

# Tropomodulin: A Cytoskeletal Protein that Binds to the End of Erythrocyte Tropomyosin and Inhibits Tropomyosin Binding to Actin

Velia M. Fowler

Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California 92041

**Abstract.** Human erythrocytes contain a  $M_r$  43,000 tropomyosin-binding protein that is unrelated to actin and that has been proposed to play a role in modulating the association of tropomyosin with spectrin-actin complexes based on its stoichiometry in the membrane skeleton of one  $M_r$  43,000 monomer per short actin filament (Fowler, V. M. 1987. *J. Biol. Chem.* 262:12792-12800). Here, we describe an improved procedure to purify milligram quantities to 98% homogeneity and we show that this protein inhibits tropomyosin binding to actin by a novel mechanism. We have named this protein tropomodulin. Unlike other proteins that inhibit tropomyosin-actin interactions, tropomodulin itself does not bind to F-actin. EM of rotary-shadowed tropomodulin-tropomyosin complexes reveal that tropomodulin ( $14.5 \pm 2.4$  nm [SD] in diameter) binds to one of the ends of the rod-like tropomyosin molecules (33 nm long). In agreement with this observation, Dixon plots of inhibition curves demonstrate that tropomodulin is a non-

competitive inhibitor of tropomyosin binding to F-actin ( $K_i = 0.7 \mu\text{M}$ ). Hill plots of the binding of the tropomodulin-tropomyosin complex to actin indicate that binding does not exhibit any positive cooperativity ( $n = 0.9$ ), in contrast to tropomyosin ( $n = 1.9$ ), and that the apparent affinity of the complex for actin is reduced 20-fold with respect to that of tropomyosin. These results suggest that binding of tropomodulin to tropomyosin may block the ability of tropomyosin to self-associate in a head-to-tail fashion along the actin filament, thereby weakening its binding to actin. Antibodies to tropomodulin cross-react strongly with striated muscle troponin I (but not with troponin T) as well as with a nontroponin  $M_r$  43,000 polypeptide in muscle and in other nonerythroid cells and tissues, including brain, lens, neutrophils, and endothelial cells. Thus, erythrocyte tropomodulin may be one member of a family of tropomyosin-binding proteins that function to regulate tropomyosin-actin interactions in non-muscle cells and tissues.

**T**ROPOMYOSINS in nonmuscle cells are thought to influence actin filament assembly and organization and to play a regulatory role in defining domains along actin filaments, based on *in vitro* biochemical experiments and on immunofluorescence localization in cells. Tropomyosin blocks the interaction of cross-linking proteins such as filamin or  $\alpha$ -actinin with actin filaments (47, 52, 65) and tropomyosin-coated actin filaments are resistant to bundling by villin (5), severing by gelsolin (16, 30, 52), and disassembly by actin-depolymerizing proteins (2, 46). Tropomyosin and  $\alpha$ -actinin are localized in a periodic, alternating fashion along actin filament bundles (stress fibers) in cultured cells (33, 35, 54) and tropomyosin is absent from actin filaments associated with cell junctions, attachment plaques and the foci of polygonal networks in spreading cells with which  $\alpha$ -actinin is associated (26, 34). Similarly, villin is associated exclusively with the actin filament bundles in the microvilli of the intestinal epithelial cell brush border, whereas tropomyosin is associated with the bundle rootlets and actin filaments in the terminal web (45). Evidence that tropomyosin

functions to regulate actin filament organization *in vivo* is provided by disruption of the single tropomyosin gene in budding yeast which leads to disappearance of the characteristic cytoplasmic actin cables and reduced cell growth (37).

Thus, modulation of tropomyosin-actin interactions is expected to be critical for the assembly, disassembly, and rearrangements of actin filaments that take place during cell locomotion, cell division, and differentiation related changes in cell shape. Previously described proteins from nonmuscle cells that can prevent the interaction of tropomyosin with actin and/or dissociate tropomyosin from actin do so by binding to actin and competing for the same binding sites on actin filaments (5, 44). The ability of these proteins to influence the association of tropomyosin with actin is correlated with the relative affinities of these proteins and tropomyosin for F-actin. For example, a  $M_r$  55,000 bundling protein from HeLa cells induces the dissociation from actin of  $M_r$  3,000–32,000 tropomyosin isoforms from cultured fibroblasts that have a low affinity for actin but has little effect on the interaction with actin of skeletal muscle tropomyosin or the  $M_r$

35,000–40,000 isotypes from cultured fibroblasts that have a high affinity for actin (45). In fact, skeletal muscle tropomyosin reduces the ability of the  $M_r$  55,000 bundling protein itself to bind or bundle actin filaments. Proteins that bind directly to tropomyosin and independently influence tropomyosin–actin interactions without themselves binding to actin have not been described in nonmuscle cells.

Troponins I and T are tropomyosin-binding proteins in skeletal and cardiac muscle that are tightly associated with each other and with troponin C (the calcium-binding component) in a troponin complex. One troponin complex is associated with each tropomyosin molecule along the actin filaments in the muscle sarcomere and functions to confer calcium sensitivity on tropomyosin regulation of actomyosin ATPase activity. In vitro, binding of troponin promotes tropomyosin head-to-tail self-association and also enhances the binding of tropomyosin to actin. Tropomyosin self-association along the actin filament is thought to be important for cooperative regulation of actomyosin ATPase during muscle contraction (for reviews, see references 8, 36, 49, 66).

To date, tropomyosin-binding proteins homologous to muscle troponins I or T have not been isolated from nonmuscle cells and tissues. Instead, a  $\text{Ca}^{2+}$ -calmodulin-regulated F-actin binding protein, caldesmon, is associated with tropomyosin along the actin filaments of smooth muscle and nonmuscle cells and is thought to participate in thin filament regulation of actomyosin ATPase activity (for reviews see references 3, 43). Smooth muscle caldesmon also associates with tropomyosin (24, 27) and binding of caldesmon to actin is strengthened in the presence of tropomyosin (55, 57). Although nonmuscle caldesmon has not been reported to bind directly to tropomyosin, it enhances binding of nonmuscle tropomyosin isotypes to F-actin and is therefore proposed to play a role in regulation of actin filament organization in nonmuscle cells (30, 65).

We show here that a previously described  $M_r$  43,000 tropomyosin-binding protein (tropomodulin) from the human erythrocyte membrane skeleton (19) is a potential candidate for a nonmuscle tropomyosin-regulatory protein based on its ability to bind to the end of tropomyosin and to inhibit tropomyosin binding to actin in vitro. Tropomodulin is immunologically related to muscle troponin I, but is unrelated to caldesmon, based on structural, functional, and immunological criteria. However, unlike troponin I and caldesmon, which bind to actin and enhance tropomyosin binding to actin, tropomodulin itself does not bind directly to actin. This, together with the identification of immunoreactive tropomodulin polypeptides in a variety of nonmuscle cells and tissues indicates that erythrocyte tropomodulin may be a representative of a new type of tropomyosin-regulatory protein in nonmuscle cells. Modulation of tropomyosin–actin associations could influence actin filament organization and function and/or actomyosin contractile activity in nonmuscle cells and tissues.

## Materials and Methods

### Membrane Preparation

Erythrocytes from 6 U of blood were isolated free of platelets, leukocytes, and other white cells by sedimentation at 1 g through 3.3% gelatin in physiological saline and were obtained as a byproduct of neutrophil isolation from Drs. G. Bokoch, A. Jesaitis, and C. Cochrane (Department of Immunology,

Research Institute of Scripps Clinic). The cells were washed four times in 4 vol of 150 mM NaCl, 5 mM sodium phosphate, pH 7.4 to remove the gelatin and were stored overnight at 0°C in an ice bucket after addition to the packed cells of one-thousandth volume of 200 mg/ml PMSF in DMSO. Packed cells were lysed by hypotonic hemolysis in 15 vol of 7.5 mM sodium phosphate, 1 mM EDTA, 2 mM DTT, 20  $\mu\text{g}/\text{ml}$  PMSF, pH 7.4 at 4°C and membranes were prepared as previously described except that the cassette system (Pellican; Millipore Corp., Bedford, MA) was equipped with 10 ft<sup>2</sup> of Durapore membrane (19). Membranes were collected by centrifugation overnight at 7,500 g (4°C), frozen in liquid nitrogen, and stored at –80°C. Approximately 300–350 ml of well-packed membranes (7–8 mg/ml protein) are obtained by this procedure from 6 U of blood.

