA encodes a protein of 348 amino acids which is 62% identical and 75% similar to chicken E-Tmod (Fig. 3). The calculated molecular mass and pI of Sk-Tmod are 39.2 kDa and 4.72, respectively, which is slightly smaller and more acidic than E-Tmod (40.3 kDa and pI of 5), and is in agreement with the different mobilities of the two isoforms observed on two-dimensional gels (Fig. 1). Interestingly, Sk-Tmod is 11 amino acids shorter than E-Tmod at the C-terminal end (Fig. 3), a region proposed to be important for pointed end capping activity (10). Notably, the recently described rat N-Tmod is also shorter than E-Tmod at the C-terminal end (351 versus 359 aa, respectively) (27).

Sk-Tmod Caps Thin Filament Pointed Ends—E-Tmod is the capping protein for the pointed ends of muscle thin filaments (5, 9, 28). To determine the location of Sk-Tmod in chicken skeletal muscle, we used polyclonal antibodies generated against Sk-Tmod to stain isolated myofibrils from adult chicken breast muscle and compared the patterns for Sk-Tmod staining and phalloidin staining for F-actin. In relaxed myofibrils, phalloidin binds along the length of the thin filaments.
Amino acid numbers are indicated on the right family.

Alignment of the predicted amino acid sequence of chicken Sk-Tmod with chicken E-Tmod (16). Identical amino acids are boxed. Amino acid numbers are indicated on the right.

Fig. 3. Amino acid sequence comparison of chicken Sk- and E-Tmod. Alignment of the predicted amino acid sequence of chicken Sk-Tmod with chicken E-Tmod (16). Identical amino acids are boxed. Amino acid numbers are indicated on the right.

and appears as a broad banding pattern with narrow gaps corresponding to the H zones in the middle of the sarcomere with no actin filaments (Fig. 5A). Sk-Tmod staining appears as a periodic doublet flanking the H zone in the middle of the sarcomere, consistent with a localization at the thin filament pointed ends (Fig. 5B, merged image in C). In contrast, when myofibrils are stained for F-actin and α-actinin (Fig. 5, D and E) the merged image clearly shows that α-actinin staining is located at the Z line in the middle of the thin filament I-Z-I array (Fig. 5F).

Among all actin capping proteins, E-Tmod is distinguished by its weak capping activity for pointed ends of pure actin filaments ($K_d \approx 0.1–0.4 \mu M$) and its strong capping activity for pointed ends of tropomyosin-actin filaments ($K_d < 1 \mu M$) (36). This is a consequence of the ability of E-Tmod to bind tropomyosin and actin (16, 36, 39). To determine whether Sk-Tmod can cap actin filament pointed ends and whether its actin capping activity is enhanced by tropomyosin, we expressed chicken Sk-Tmod in E. coli and purified it to homogeneity (see “Experimental Procedures”). The pointed end capping activity of recombinant Sk-Tmod was evaluated from its ability to inhibit elongation of actin filaments capped at their barbed ends by gelsolin, using the fluorescence increase of pyrenyl actin to measure actin polymerization (36). In the absence of tropomyosin, Sk-Tmod inhibits the initial rate of elongation at the pointed filament end with an estimated $K_d$ of $0.2 \mu M$ (Fig. 6A). This indicates that Sk-Tmod can bind directly to pure actin filaments at their pointed ends with about the same affinity as E-Tmod ($0.1–0.4 \mu M$) (36). Furthermore, Sk-Tmod also increases the critical concentration at the pointed end as demonstrated by a decrease in the total F-actin at steady state (i.e., lower end point of polymerization) (Fig. 6A), as previously observed for E-Tmod (36).

In the presence of skeletal muscle tropomyosin, nanomolar concentrations of Sk-Tmod completely block elongation from the pointed ends of the actin filaments and do not increase the critical concentration (Fig. 6B), similarly to E-Tmod (36).² The $K_d$ for Sk-Tmod capping of tropomyosin-actin filaments is estimated to be less than $7 \text{nm}$, from this experiment (Fig. 6B). The much higher capping activity of Sk-Tmod for a tropomyosin-actin filament than for pure actin is similar to E-Tmod and suggests that Sk-Tmod also binds tropomyosin in addition to actin at the pointed filament end. In fact, a tropomyosin blot overlay assay (16, 24) demonstrates that skeletal muscle tropomyosin binds directly to Sk-Tmod (data not shown).