### Purification of Proteins

**Tropomodulin.** Two batches of frozen membranes (600–700 ml; 12 U of blood) were combined and extracted with Triton X-100 as described by Gardner and Bennett (25). Triton X-100-extracted membranes were resuspended to a final volume of 390 ml by homogenization in the buffer used for hypotonic hemolysis (see above) and tropomodulin was solubilized by addition of an equal volume of 1.6 M sodium bromide, 10 mM sodium phosphate, 1 mM EDTA, 2 mM DTT, 20  $\mu\text{g}/\text{ml}$  PMSF, pH 7.5. After incubation for 30 min at 4°C, insoluble material was removed by centrifugation for 60 min at 235,000 g. The supernatant was collected, dialyzed into 20 mM Tris, 1 mM EDTA, 2 mM DTT, pH 8.0 at 4°C, clarified by centrifugation for 120 min at 186,000 g, and loaded onto a 16 × 1.5 cm DEAE-cellulose (DE53; Whatman, Inc., Clifton, NJ) column equilibrated in the same buffer. The column was washed with 5 column vol of this buffer and then eluted at 24 ml/h with 500 ml of a linear NaCl gradient to 400 mM, collecting 4-ml fractions. Fractions containing tropomodulin (ascertained by SDS-gel electrophoresis using a pH 9.1 separating gel [19]) eluted between 90 and 120 mM NaCl and were pooled and concentrated by addition of solid ammonium sulfate to 65% saturation. The precipitate was collected by centrifugation for 20 min at 30,000 g, resuspended in 0.6 ml of 100 mM NaCl, 20 mM Tris, 1 mM EDTA, 2 mM DTT, 0.02% sodium azide, pH 8.0 and dialysed overnight at 4°C against 1 liter of the same buffer. After centrifugation for 20 min at 150,000 g to remove aggregated material, the supernatant was loaded onto a 0.9 × 54 cm Ultrogel Aca 34 (Pharmacia-LKB Instruments, Gaithersburg, MD) gel filtration column and eluted at 2 ml/h in the same buffer, collecting 0.5 ml fractions. The tropomodulin eluted in the second major protein peak (determined by  $A_{280}$  and SDS-gel electrophoresis of column fractions) and peak fractions were pooled and concentrated by vacuum dialysis (Colloidion membranes; Schleicher & Schuell, Keene, NH) to a final volume of 0.5–0.8 ml. After dialysis overnight at 4°C into 80 mM KCl, 20 mM Hepes, 0.1 mM EDTA, 2 mM DTT, 0.02% sodium azide, pH 7.3, the tropomodulin was centrifuged at 150,000 g. For rotary shadowing, tropomodulin was purified by an additional step of sedimentation on a 5–20% linear sucrose gradient. Purified tropomodulin was stored at 0°C in an ice bucket for up to 1 mo before any proteolysis or change in its properties was detectable.

**Tropomyosin.** Membranes used for purification of erythrocyte tropomyosin were prepared as described above, except that the lysis buffer contained 2 mM  $\text{MgCl}_2$  and 1 mM EGTA instead of 1 mM EDTA (20). These membranes were lyophilized and tropomyosin was extracted and purified as described by Mak et al. (41) except that the lyophilized membranes were preextracted with 0.1 M KCl, 1 mM EDTA, 20 mM Tris-HCl, 1 mM DTT for 60 min at 0°C to remove magnesium before extracting the tropomyosin with the 1.0 M KCl buffer. For rotary shadowing, tropomyosin was purified by an additional step of gel filtration over an Ultrogel Aca 34 gel filtration column in 1 M NaCl, 10 mM Tris, 0.1 mM EDTA, 2 mM DTT, 0.02% sodium azide, pH 8.0.

**Actin.** Rabbit skeletal muscle actin was prepared from an acetone powder of muscle by the method of Spudich and Watt (56) and further purified by gel filtration over an Ultrogel Aca 34 column as previously described (20). Actin was stored as monomer at –80°C and put through one cycle of polymerization and depolymerization before using in experiments.

**Troponins.** The troponin complex was isolated from an acetone powder of rabbit skeletal muscle by high salt extraction and ammonium sulfate fractionation (51) followed by chromatography on hydroxylapatite (14). Troponins I and T were purified by sequential chromatography on DEAE-cellulose (Whatman Inc.) and CM-Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ) in 6 M urea as described by Potter (51).

### Protein Concentrations

The protein concentrations of erythrocyte tropomodulin, tropomyosin and

the muscle troponins were determined by the method of Lowry (39) after precipitation with TCA as described by Fowler and Bennett (20), with bovine serum albumin as a standard. For very dilute samples, 50  $\mu$ g of tRNA (*Escherichia coli*, Strain B; Calbiochem-Behring Corp., La Jolla, CA) was added as a carrier. The protein concentration of monomeric actin was determined from the absorbance at 290 nm using an extinction coefficient of 0.617 cm<sup>2</sup>/g and corrected for light scattering by subtracting the absorbance at 320 nm.

## Antibodies

**Tropomodulin.** Antibodies to tropomodulin were generated in rabbits by a primary injection into the popliteal lymph nodes and into multiple intradermal sites on the back of two rabbits, with 35  $\mu$ g of electrophoretically purified tropomodulin (in the gel slice) emulsified with CFA for each rabbit. Rabbits were boosted after 4 and after 8 wk by injections at multiple intradermal sites and in the subscapular area, with 40  $\mu$ g tropomodulin emulsified in incomplete Freund's adjuvant for each rabbit. Serum was collected 1, 2, and 3 wk after each boost, and antibody response was monitored by immunoblots. Antibodies were affinity purified as described by Burke et al. (6) by adsorption and elution from 100  $\mu$ g of purified tropomodulin that had been electrophoretically transferred to nitrocellulose (19).

**Troponins.** Antibodies to rabbit skeletal muscle troponin I were affinity purified on a troponin I-Sepharose column (prepared according to instructions from Pharmacia Fine Chemicals) from immune goat serum generously supplied by Dr. Marion L. Greaser (University of Wisconsin, Madison, WI). Affinity-purified guinea pig antibodies to rabbit skeletal muscle troponin T were a gift from Dr. Larry B. Smillie (University of Alberta, Edmonton, Canada).

## Actin-binding Assays

Tropomyosin binding to F-actin was determined by measuring the amount of <sup>125</sup>I-labeled tropomyosin cosedimenting with the F-actin. Tropomyosin was radiiodinated with <sup>125</sup>I-labeled Bolton and Hunter reagent (New England Nuclear, Boston, MA) and the protein concentration (0.05–0.1 mg/ml) and specific activity (2–3  $\times$  10<sup>6</sup> cpm/ $\mu$ g) determined as previously described (20). The F-actin binding properties of the radiiodinated tropomyosin were identical to unlabeled tropomyosin in terms of apparent affinity and stoichiometry of binding (not shown), when used within 3–4 d of iodination and all of the radioiodine was associated with tropomyosin, as determined by SDS-gel electrophoresis and autoradiography. Before use in actin-binding assays, <sup>125</sup>I-tropomyosin and tropomodulin were centrifuged for 20 min at 150,000 g at 4°C, and the actin was polymerized for 2 h at 22°C by addition of 2 M KCl to 80 mM and 0.1 M MgCl<sub>2</sub> to 2 mM. Tropomyosin was mixed with F-actin (added using a Microman pipettor; Rainin Instrument Co., Woburn, MA) in the presence or absence of tropomodulin at the indicated concentrations in a 50- $\mu$ l assay volume in a buffer containing 80 mM KCl, 20 mM Hepes, 2 mM MgCl<sub>2</sub>, 2 mM DTT, 10 mg/ml BSA, pH 7.3. Components were mixed directly in 250  $\mu$ l centrifuge tubes (model TLA 100; Beckman Instrument Co., Fullerton, CA), incubated 90 min at 25°C and centrifuged for 25 min at 150,000 g in a rotor (TLA 100; Beckman Instruments). The supernatant was aspirated off using a vacuum aspirator and the amount of <sup>125</sup>I-tropomyosin sedimenting was determined by counting the pellets (in the tubes) directly in a Beckman gamma counter. The amount of <sup>125</sup>I-tropomyosin bound to F-actin was calculated by subtracting the amount of <sup>125</sup>I-tropomyosin sedimenting in the absence of F-actin for each concentration of tropomyosin, which never amounted to >5% of the total counts sedimenting in the presence of F-actin. The amount of F-actin sedimenting was determined independently in each assay by SDS-gel electrophoresis of supernatants and pellets as previously described (21), and was not influenced by the presence of tropomyosin or tropomodulin (not shown).

## Electrophoresis Procedures

SDS-gel electrophoresis was according to Laemmli (33), using 10% acrylamide or 7.5–15% linear gradient acrylamide gels with a 5% stacking gel except that a pH 9.1 or 8.6 separating gel was used to resolve tropomodulin from actin, as indicated in figure legends (see reference 20). Electrophoretic transfer of polypeptides from the SDS gels to nitrocellulose paper (0.2  $\mu$ M; Schleicher & Schuell, Keene, NH) was as previously described (20). Nitrocellulose gel transfers were pretreated before antibody labeling by incubation for 1 h in 150 mM NaCl, 10 mM sodium phosphate, pH 7.5 at 65°C (20). Transfers were labeled overnight at 4°C on a rotating shaker with affinity-purified antibodies (1–2  $\mu$ g/ml) in a solution containing 150 mM

NaCl, 10 mM sodium phosphate, 0.2% Triton X-100, 1 mM EDTA, 0.02% sodium azide and 40 mg/ml BSA, pH 7.5. To detect the goat anti-troponin I antibodies, transfers were subsequently incubated for 2 h at room temperature with a 1/1,000 dilution of rabbit anti-goat antiserum in the same solution. Blots were then washed five times each 2 min at 4°C in the same buffer (without the serum albumin), and then labeled for 2 h at 4°C with 10<sup>6</sup> cpm/ml of <sup>125</sup>I-labeled protein A (*Staphylococcus aureus*) in the same buffer without the serum albumin. Transfers were washed five times each 2 min, dried, and exposed at –80°C to film (X-AR; Eastman Kodak Co., Rochester, NY) for autoradiography.

## Electron Microscopy

To prepare samples for rotary shadowing, proteins were dialysed for 1–2 h against two changes of 0.1 M ammonium acetate, 1 mM DTT, pH 7.0 in glass-distilled water, and diluted to 5–10  $\mu$ g/ml. Glycerol was added to a final concentration of 50–60%, samples were sprayed onto freshly cleaved mica, and rotary shadowed replicas were prepared by George Klier (La Jolla Cancer Research Foundation), using a tungsten filament with a twin-beam electron gun in an evaporator (Edwards High Vacuum, Inc., Grand Island, NY), as previously described (15). Replicas were examined at 75V with a Hitachi-600 electron microscope and photomicrographs taken by V. M. Fowler or G. Klier at magnifications of 60,000 or 100,000.

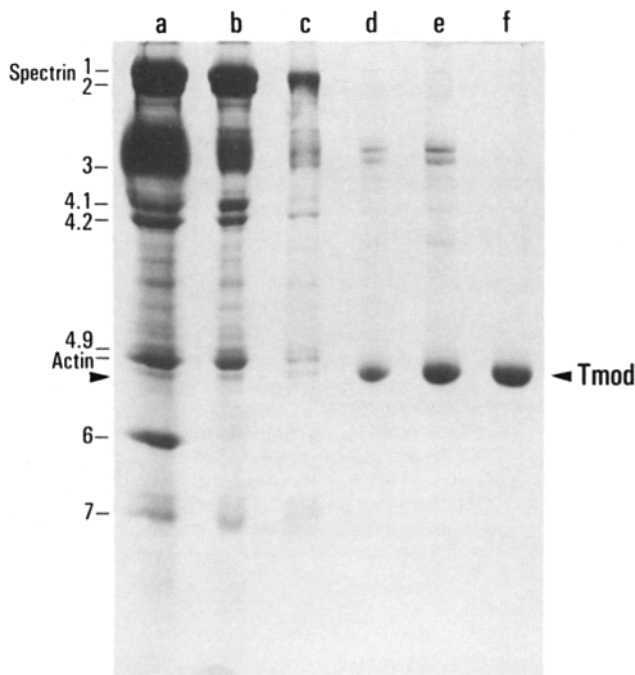
## Results

### Purification of Tropomodulin

Originally, tropomodulin was purified from a 1 M Tris extract of tropomyosin-depleted erythrocyte membranes (19). However, the yields of protein obtained by this procedure were poor (~50  $\mu$ g from 300 ml of membranes prepared from 2 U of blood), primarily due to inefficient extraction of the tropomodulin from the membranes. We have devised an improved purification procedure based on the observation that tropomodulin can be selectively and completely solubilized from membrane skeletons by incubation at 0°C with high concentrations of the chaotropic salt, sodium bromide. This procedure also solubilizes ankyrin, protein 4.9, and other minor components, without appreciably solubilizing spectrin, proteins 4.1, 4.2, or actin (Fig. 1, lane c). Sodium chloride is not as effective at solubilizing tropomodulin, whereas substitution of the iodide salt for the bromide solubilizes practically all of the components of the membrane skeleton, including spectrin, actin and protein 4.1 (not shown).

The solubilized protein is purified from the sodium bromide extract by adsorption to DEAE-cellulose and elution with a linear NaCl gradient from 0 to 400 mM, followed by gel filtration on Ultrogel AcA 34 (Fig. 1). At this stage the tropomodulin is ~80–90% pure, depending on the preparation (Fig. 1, lane f). If more highly purified material is desired, the tropomodulin can be further purified to 98% homogeneity by centrifugation on a 5–20% linear sucrose gradient (not shown). Approximately 1.5–2 mg of tropomodulin are obtained from 600–700 ml of membranes isolated from 12 U of blood, which represents a ~10–15% yield with respect to the amount of tropomodulin present in the original membranes (estimated by quantitative immunoblotting using purified tropomodulin as a standard).

The amino acid composition of tropomodulin is characterized by a low content of aromatic amino acids and a high proportion of acidic residues (28.1%) (Table I), consistent with the pI of 5.1–5.2 determined from isoelectric focusing gels in 9 M urea (not shown). Comparison of tropomodulin's amino acid composition with that of erythrocyte tropomyo-



**Figure 1.** Summary of purification of tropomodulin from human erythrocyte membranes. Lane *a*, membranes (95  $\mu$ g); lane *b*, Triton X-100-extracted membranes (45  $\mu$ g); lane *c*, NaBr-solubilized material from Triton-extracted membranes (DEAE-column load) (18  $\mu$ g); lanes *d* and *e*, pooled fractions from the DEAE-column (4 and 12  $\mu$ g, respectively); lane *f*, purified tropomodulin obtained from the Ultrogel AcA 34 column (8  $\mu$ g). Tropomodulin was solubilized and purified from Triton X-100-extracted erythrocyte membranes as described in Materials and Methods. To compare the relative amounts of tropomodulin in the starting membranes (lane *a*), Triton-extracted membranes (lane *b*) and the NaBr-solubilized material (lane *c*), samples were normalized to the original volume of the membranes and equivalent amounts electrophoresed on 10% acrylamide, pH 9.1 gels (19). The remaining samples were loaded to demonstrate presence (or absence) of contaminants.

sin, rabbit skeletal muscle actin and rabbit skeletal muscle troponin I (see below) demonstrates that tropomodulin is distinct from any of these proteins (Table I).

#### Localization of the Tropomodulin Binding Site on Tropomyosin

In the electron microscope, erythrocyte tropomodulin molecules visualized after rotary shadowing with tungsten and carbon are roughly globular in shape and somewhat heterogeneous in size (Fig. 2). Measurements of individual particles give a mean diameter of 14.5 nm  $\pm$  2.4 nm (SD,  $n = 500$ ), with some trailing towards the low end of the size range (not shown). In contrast, rotary shadowed erythrocyte tropomyosin molecules are slender rod-like molecules,  $\sim$ 33–34 nm long (Fig. 3). This length is characteristic of nonmuscle tropomyosins with native and subunit molecular weights of  $M_r$  60,000 and 30,000, respectively (8, 49). Longer molecules (*arrowheads*) are presumed to represent end-to-end polymers of individual molecules, and are characteristic of tropomyosin from skeletal muscle (17) and from some nonmuscle cells (16, 53).

Electron micrographs of rotary shadowed tropomodulin-tropomyosin complexes show images of rods with a globular

**Table I.** Amino Acid Composition of Human Erythrocyte Tropomodulin, Human Erythrocyte Tropomyosin, Rabbit Skeletal Muscle Actin, and Rabbit Skeletal Muscle Troponin I (Moles Percent)

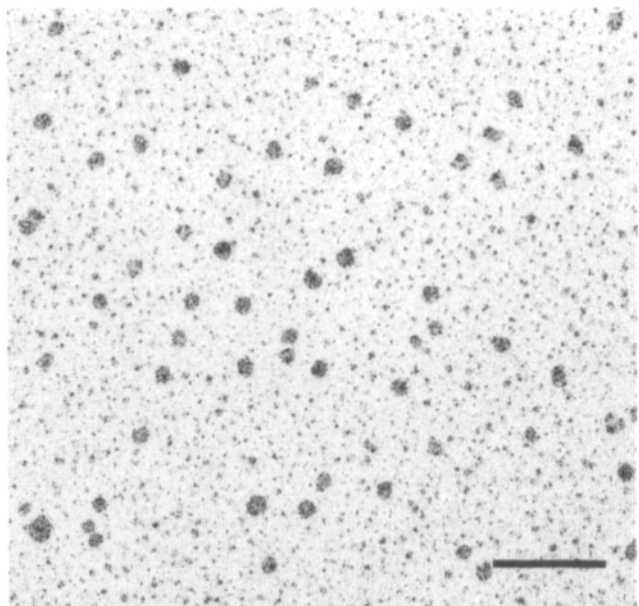
Amino acid	Human erythrocyte tropomodulin*	Human erythrocyte tropomyosin <sup>‡</sup>	Rabbit skeletal muscle actin <sup>§</sup>	Rabbit skeletal muscle troponin I
Asx	12.7	8.4	9.3	8.7
Glx	15.4	27.6	10.6	16.9
Ser	4.5	3.2	6.3	5.8
Gly	4.6	2.2	7.6	4.3
His	0.9	1.3	2.5	2.4
Thr	4.8	3.4	7.7	1.9
Ala	7.0	11.8	7.7	11.1
Arg	5.3	9.5	4.9	9.7
Pro	6.9	0	5.2	3.9
Tyr	3.0	1.3	4.4	1.4
Val	5.6	3.6	6.0	3.9
Met	2.0	2.2	4.4	4.3
Cys	0.6	ND	1.3	1.4
Ile	4.1	4.1	7.9	2.4
Leu	13.5	11.2	7.1	8.2
Phe	1.7	0.5	3.3	1.4
Lys	6.9	9.0	5.2	11.6
Trp	ND	ND	1.1	0.5

\* Tropomodulin was dialyzed versus 1 liter of 0.1 M NaCl, 2 mM sodium phosphate, pH 7.5 and then versus three changes of 1 liter each deionized water (pH  $\sim$ 7.0) at 4°C. Samples were hydrolysed for 20–24 h at 110°C in 6 N HCl and analyzed on a high performance analyzer (model 6300; Beckman Instruments) by Kerry Gwinn (Dr. Tony Hugli's laboratory, Research Institute of Scripps Clinic). Cysteine was determined as cysteic acid after performic acid oxidation. Values shown are the means from two replicate analyses.

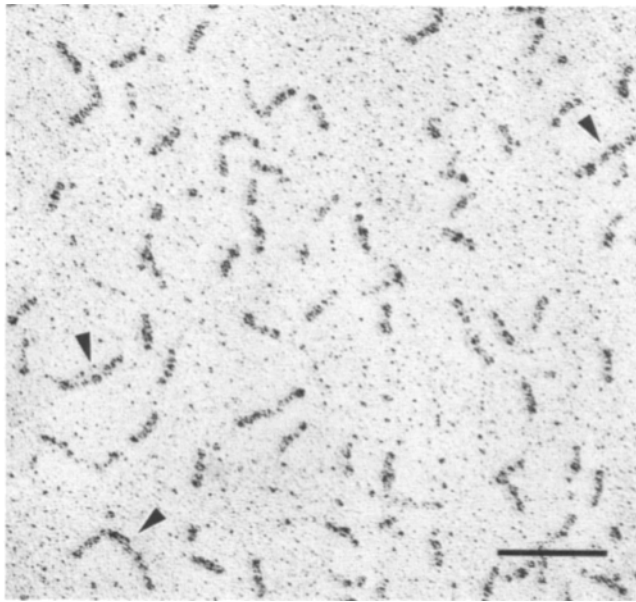
<sup>‡</sup> From Fowler and Bennett (20).