Tissue Expression Pattern of Sk-Tmod.—A Sk-Tmod probe detects a single band of approximately 1.3 kb in adult breast muscle (PM) but not in heart, brain, cerebellum, spleen, lung, gizzard, liver, intestine, ovary, or thymus (Fig. 7, A and B). In contrast, an E-Tmod probe detects three bands of approximately 1.4, 2.1, and 2.6 kb in heart but not in breast muscle (PM) (Fig. 7A, right panel, asterisks) or in the other tissues (data not shown). Similar sizes for E-Tmod messages have been reported previously and are most likely due to utilization of different polyadenylation signals (15, 16). Although Sk-Tmod is the predominant tropomodulin isoform in adult chicken breast muscle, E-Tmod is also present in breast muscle, albeit in low amounts, and can be detected by Northern blots of poly (A)⁺ RNA (16) or by grossly overexposing the Western blots of two-dimensional gels (data not shown).

E-Tmod was originally identified in human erythrocytes and later shown to be in the rat lens (data not shown, and Refs. 23 and 39). The tropomodulin isoform composition of chicken lens fiber cells and erythrocytes was characterized by two-dimensional gel electrophoresis followed by Western blotting, because the very low amounts of mRNA contained in these biosynthetically inactive tissues make Northern blot analysis difficult. Fig. 7C shows that a single tropomodulin spot is detected in both adult chicken lens and erythrocytes (RBC) (Fig. 7C, left panel). Mixing experiments of lens or erythrocytes (RBC) with adult breast (PM) muscle (Fig. 7C, right panel) demonstrate that the tropomodulin spots in these tissues comigrate, indicating that Sk-Tmod rather than E-Tmod predominates in chicken lens and erythrocytes.

Sk-Tmod replaces E-Tmod during development of chicken Skeletal Breast Muscle.—Muscle growth and an increase in
muscle activity (as seen in flapping movement) are remarkable during the postnatal development of chicken breast muscle. The changes in the physiological properties of the muscle during development are paralleled by shifts in the expression of distinct isoforms for each sarcomeric component (13). To determine whether tropomodulin isoform expression changes during breast muscle development in vivo, we first compared message levels of E- and Sk-Tmod by Northern blotting. Sk-Tmod messages are detected exclusively in adult breast muscle and not in either day 12 or day 18 embryonic breast muscle (Fig. 8A, left panel). When the same blot is reprobed with the E-Tmod specific probe, the three characteristic E-Tmod messages of approximately 1.4, 2.1, and 2.6 kb are detected in both day 12 or 18 day embryonic breast muscle but not in adult breast muscle (Fig. 8A, right panel).

In agreement with the Northern analysis, Western blots of two-dimensional gels show that E-Tmod is the most abundant tropomodulin isoform detected in breast muscle from day 12 embryos (Fig. 8B) and from day 20 embryos (just before hatching) (data not shown). Soon after hatching (2 days), the more acidic Sk-Tmod isoform starts to be detected (Fig. 8B), and, as development proceeds, the E-Tmod spot disappears and is replaced by the Sk-Tmod isoform. Thus, in breast muscle from chickens 1 week old or older, the Sk-Tmod isoform is the predominant tropomodulin isoform detected (Fig. 8B).

**Sk-Tmod Is Enriched in Adult Fast Skeletal Muscle Fibers, Whereas E-Tmod Is Enriched in Slow Skeletal Muscle Fibers—** Adult breast muscle consists mainly of fast muscle fiber types, while embryonic breast muscle resembles adult slow fiber types in its physiological properties (40). Studies of isoform expression for other sarcomeric proteins have shown that the embryonic isoform often predominates in adult chicken slow fiber types as well as in cardiac muscle, while a different isoform is expressed in adult fast fibers (e.g. myostin light chain or C-protein) (13). To further characterize the expression of Sk-Tmod and E-Tmod in different fiber types, we immunolocalized tropomodulins in serial cross sections of frozen adult chicken BF muscle which contains both fast and slow muscle fiber types. A monoclonal antibody specific for fast twitch C-protein (MF1) was used as a marker for fast muscle fiber types (19) and its staining pattern was compared with that of mAb 95, which specifically recognizes E-Tmod, and mAb 17, which preferentially recognizes Sk-Tmod by immunofluorescence (see “Experimental Procedures”).