<sup>§</sup> Values calculated from the amino acid sequence for actin (7) and for rabbit fast muscle skeletal troponin I (60).

head at one end, demonstrating that tropomodulin binds to one of the ends of the tropomyosin molecule (Fig. 4). In examination of >100 such complexes, rods with a globular head in the middle or on both ends were never observed.



**Figure 2.** Electron micrograph of rotary shadowed erythrocyte tropomodulin molecules. Bar, 100 nm.



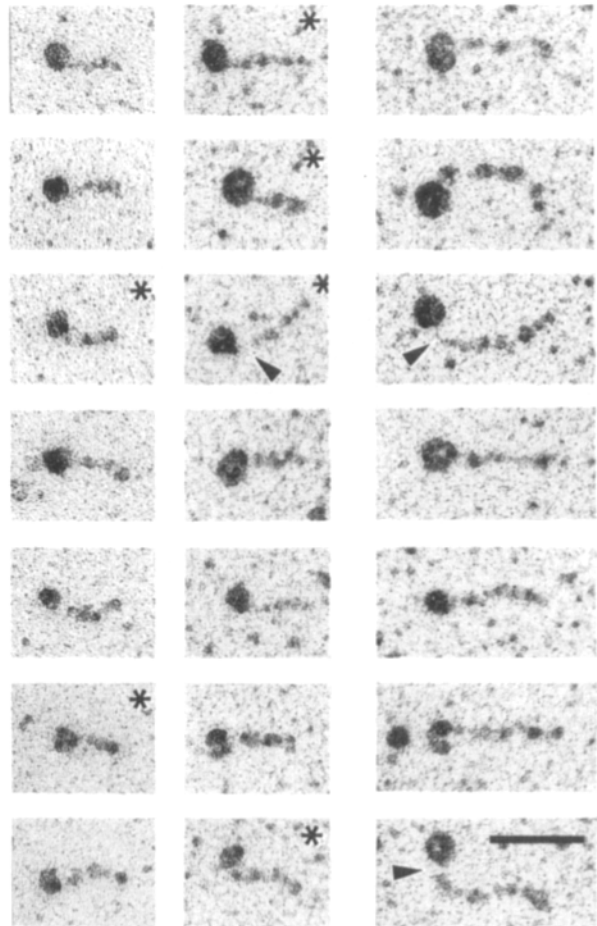
**Figure 3.** Electron micrograph of rotary shadowed erythrocyte tropomyosin molecules. The arrowheads indicate examples of end-to-end polymers of tropomyosin. Bar, 100 nm.

Some complexes appear to consist of an individual tropomyosin molecule associated with tropomodulin (Fig. 4, *left and middle columns*), whereas others appear to consist of end-to-end polymers of tropomyosin to which tropomodulin is bound (Fig. 4, *right column*). The rotary shadowed tropomodulin-tropomyosin complexes also exhibit considerable heterogeneity in the sizes of the tropomodulin molecules attached to the tropomyosin rods (Fig. 4), suggesting that tropomodulin may self-associate upon attachment to tropomyosin (see Discussion).

In some images, the globular tropomodulin head appears to be connected to the rod-like tropomyosin molecule by an extremely thin strand, or to be separated by a short gap (Fig. 4, *arrowheads*). In others, the length of the tropomyosin rod appears to be longer or shorter than 33–34 nm (Fig. 4, *asterisks*). Although we have not systematically quantitated these features, the variability in length of the rod portion of these complexes suggests that tropomodulin may be attached to tropomyosin by a short flexible domain, allowing the bulk of the tropomodulin molecule to extend away from the tropomyosin, or alternatively to roll back onto the tropomyosin rod.

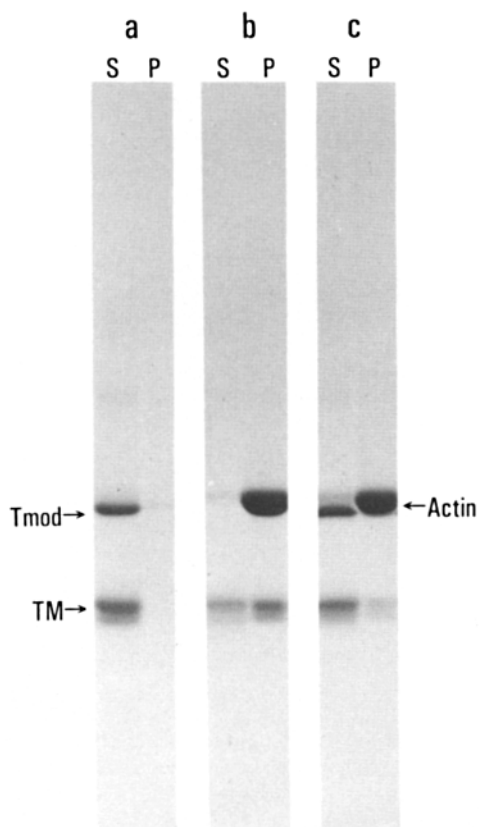
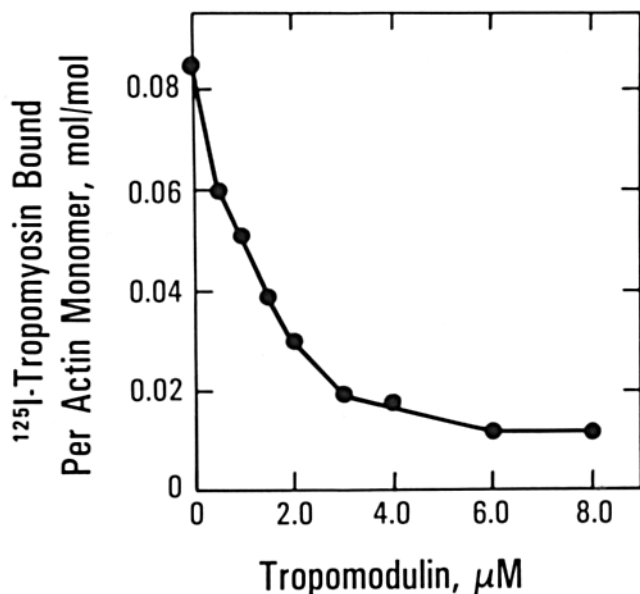
#### ***Effect of Tropomodulin on Tropomyosin Binding to F-Actin***

Erythrocyte membrane tropomyosin, like other nonmuscle tropomyosins, binds to F-actin and spans between five and six actin monomers along the actin filament at saturation (20, 41). Because both tropomodulin and tropomyosin are associated with the erythrocyte membrane skeleton (19), we expected that tropomodulin would bind to tropomyosin-coated actin filaments. To our surprise, inclusion of increasing amounts of tropomodulin dramatically reduced tropomyosin binding to F-actin (Fig. 5 *a*). Tropomodulin is equally effective at inhibiting tropomyosin binding to F-actin whether or not the tropomyosin is preincubated with the F-actin before addition of the tropomodulin (Table II), and tropomodulin itself does not bind to F-actin (Fig. 5 *b*).



**Figure 4.** Electron micrographs of rotary shadowed tropomodulin-tropomyosin complexes. To form the complex, tropomodulin was mixed with tropomyosin in a 1:1 molar ratio (2.5  $\mu\text{M}$  each protein) and incubated for 2 h at room temperature in a buffer consisting of 80 mM KCl, 1 mM  $\text{MgCl}_2$ , 1 mM DTT, 20 mM Hepes, pH 7.3. Samples were then prepared for shadowing as described in Materials and Methods. Complexes in the left-hand column were more lightly shadowed than the complexes in the middle and right-hand columns which were from another preparation. Complexes grouped in the right-hand column appear to consist of an end-to-end dimer of tropomyosin (60–70 nm) attached to a globular tropomodulin molecule, whereas those grouped in the left and middle columns appear to consist of a single tropomyosin rod attached to a globular tropomodulin molecule. Asterisks indicate examples of complexes in the left and middle columns in which the tropomyosin rod portion appears to be shorter or longer than a single tropomyosin molecule (33–34 nm), but not long enough to be an end-to-end dimer of tropomyosin, as in the far right column. Arrowheads indicate examples of a thin strand or short gap connecting the globular tropomodulin head to the tropomyosin rod. Bar, 50 nm.