The staining pattern obtained with the fast C-protein antibody reveals the heterogeneity in the muscle fibers in BF (Fig. 9B). Most fibers stain brightly for fast C-protein (Fig. 9B, double arrowhead), a subset of fibers appear moderately stained (Fig. 9B, short arrow), while in a few fibers fast C-protein is hardly or not at all detected (Fig. 9B, long arrow). Overall, the intensity of the fiber staining for E-Tmod and fast C-protein is complementary to each other. For instance, the minority of the fibers that appear brightly stained for E-Tmod are not stained for fast C-protein (Fig. 9, A and B, long arrow), indicating that E-Tmod is enriched in slow muscle fiber types. Conversely, most fibers that do not stain for E-Tmod stain brightly for fast C-protein (compare Fig. 9, A and B, double arrowhead). On the other hand, fibers stained for Sk-Tmod also contain fast C-protein (Fig. 9, B and C, short arrow, double arrowhead) and fibers with hardly any detectable Sk-Tmod are brightly stained for E-Tmod but not fast C-protein (Fig. 9, A–C, long arrow). Additionally, we found that by Western blot of two-dimensional gels, Sk-Tmod and E-Tmod are both present in BF, whereas Sk-Tmod is the only tropomodulin isoform detected in posterior latissimus dorsi muscle, which consists of fast muscle fiber types (data not shown).

Together, these results suggest that Sk-Tmod predominates in fast twitch fiber types. However, fibers that are moderately stained for fast C-protein are also stained for E- and Sk-Tmod (Fig. 9, A–C, short arrow) indicating that some fibers contain both tropomodulin isoforms. Hybrid fibers containing both fast and slow isoforms of different myofibrillar protein isoforms are reported to be quite frequent and may indeed represent the rule rather than the exception (13).

**E-Tmod Colocalizes with α-Spectrin in Distinct Subsarcolemmal Domains—** E-Tmod was first identified as a component of the spectrin-actin membrane skeleton in human erythrocytes (15, 39). Interestingly, we noticed E-Tmod staining associated with the sarcolemma of many muscle fibers in BF muscle (Fig. 9A, arrowhead). This staining is specific for E-Tmod because it is eliminated by pre-incubating mAb 95 with a 100-fold molar excess of purified E-Tmod, but not Sk-Tmod, prior to incubation with the muscle sections (data not shown). Furthermore, we obtained the same sarcolemmal staining using another E-Tmod specific monoclonal antibody (mAb 9) (Fig. 10A), which recognizes an epitope of E-Tmod located in a different region of the molecule than the epitope recognized by mAb 95 (data not shown). These observations suggested that E-Tmod might be a component of the subsarcolemmal spectrin-actin membrane skeleton in skeletal muscle.

α-Spectrin has been described to be concentrated at subdomains along the sarcolemma that overlay the I bands in skeletal muscle and are termed costameres (41). To explore the association of E-Tmod with the subsarcolemmal spectrin lattice, we double-stained cross-sections of BF muscle for E-Tmod and α-spectrin. Fig. 10 shows that E-Tmod as well as α-spectrin are associated with the sarcolemma of all muscle fiber types contained in this muscle (Fig. 10, A and B, arrows). A higher magnification view of an oblique BF section demonstrates that E-Tmod staining is not uniformly distributed along the sarcolemma, and instead appears to be concentrated in distinct and periodic puncta ~2 μm apart, which colocalize with the α-spectrin puncta (Fig. 10, compare C with D, arrowheads).