To examine the mechanism whereby tropomodulin inhibits tropomyosin binding to F-actin, a series of tropomodulin-inhibition curves were generated at several different concentrations of tropomyosin. A Dixon plot (12) of these data demonstrates that tropomodulin is a non-competitive inhibitor of tropomyosin binding to F-actin with a  $K_i$  of 0.7  $\mu\text{M}$  (Fig. 6), similar to the  $K_i$  for tropomyosin binding to tropomodulin (0.5  $\mu\text{M}$ , see ref. 19). This implies that tropomodulin binds to a site on tropomyosin distinct from the actin-binding sites that are located along the length of the tropomyosin rod



**Figure 5.** Effect of purified tropomodulin on binding of human erythrocyte tropomyosin to muscle F-actin. (A) Inhibition of <sup>125</sup>I-labeled tropomyosin binding to F-actin by increasing concentrations of tropomodulin. <sup>125</sup>I-Bolton Hunter-labeled tropomyosin ( $8.31 \times 10^4$  cpm/ $\mu$ g,  $1.0 \mu$ M) was incubated with muscle F-actin ( $2.0 \mu$ M) and varying amounts of tropomodulin, and the amount of <sup>125</sup>I-tropomyosin binding to F-actin was determined as described in Materials and Methods. (B) SDS-gels of supernatants (S) and pellets (P) from mixtures of (a) tropomodulin and tropomyosin (unlabeled), (b) tropomyosin and F-actin, (c) tropomodulin, tropomyosin, and F-actin. Components were mixed and incubated as described in Materials and Methods (final concentrations of  $2.0 \mu$ M for tropomodulin,  $1.1 \mu$ M for tropomyosin, and  $4.0 \mu$ M for F-actin)

**Table II.** Effect of Order of Addition of Components on Tropomodulin Inhibition of Tropomyosin Binding to F-Actin

Condition	TM bound	
	mol/mol actin monomer	%
A TM + F-actin (90 min)	0.023	100
B TM + F-actin (45 min)	0.018	78
C TM + F-actin (45 min) + Tmod (45 min)	0.006	26
D TM + Tmod (45 min) + F-actin (45 min)	0.005	22

(A and B) <sup>125</sup>I-Bolton Hunter labeled tropomyosin (TM) ( $1.03 \times 10^5$  cpm/ $\mu$ g;  $0.4 \mu$ M) was incubated with rabbit skeletal muscle F-actin ( $4.0 \mu$ M) for 45 or 90 min, and the amount of <sup>125</sup>I-tropomyosin binding to F-actin was determined as described in Materials and Methods. In C, <sup>125</sup>I-tropomyosin was preincubated with F-actin before addition of tropomodulin (Tmod) ( $2.0 \mu$ M) and in D, <sup>125</sup>I-tropomyosin was preincubated with tropomodulin before addition of the F-actin.

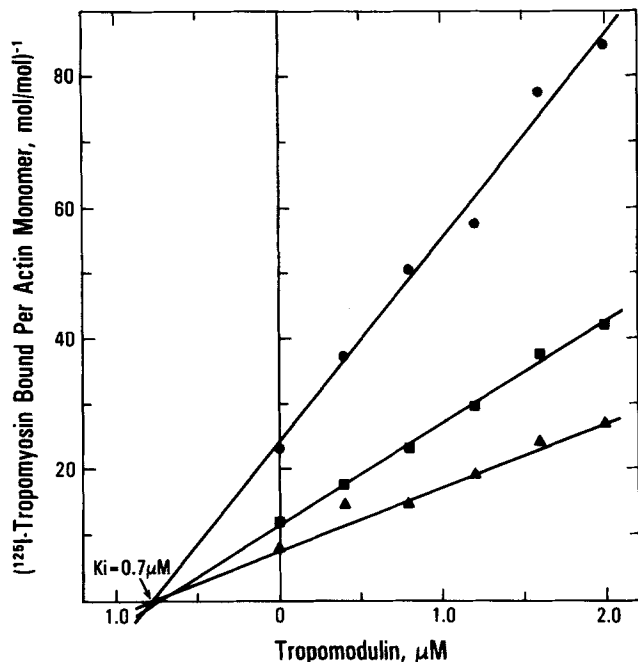
and agrees with the morphological localization of the tropomodulin binding site to the end of tropomyosin (Fig. 4).

To estimate the affinity of the tropomodulin-tropomyosin complex for F-actin, a binding curve for tropomyosin binding to F-actin was measured in the presence of a large excess of tropomodulin ( $8 \mu$ M) (Fig. 7 a). This amount of tropomodulin was sufficient to saturate the tropomyosin because further increases in the amount of tropomodulin caused no further reduction in the amount of tropomyosin bound to F-actin (not shown, but see Fig. 5 a). Hill plots (29) of the tropomyosin binding curves in the presence and absence of tropomodulin (Fig. 7 b) show that the apparent affinity of the tropomodulin-tropomyosin complex for F-actin is reduced 20-fold ( $K_a = 1.0 \times 10^5$  M<sup>-1</sup>) compared with that of tropomyosin for F-actin ( $K_a = 1.9 \times 10^6$  M<sup>-1</sup>). The Hill plots of these binding curves also demonstrate that binding of the tropomodulin-tropomyosin complex to F-actin does not exhibit any positive cooperativity ( $n = 0.9$ ) in contrast to tropomyosin ( $n = 1.9$ ). This suggests that, unlike erythrocyte tropomyosin, the tropomodulin-tropomyosin complex is incapable of self-association along the actin filament (see Discussion).

#### Immunological Relationship of Tropomodulin to Muscle Troponin I

Affinity-purified rabbit antibodies to purified erythrocyte tropomodulin are monospecific for tropomodulin on immunoblots of erythrocyte membrane proteins (Fig. 8 B, lane a). These antibodies cross-react strongly with purified rabbit skeletal muscle troponin I (Fig. 8 B, lane d) and with a comigrating  $M_r$  22,000 polypeptide in extracts of rat striated muscle (diaphragm) Fig. 8 B, lane e). The  $M_r$  22,000 polypeptide in rat muscle indeed appears to be rat troponin

except with the omission of the bovine serum albumin and in a final volume of  $75 \mu$ l. After centrifugation, supernatants were removed, TCA precipitated, and solubilized for SDS-gel electrophoresis along with the pellets. Equivalent amounts of supernatants and pellets were electrophoresed on 7.5–15% linear gradient, pH 9.1 acrylamide gels (19). In this experiment, ~50% of the tropomyosin cosediments with the F-actin and tropomodulin reduces this amount to approximately 10%, as determined by pyridine elution of dye from the Coomassie blue stained bands (20).

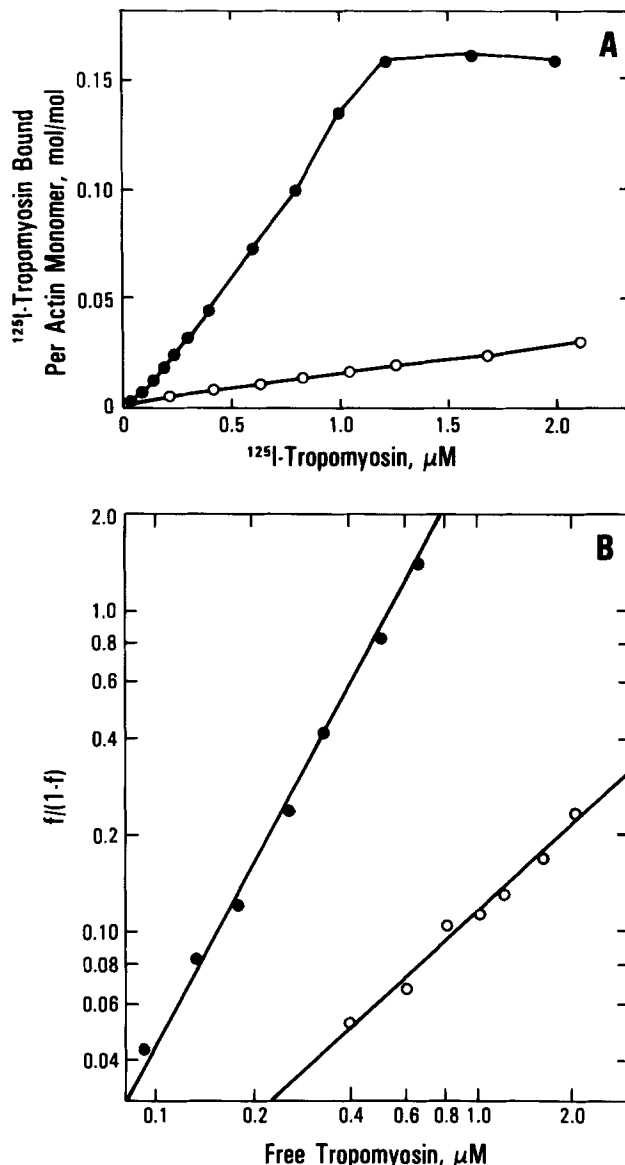


**Figure 6.** Dixon plot (12) of inhibition of binding of  $^{125}\text{I}$ -erythrocyte membrane tropomyosin to muscle F-actin in the presence of increasing concentrations of purified tropomodulin. Binding of 0.4 ( $\bullet$ ), 0.6 ( $\blacksquare$ ), and 0.8 ( $\blacktriangle$ )  $\mu\text{M}$   $^{125}\text{I}$ -tropomyosin ( $1.27 \times 10^5$  cpm/ $\mu\text{g}$ ) to F-actin (4.0  $\mu\text{M}$ ) was determined in the presence of various concentrations of unlabeled tropomodulin, as described in Materials and Methods.

I since it is also labeled with affinity-purified antibodies to rabbit skeletal troponin I (Fig. 8 C, lane e). A relationship between tropomodulin and troponin I is further indicated since antibodies to troponin I also cross-react (albeit rather weakly) with purified erythrocyte tropomodulin (Fig. 8 C, lane b). In contrast, antibodies to tropomodulin do not cross-react at all with purified rabbit skeletal troponin T (Fig. 8 B, lane c), and conversely, antibodies to rabbit troponin T do not cross-react with tropomodulin (Fig. 8 D, lane b).

Anti-tropomodulin antibodies also cross-react strongly with a set of polypeptides in rat striated muscle that approximately comigrate with erythrocyte tropomodulin (Fig. 8 B, lane e). Like erythrocyte tropomodulin (19), the apparent mobility of these immunoreactive polypeptides on SDS-gels is inversely dependent on the pH of the separating gel buffer;  $M_r$  45,000 at pH 8.6 (Fig. 8),  $M_r$  43,000 at pH 8.8 (not shown), and  $M_r$  39,000 at pH 9.1 (Fig. 9, lane 4).<sup>1</sup> Interestingly, antibodies to troponin I also cross-react weakly with a similar (the same?) set of  $M_r \sim 45,000$  polypeptides in rat muscle (Fig. 8 C, lane e). These polypeptides do not comigrate with rat muscle troponin T on pH 8.6 gels (Fig. 8 D, lane e), or gels run at any other pH (not shown). Thus, striated muscle appears to contain tropomodulin-like protein(s) that are immunologically related yet distinct from authentic troponin I. We are currently purifying and characterizing the putative muscle tropomodulin analogue(s) as well as cloning and sequencing cDNAs for erythrocyte tropomodulin to pursue these questions.

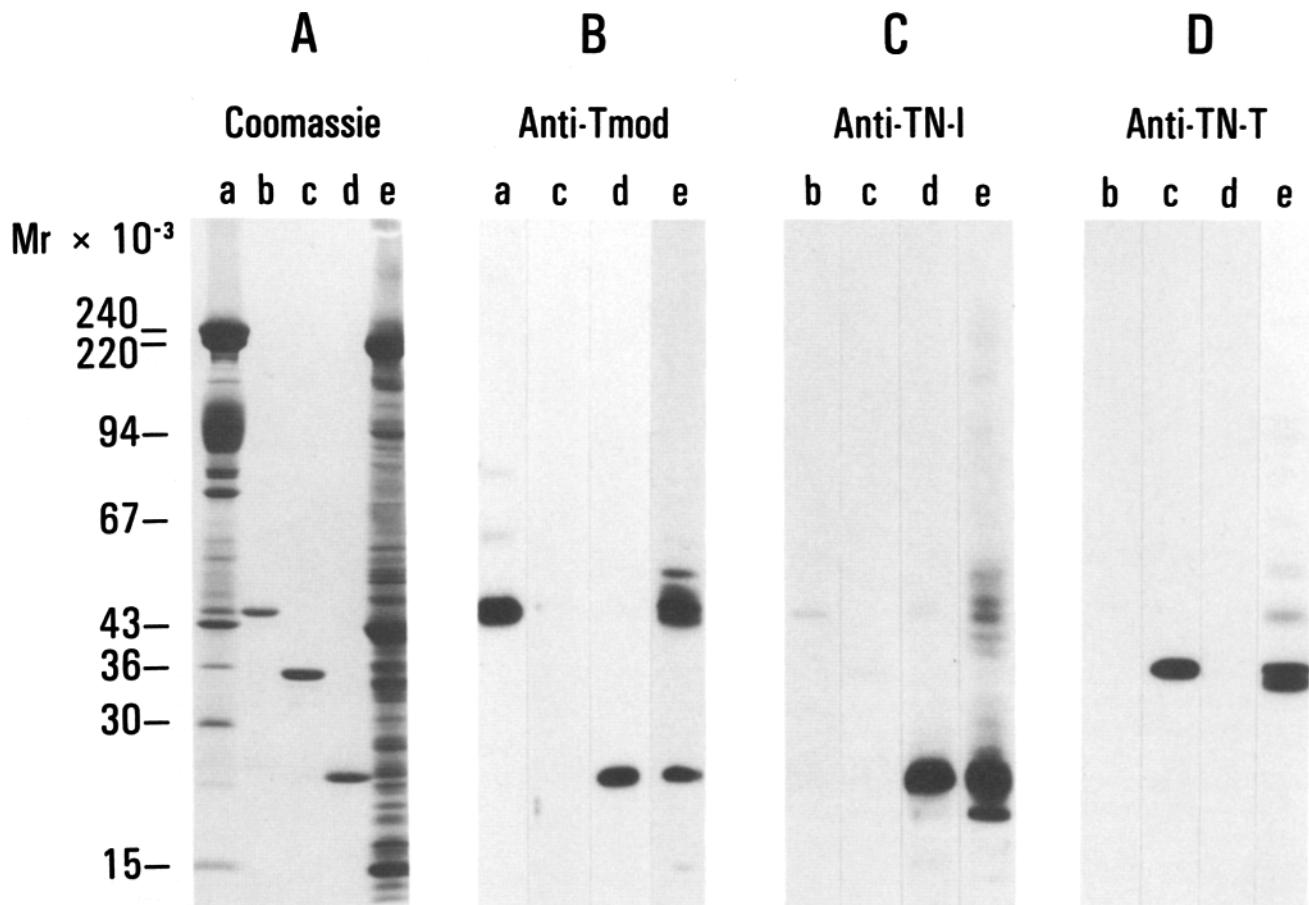
1. Unless noted otherwise, the apparent mobility ( $M_r$ ) of tropomodulin on SDS gels (43,000) is as determined using a standard Laemmli separating gel with a pH of 8.8 (32).



**Figure 7.** (A) Binding of  $^{125}\text{I}$ -erythrocyte tropomyosin to muscle F-actin in the absence ( $\bullet$ ) or presence ( $\circ$ ) of excess tropomodulin. (B) Hill plot (29) of the data presented in A.  $^{125}\text{I}$ -tropomyosin ( $5.35 \times 10^4$  cpm/ $\mu\text{g}$ ) at the indicated concentrations was preincubated with ( $\circ$ ) or without ( $\bullet$ ) tropomodulin (8.0  $\mu\text{M}$ ) for 1 h at 25°C before addition of muscle F-actin (2.0  $\mu\text{M}$ ) (final concentrations) and the amount of  $^{125}\text{I}$ -tropomyosin binding to F-actin was determined as described in Materials and Methods. In B, the binding data were plotted according to the Hill equation (29);  $\log(f/1-f)$  versus  $\log$  free tropomyosin concentration, where  $f$  is the fraction of binding sites on F-actin that are occupied by tropomyosin. The total number of sites was estimated from the saturation point of the tropomyosin binding curve ( $\bullet$ ) in A.

### Immunoreactive Tropomodulin Polypeptides in Nonmuscle Cells and Tissues

Polypeptides that comigrate with erythrocyte tropomodulin and cross-react with antibodies to tropomodulin are also present in rat brain and bovine endothelial cells (Fig. 9, lanes 5 and 6), as well as in rat liver, ileum, kidney and bovine lens (not shown). These cross-reactive polypeptides also exhibit a pH-dependent mobility shift on SDS gels (not



**Figure 8.** Immunological relationship of erythrocyte tropomodulin and muscle troponins. Samples of human erythrocyte membranes (lane *a*), erythrocyte tropomodulin (lane *b*), troponin T from rabbit skeletal muscle (lane *c*), troponin I from rabbit skeletal muscle (lane *d*), and rat striated muscle (diaphragm) (lane *e*) were electrophoresed on a 7.5–15% acrylamide linear gradient, pH 8.6 gel and either stained with Coomassie blue (*A*) or electrophoretically transferred to nitrocellulose paper and labeled with affinity-purified antibodies to erythrocyte tropomodulin (*B*), rabbit skeletal muscle troponin I (*C*), or rabbit skeletal muscle troponin T (*D*) as described in Materials and Methods. Amounts of proteins were 40  $\mu\text{g}$  of erythrocyte membranes in *A* and 4  $\mu\text{g}$  in *B*; 2.0  $\mu\text{g}$  each of purified tropomodulin, troponin I, and troponin T in *A* and 0.2  $\mu\text{g}$  each in *B*, *C*, and *D*;  $\sim 60$   $\mu\text{g}$  of rat diaphragm muscle in all panels. All blots were exposed to x-ray film for the same amount of time (3 h).

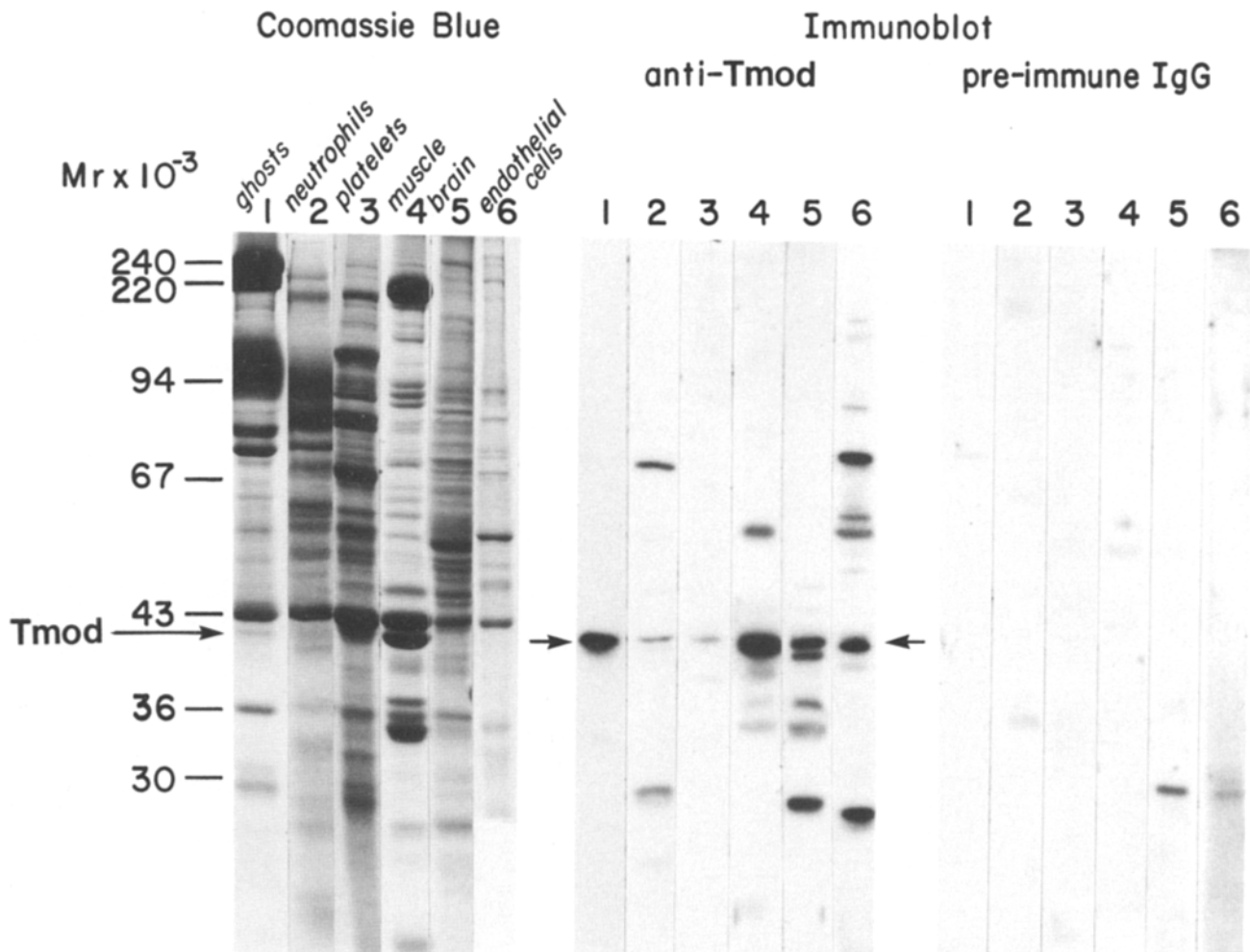
shown). In platelets and neutrophils, this comigrating polypeptide is barely detectable and could be explained by a small amount of contaminating erythrocytes (Fig. 9, lanes 2 and 3). However, antibodies to tropomodulin also cross-react with  $M_r$  70,000 polypeptides in human neutrophils as well as in bovine endothelial cells (Fig. 9, lanes 2 and 6) and with  $M_r$  27,000–28,000 polypeptides in rat brain and bovine endothelial cells (Fig. 9, lanes 5 and 6).<sup>2</sup> Preimmune IgG does not label any of these polypeptides. These results suggest that tropomodulin may be one member of a family of related tropomyosin-binding proteins present in both muscle and nonmuscle cells and tissues. Experiments are in progress to determine whether any of these cross-reactive polypeptides are also tropomyosin-binding proteins.