This result suggests that E-Tmod is associated with the
FIG. 6. Sk-Tmod inhibits elongation from the pointed ends of actin and tropomyosin-actin filaments. A, effect of Sk-Tmod on the elongation rate of gelsolin-capped actin filaments. Elongation was initiated by the simultaneous addition of gelsolin-capped actin filaments (final concentration 10 nM; gelsolin:actin, 1:10) and polymerizing salts to a 2.5 μM solution of rabbit skeletal muscle G-actin (10% pyrenylactin), containing 0.1% Triton X-100, 0.3 mM MgCl₂, 0.6 mM ATP, and 1.5 μM μM F-actin in the absence of Sk-Tmod (i.e. 0.69 μM G-actin, which is the pointed end critical concentration), and 1.45, 1.21, and 1.23 μM F-actin for 0.3, 0.6, and 1.5 μM Sk-Tmod, respectively. This corresponds to a maximal increase in the G-actin concentration to ~1.38 μM at steady state (i.e. about a 2-fold increase in the pointed end critical concentration), as described previously for N-Tmod (Ref. 36 and Footnote 2). B, effect of Sk-Tmod on the elongation rate at the pointed ends of tropomyosin-actin filaments. The elongation assay was carried out as for A, except that the assay medium contained 1.5 μM G-actin, 1.15 μM rabbit skeletal muscle tropomyosin, and 6 nM gelsolin:actin seeds (0.9 μM F-actin) (gelsolin:actin, 1:150) (●, ▲), with the addition of 7 (●), 15 (▲), and 30 (●) nM Sk-Tmod. The end points of F-actin polymerization were 1.74 μM for the control in the absence of tropomodulin (corresponding to ~0.66 μM G-actin; the pointed end critical concentration), and 1.62, 1.75, and 1.65 μM F-actin for 7, 15, and 30 nM Sk-Tmod. These are not significantly different from the end point in the absence of tropomodulin, as described previously for E-Tmod (Ref. 36 and Footnote 2).

α-spectrin-containing costameres overlying the I bands in chicken skeletal muscles.

DISCUSSION

Tropomodulins are a family of actin filament pointed end capping proteins. The first tropomodulin isoform identified in vertebrates was E-Tmod, a ~40-kDa protein whose expression in mammals is restricted to terminally differentiated cells including erythrocytes, lens fibers, neurons and striated muscle (9, 28, 42). Subsequently, another ~40-kDa tropomodulin isoform, N-Tmod, was identified in embryonic and adult neurons (27). ~40-kDa tropomodulin homologs have also been identified in flies (Sanpodo) (31) and in C. elegans (28, 31). Larger, more distantly related proteins with limited regions of similarity to portions of the ~40-kDa tropomodulins have also been identified, including a ~64-kDa protein in smooth muscle and extraocular muscle (43, 44) as well as a longer ~60-kDa (predicted) protein from C. elegans (28, 31). In this study, we report the identification of a third ~40-kDa tropomodulin isoform from vertebrates, which we have named Sk-Tmod, based on its high level of expression in skeletal muscle.

The three known ~40-kDa vertebrate tropomodulin isoforms are products of different genes and are all about 60–65% identical (75–90% similar) to each other at the amino acid level. The high sequence homology between tropomodulin isoforms implies that they most likely share functional properties. Indeed, here we show that Sk-Tmod shares all the known functional properties described for E-Tmod (9); it is associated with the pointed ends of muscle thin filaments in vitro and completely blocks actin elongation from the pointed ends of tropomyosin-actin filaments in vivo (Kₐ < 7 nM). Like E-Tmod, Sk-Tmod also inhibits elongation from the pointed ends of pure actin filaments but with considerably lower affinity (Kₐ ~ 0.2 μM). Thus, we propose that a defining feature of this family of proteins is the ability to block the elongation of tropomyosin-actin filaments from their pointed ends, mediated by their binding to both actin and tropomyosin (9). N-Tmod has been shown previously to interact with tropomyosin similarly to E-Tmod, but its actin capping activity has not yet been investigated (27). Thus, verification of this hypothesis will require determination of the tropomyosin-actin filament capping activities for N-Tmod and the other recently identified tropomodulin homologs from invertebrates.

On the other hand, the sequences of tropomodulins in each independent group in the tropomodulin family (E-, N-, and Sk-Tmod groups) are highly conserved across vertebrates (28 and Fig. 4 of this study). This might indicate that different tropomodulin isoforms have acquired minor but functionally significant differences in their sequences that have been preserved through evolution. This study and those of others have described several observations supporting this idea. First, tropomodulin isoforms exhibit strikingly different tissue expression patterns (28, 42). For example, in adult chickens, Sk-Tmod is found in fast skeletal muscle, erythrocytes, and lens. In contrast, E-Tmod is found in heart and slow skeletal muscle while N-Tmod is expressed in brain.4 A second observation, and probably the one that most strongly supports the idea of functional specificity for each tropomodulin isoform, is the sorting of Sk- and E-Tmod isoforms to different cytoskeletal structures within the same skeletal muscle cell (e.g. myofibrils and costameres, respectively). This may indicate that other regions of the molecule, not involved in tropomyosin or actin binding, might be responsible for interactions with other sarcomeric or cytoskeletal components and determine unique functions, which are as yet undescribed.