2. The cross-reactive  $M_r$  22,000 troponin I polypeptide in rat muscle is not evident in Fig. 9 because these gels were electrophoresed to obtain maximum resolution of tropomodulin from actin, which caused the troponin I to run off of the gel.

## Discussion

We describe here the properties of a novel  $M_r$  43,000 tropomyosin-binding protein, tropomodulin, from the erythrocyte membrane skeleton that binds to the end of erythrocyte tropomyosin and inhibits tropomyosin binding to F-actin without itself binding to F-actin. Tropomodulin is unrelated to actin, a tropomyosin-binding protein with a similar subunit molecular weight, based on amino acid composition, hydrodynamic properties, pH-dependent mobility on SDS-gels, stoichiometry and site specificity for tropomyosin binding, and inability to polymerize (this paper and see reference 19). Tropomodulin also appears to be distinct from caldesmon, a  $M_r$  70,000–80,000 tropomyosin-binding protein from nonmuscle cells (3), based on amino acid composition and molecular weight (this paper), lack of heat stability and inability to bind calmodulin (Fowler, V. M., unpublished observations), property of weakening rather than enhancing tropomyosin binding to actin, and inability to bind to actin (this paper). Antibodies to tropomodulin do not cross-react





**Figure 9.** Identification of immunoreactive erythrocyte tropomodulin polypeptides in selected non-erythroid cells and tissues. Lane 1, human erythrocyte ghosts; lane 2, human neutrophils; lane 3, human platelets; lane 4, rat striated muscle (diaphragm); lane 5, rat brain; lane 6, bovine aorta endothelial cells. Outdated platelet-rich plasma was obtained from the Red Cross and platelets were freed of erythrocytes by low-speed centrifugation and then washed twice with 0.9% NaCl, 0.3% sodium citrate. Isolated human neutrophils were kindly supplied by Dr. John Curnutte (Scripps Research Institute), and cultured bovine aorta endothelial cells by Dr. Paul McNeil (Harvard Medical School). Cells and tissues were solubilized directly in SDS-sample buffer and  $\sim 50$ – $100$ - $\mu$ g amounts were electrophoresed on triplicate 10% acrylamide, pH 9.1 gels (19) and either stained with Coomassie blue (*left*), or transferred to nitrocellulose and labeled with affinity-purified antibodies to tropomodulin (*middle*) or preimmune IgG (*right*), followed by  $^{125}$ I-Protein A to detect the immunoreactive polypeptides.

with actin or caldesmon and conversely, antibodies to actin or caldesmon do not cross-react with erythrocyte tropomodulin (Fowler, V. M., unpublished data). Furthermore, comparison of the sequences of two different 21- and 25-amino acid tryptic peptides from tropomodulin with the sequences of muscle and nonmuscle actins or smooth muscle caldesmon reveals no significant homologies (not shown).

### Physical Properties of Tropomodulin

Tropomodulin is an acidic (pI 5.1–5.2)  $M_r$  43,000 protein with an amino acid composition unlike other previously characterized  $M_r$  40,000–43,000 proteins (48). In solution, tropomodulin appears to be an asymmetric monomer ( $f_{f_0} = 1.5$ ) with a Stokes radius of 3.9 nm and a sedimentation coefficient of 2.8 S (19). These hydrodynamic data are surprising considering the roughly globular morphology and

large size of rotary shadowed tropomodulin molecules (average diameter = 14.5 nm [Fig. 2]). Assuming a shadow thickness of 2–3 nm,<sup>3</sup> and a volume per dalton of  $1.3 \text{ \AA}^3$  (39a), a 14.5-nm spherical particle would be expected to have a molecular mass on the order of 250,000–450,000 D. It is possible that the buffer conditions used for adsorption of tropomodulin to mica followed by rotary shadowing leads to self-association and distortion of tropomodulin molecules, thus altering their true size and morphology. Additionally, in preliminary experiments we have observed that tropomodulin exhibits a concentration-dependent self-association to tetramers, based on chemical cross-linking of tropomodulin in solution by copper-phenanthroline catalysed oxidation (un-

3. Shadow thickness was estimated by subtracting the actual width of a tropomyosin molecule (2 nm, see reference 23) from the apparent width measured for shadowed tropomyosin molecules.

published observations). Alternative approaches to determine the molecular mass, shape, and association states of tropomodulin (e.g., analytical ultracentrifugation, laser light scattering) will be required to definitively reconcile these disparate observations.

### *Interaction of Tropomodulin with Tropomyosin*

EM of rotary shadowed tropomodulin-tropomyosin complexes demonstrates clearly that tropomodulin binds to only one of the ends of the tropomyosin rod, since we never see complexes with globular heads at both ends or in the middle (Fig. 4). This morphological observation is in agreement with the conclusion from Dixon plots (Fig. 6) that tropomodulin is a noncompetitive inhibitor of tropomyosin binding to actin, binding to a site on tropomyosin distinct from the actin-binding sites located along the length of the tropomyosin rod. The images of rotary shadowed tropomyosin-tropomodulin complexes also show that tropomodulin does not bind to the head-to-tail junction region of tropomyosin dimers (Fig. 4, *right column*), suggesting that tropomodulin-tropomyosin interactions may be incompatible with tropomyosin self-association. This idea is supported by Hill plots (Fig. 7 *b*) which show no evidence of positive cooperativity for tropomodulin-tropomyosin binding to actin and imply that the tropomodulin-tropomyosin complex is incapable of head-to-tail self-association along the actin filament (also see below). (Since subsaturating concentrations of tropomodulin were used to prepare the complexes in the experiment presented in Fig. 4, we would not expect that all tropomyosin molecules would have a tropomodulin molecule attached at one end.)

Tropomyosin molecules are coiled-coil, alpha-helical parallel dimers with the amino terminus of each subunit at one end of the molecule and the carboxy terminus at the other (8, 36, 49). We are currently investigating whether tropomodulin binds to the NH<sub>2</sub>- or COOH-terminal end of tropomyosin. The variability in the lengths of tropomyosin rods in the rotary shadowed tropomodulin-tropomyosin complexes (Fig. 4) may mean that tropomodulin interacts with upstream portions of the tropomyosin molecule in addition to the extreme COOH-terminal end.

### *Mechanism of Tropomodulin Inhibition of Tropomyosin Binding to F-Actin*

Differences in the actin-binding affinity of different tropomyosin isoforms are thought to be due at least in part to differences in their ability to self-associate head-to-tail along the actin filament (8, 49). This is because binding of an individual tropomyosin molecule to F-actin is much weaker than binding of two or three contiguous tropomyosin molecules, linked through contacts at their ends, to actin (58). Although it has been generally assumed that non-muscle tropomyosins are incapable of head-to-tail self-association because they bind to F-actin more weakly than muscle tropomyosins (8, 49), nonmuscle tropomyosins purified from different cells and tissues vary considerably in their actin-binding affinity (4, 9, 20, 38, 53). In addition, Pruliere et al. (54) have shown directly that pig platelet tropomyosin self-associates head-to-tail, using viscometry and negative staining EM.