On the other hand, the tropomodulin isoform composition in the same tissue appears to differ between species. For instance, chicken lens and erythrocytes contain Sk-Tmod (Fig. 7) while mouse lens and erythrocytes contain E-Tmod.4 This may indicate that tropomodulins could have redundant functions and that their differential expression in tissues and between species may reflect the regulatory constraints of different developmental programs. Future studies on the genomic organiza-
tion and the intron/exon structure of tropomodulins may also provide an understanding of the role that regulatory or structural regions play in generating and maintaining this tropomodulin isoform sequence diversity.

**Expression of Different Tropomodulin Isoforms in Muscle Correlates with Changes in Thin Filament Lengths**—Our results show an interesting correlation between the tropomodulin isoform expressed and the changes in thin filament lengths that take place during the development of chicken breast muscle. Decoration of thin filaments from adult chicken breast muscle with antibodies to troponin invariably revealed 24 striations at regular intervals of 38 nm, whereas in embryonic muscle the number of striations varied between 25 and 29 (45). This suggests that the thin filaments are all ~0.9 μm long in adult breast muscle, whereas they vary from ~0.95 to 1.1 μm in embryonic breast muscle. Our results show that E-Tmod is present in embryonic breast muscle and thus appears to be associated with sarcomeres in which the thin filaments are shorter and have a wider range of lengths. In contrast, Sk-Tmod is present in adult breast muscle and thus appears to be associated with sarcomeres in which the thin filaments are shorter and have a more precise length distribution. To further explore this idea, it will be necessary to compare directly the expression of tropomodulin isoforms in other muscles in which thin filament lengths have been measured.

How might different tropomodulin isoforms influence the
The puncta along the sarcolemma (higher magnification images show that E-Tmod is localized in distinct
fibers contain a moderate amount of E-Tmod (A, short arrow), fast twitch C-protein (B, short arrow), and Sk-Tmod (C, short arrow). Sk-Tmod is most abundant in the fast muscle fiber types that are brightly stained for fast C-protein (B and C, double arrowheads) and do not contain E-Tmod (A, double arrowhead). E-Tmod staining is also detected at the sarcolemma of muscle fibers (A, arrowhead). The brighter staining obtained for fast C-protein on the right edge of the tissue section is most likely due to a fold or a difference in thickness of the section. Bar, 50 μm.

Fig. 9. Sk-Tmod predominates in skeletal fast muscle fiber types while E-Tmod predominates in slow muscle fiber types. Serial cross-sections of frozen adult chicken BF muscle were stained for E-Tmod (A), fast twitch C-protein (B), or Sk-Tmod (C). E-Tmod is most abundant in slow muscle fiber types (A, long arrow), where fast twitch C-proteins (B, long arrow) and Sk-Tmod (C, long arrow) are hardly detected. Some
fibers contain a moderate amount of E-Tmod (A, short arrow), fast twitch C-protein (B, short arrow), and Sk-Tmod (C, short arrow). Sk-Tmod is most abundant in the fast muscle fiber types that are brightly stained for fast C-protein (B and C, double arrowheads) and do not contain E-Tmod (A, double arrowhead). E-Tmod staining is also detected at the sarcolemma of muscle fibers (A, arrowhead). The brighter staining obtained for fast C-protein on the right edge of the tissue section is most likely due to a fold or a difference in thickness of the section. Bar, 50 μm.

Fig. 10. E-Tmod colocalizes with α-spectrin at discrete subsarcolemmal domains in skeletal muscle fiber cells. Serial cross-sections of frozen adult chicken BF muscle were double stained for E-Tmod (A), or α-spectrin (B). E-Tmod together with α-spectrin localizes at the sarcolemma of all the muscle fiber types (A and B, arrow). Higher magnification images show that E-Tmod is localized in distinct puncta along the sarcolemma (C, arrowhead), which colocalize with α-spectrin (E, arrowhead). The merged image is shown in D (merged). The asterisk in D and E indicates a capillary brightly stained for α-spectrin. Bar in A and B, 25 μm. Bar in C–E, 2 μm.

hatching (46). Thus, in adult breast muscle, α/α is the principal isoform found (47–50). Furthermore, the ratio of α/α, α/β, and β/β dimers differs between mature muscle fiber types (51, 52).

Nebulin is a long actin-binding protein that extends from the Z line to the thin filament pointed end and has been proposed to function as a molecular template to specify thin filament lengths in skeletal muscle (53–55). The existence of different nebulin isoforms which differ in their molecular size has been correlated with the length of thin filaments in different skeletal muscle types and during differentiation of chicken breast muscle (56–58). One can speculate that distinct nebulin isoforms might interact with specific tropomodulin isoforms to regulate tropomodulin affinity for the pointed ends and thereby produce thin filaments of different lengths for physiologically distinct types of muscle (5). However, cardiac muscle does not contain nebulin (55), yet it contains E-Tmod, which might suggest that nebulin is not necessarily required by E-Tmod to regulate the length of thin filaments.

Sorting of Tropomodulin Isoforms to Different Cytoskeletal Structures—Interestingly, in fast skeletal muscle fibers, which coexpress both E and Sk-Tmod, E-Tmod appears to be associated with the α-spectrin-containing costameric network overlying the I bands at the sarcolemma while Sk-Tmod is associated with the myofibrils within the same cell. What can account for restriction of E-Tmod to the sarcolemma in hybrid muscle fibers which express both E and Sk-Tmod? One possibility is that Sk-Tmod binds thin filament pointed ends more tightly and thus competes for E-Tmod binding to this site. Similarly, E-Tmod may bind more tightly to costameric actin filaments than Sk-Tmod. In this case, we might expect the existence of specific sequences in tropomodulin isoforms to target the tropomodulins to their appropriate binding partners in each structure. However, it is important to point out that the basis for sorting of different tropomodulin isoforms to different cytoskeletal structures is likely to be complex since in some muscle fibers (i.e. slow and mixed) E-Tmod is detected associated with myofibrils and at the sarcolemma.

Costameres were first described as vinculin-rich areas forming rib-like elements flanking the Z lines of underlying myofibrils in skeletal muscle (59, 60). Subsequently, spectrin was found to be enriched in costameres overlying the I bands, in transverse elements over the M line and in fine longitudinal strands connecting the costameres (41, 61–65). To date, a variety of other proteins including desmin, integrins, dystrophin, and components of the spectrin-based membrane skeleton have also been localized to elements of the costameric network (64, 66), but very little is known about their molecular organization. Our observation that E-Tmod is a component of the costameric
network suggests that similar to erythrocytes, the spectrin-based membrane skeleton underlying the costameric network is built by short actin filaments capped by E-Tmod at their pointed ends and cross-linked by spectrin (9).

It has been proposed that costameres could play a mechanical role in anchoring the sarcomeric cytoskeleton of the most peripheral myofibrils to the membrane via intermediate filaments and also in linking the sarcolemma to the extracellular matrix via integral membrane proteins (60, 63, 64, 67, 68). In addition to a mechanical role, costameres have also been proposed to be involved in signal transduction across the membrane through focal adhesion proteins such as integrins, talin, and vinculin, or through the dystrophin-based membrane skeleton, which appears to interact with enzymatic or regulatory proteins (64). Thus, E-Tmod in costameres may function in a structural capacity to stabilize and limit the lengths of actin filaments in a spectrin-actin network, thereby influencing the clustering of focal adhesion components and/or dystrophin-associated proteins at costameres. This could regulate, in turn, the signaling pathways controlling skeletal muscle fiber growth or atrophy.

Prospects—While studies of isolated proteins have been useful in identifying protein function, they may be less helpful in distinguishing properties of closely related isoforms. The correlation of tropomodulin isoform expression with differences in the contractile properties of different muscle types suggests the possibility that tropomodulins might also be involved in thin filament linked regulation of contraction together with tropomyosin and troponins (for a discussion, see Ref. 5). In vivo, it is likely that the functional advantages produced by each molecular phenotype have contributed to preserve certain combinations of sarcomeric isoforms in each muscle. Recently, several functional studies have been published reporting that closely related sarcomeric isoforms of tropomyosin and tropo-

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