Erythrocyte tropomyosin exists as homodimers of *M*,

29,000 and 27,000 polypeptides, based on oxidative cross-linking experiments (Fowler, V. M., unpublished data), and the *M*<sub>r</sub> 27,000 tropomyosin has a higher apparent affinity and lower Mg<sup>2+</sup> requirement for binding to F-actin than does the *M*<sub>r</sub> 29,000 tropomyosin (20). The actin-binding properties of the *M*<sub>r</sub> 27,000 isoform more closely resemble those of muscle tropomyosin (64), and binding of erythrocyte tropomyosin to F-actin is highly cooperative (Fig. 7 *b*, and see reference 20), as is muscle tropomyosin (58, 64), consistent with the possibility that erythrocyte tropomyosin self-associates along the actin filament. Indeed, images of rotary shadowed molecules of erythrocyte tropomyosin reveal numerous examples of end-to-end polymers of individual tropomyosin molecules (Fig. 3).<sup>4</sup>

In the presence of excess tropomodulin, tropomyosin binding to F-actin no longer exhibits any positive cooperativity (Hill coefficient = 0.9) and its apparent affinity for F-actin is reduced by ~20-fold (Fig. 7 *b*). This, together with the ability of tropomodulin to bind to the end of erythrocyte tropomyosin, suggests that tropomodulin may weaken tropomyosin-actin interactions by interfering with head-to-tail self-association of tropomyosin along the actin filament. Alternatively, since recent experiments with recombinant tropomyosins indicate that ability to self-associate head-to-tail may not necessarily be linked to cooperativity in F-actin binding (29), it is also conceivable that binding of tropomodulin to tropomyosin could induce a conformational change in the tropomyosin rod that results in a qualitative change in its interaction with F-actin, reducing both its binding affinity and cooperativity. To evaluate these ideas, it will be necessary to examine directly the effect of tropomodulin on tropomyosin self-association in the absence of F-actin.

### *Relationship of Tropomodulin to Muscle Troponin I*

The immunological cross-reactivity between erythrocyte tropomodulin and striated muscle troponin I (but not troponin T) is surprising (Fig. 8). Troponin I is a basic (pI 9.3) *M*<sub>r</sub> 20,000–24,000 protein which is tightly associated both with troponin C (the calcium-binding component) and troponin T in the troponin complex (for reviews, see references 36, 66). Purified troponin I also binds to tropomyosin (10, 50) and strengthens the binding of both muscle (13) and non-muscle (equine platelet) (9) tropomyosins to F-actin. In contrast, tropomodulin is an acidic (pI 5.1–5.2) *M*<sub>r</sub> 43,000 tropomyosin-binding protein which weakens the binding of tropomyosin to F-actin. Furthermore, troponin I interacts directly with troponin C, as well as with calmodulin, whereas we have been unable to detect any binding of tropomodulin to calmodulin (Fowler, V. M., unpublished observations). Troponin I can also bind directly to F-actin in the absence of tropomyosin, unlike tropomodulin (Fig. 5 *b*). Moreover, the sequences of two different 21- and 25-amino acid tryptic peptides from tropomodulin do not exhibit any significant similarities with the sequence of troponin I (not shown).

Together, these observations suggest that any potential similarity between tropomodulin and troponin I must be

4. A previous report that erythrocyte tropomyosin is incapable of head-to-tail self-association using a viscometric assay (41) may be explained by the relative insensitivity of this assay, together with the threefold greater amount of the weaker actin-binding *M*<sub>r</sub> 29,000 isoform in purified preparations of erythrocyte tropomyosin (20).

confined to a limited region of each molecule, perhaps the tropomyosin-binding domain(s). The presence of variable, isotype-specific sequences in the COOH-terminal portion of muscle and non-muscle tropomyosin molecules (8, 40) could explain our previous observations that the muscle troponin complex is unable to compete for  $^{125}\text{I}$ -tropomyosin binding to tropomodulin (19). Alternatively, purified troponin I may interact differently with erythrocyte tropomyosin than does the intact troponin complex.

### **Role of Tropomodulin in the Erythrocyte Membrane Skeleton**

The human erythrocyte membrane skeleton consists of many short actin filaments (33–37 nm long) cross-linked into a strikingly regular hexagonal lattice by long, flexible spectrin molecules (for reviews see references 1, 18, 42). Both tropomyosin and tropomodulin are components of the erythrocyte membrane skeleton and are present in stoichiometric amounts with respect to the erythrocyte actin filaments (tropomodulin/tropomyosin/actin filament = 1:2:1) (19–21). The erythrocyte tropomyosin molecules (33–34 nm) are almost long enough to span the length of the short actin filaments, one on each strand of the filament (in vitro, tropomyosin/actin = 1:5–6). Based on the ability of tropomyosin to inhibit purified spectrin binding to F-actin (21, 41), tropomyosin was proposed to play a role in regulating spectrin-actin interactions. In addition, tropomyosin was also proposed to play a role in mechanically stabilizing the erythrocyte actin filaments based on in vitro observations that tropomyosin-coated actin filaments are resistant to fragmentation (59).

The inhibitory effect of tropomodulin on tropomyosin-actin interactions is surprising considering the relative amounts of these components in the erythrocyte membrane skeleton. However, at present there is no direct evidence that tropomodulin is in fact associated with tropomyosin on the erythrocyte actin filaments. It is possible that in the mature erythrocyte, tropomodulin is sequestered from tropomyosin by its association with other membrane proteins so as not to inhibit tropomyosin binding to actin. This is consistent with observations that tropomodulin remains associated with the membrane after extraction of tropomyosin (19). Therefore, we speculate that tropomodulin may be bound to tropomyosin only in immature erythrocytes and function to regulate tropomyosin-actin associations during erythroid differentiation, for example during the massive reorganization of actin filaments that occurs during enucleation (31). Titration of tropomyosin-actin associations by limiting amounts of tropomodulin could potentially result in selective disassembly of non-tropomyosin-coated actin filaments by actin filament-fragmenting and/or depolymerizing proteins. We are exploring these ideas by characterizing the interaction of tropomodulin with other erythrocyte membrane proteins and by examining the biosynthesis and assembly of tropomodulin, tropomyosin and actin in the membrane skeleton during terminal differentiation of erythrocytes.

Although tropomodulin is unlike muscle troponin I by numerous criteria (see above), the presence of myosin in erythrocytes (22, 63) makes it conceivable that tropomodulin could be a functional analogue of muscle troponin I and interact with erythrocyte tropomyosin to regulate erythrocyte actomyosin ATPase activity. If so, we would expect that additional tropomyosin and/or tropomodulin binding com-

ponents will be required to confer calcium-sensitivity on tropomyosin regulation of actomyosin ATPase since tropomodulin-tropomyosin interactions are not calcium-sensitive (19). In this regard, it may be significant that erythrocytes have been recently reported to contain a  $M_r$  70,000 polypeptide that cross-reacts specifically with antibodies to non-muscle caldesmon (11).

I would like to thank Constance M. Smith for her technical assistance in working out the purification procedures for erythrocyte tropomodulin and for her help in preparing antibodies to tropomodulin. I am particularly grateful to George Klier (La Jolla Cancer Research Institute) for his patience and skill in preparing rotary shadowed replicas of tropomodulin and tropomyosin. I would also like to thank Dr. Marion Greaser for his generous gift of anti-troponin I antisera together with purified troponin I, Dr. Larry B. Smillie for affinity purified anti-troponin T antibodies, and Drs. Ira M. Herman and Fumio Matsumura for their gifts of anti-actin and anti-caldesmon antibodies, respectively.

Received for publication 23 March 1990 and in revised form 27 April 1990.

### **References**

1. Bennett, V. 1985. The membrane skeleton of human erythrocytes and its implications for more complex cells. *Annu. Rev. Biochem.* 54:273–304.
2. Bernstein, B. W., and J. R. Bamberg. 1982. Tropomyosin binding to F-actin protects the F-actin from disassembly by brain actin-depolymerizing factor (ADF). *Cell Motil.* 2:1–8.
3. Bretscher, A. 1986. Thin filament regulatory proteins of smooth- and non-muscle cells. *Nature (Lond.)* 321:726–727.
4. Broschat, K. O., and D. R. Burgess. 1986. Low  $M_r$  tropomyosin isoforms from chicken brain and intestinal epithelium have distinct actin-binding properties. *J. Biol. Chem.* 261:13350–13359.
5. Burgess, D. R., K. O. Broschat, and J. M. Hayden. 1987. Tropomyosin distinguishes between the two actin-binding sites of villin and affects actin-binding properties of other brush border proteins. *J. Cell Biol.* 104:29–40.
6. Burke, B., G. Griffiths, H. Reggio, D. Louvard, and G. Warren. 1982. A monoclonal antibody against a 135-K Golgi membrane protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:1621–1628.
7. Collins, J. H., and M. Elzinga. 1975. The primary structure of actin from rabbit-skeletal muscle. *J. Biol. Chem.* 250:5915–5920.
8. Côté, G. P. 1983. Structural and functional properties of the non-muscle tropomyosins. *Mol. Cell. Biochem.* 57:127–146.
9. Côté, G. P., and L. B. Smillie. 1981. The interaction of equine platelet tropomyosin with skeletal muscle actin. *J. Biol. Chem.* 256:7257–7261.
10. Dabrowska, R., Z. Podlubnaya, E. Nowak, and W. Drabikowski. 1976. Interaction of tropomyosin with troponin components. *J. Biochem. (Tokyo)* 80:89–99.
11. der Terrossian, E., C. Deprette, and R. Cassoly. 1989. Caldesmon is present in human and pig erythrocytes. *Biochem. Biophys. Res. Commun.* 159:395–401.
12. Dixon, M. 1953. The determination of enzyme inhibitor constants. *Biochem. J.* 55:170–171.
13. Eaton, B. L., D. R. Koming, and E. Eisenberg. 1975. Correlation between the inhibition of the acto-heavy meromyosin ATPase and the binding of tropomyosin to F-actin: effects of  $\text{Mg}^{++}$ , KCl, troponin I and troponin C. *Biochemistry* 14:2718–2725.
14. Eisenberg, E., and W. W. Kielley. 1974. Troponin-tropomyosin complex: Column chromatography separation and activity of the three active troponin components with and without tropomyosin present. *J. Biol. Chem.* 249:4742–4748.
15. Engvall, E., H. Hesse, and G. Klier. 1986. Molecular assembly, secretion, and matrix deposition of type VI collagen. *J. Cell Biol.* 102:703–710.
16. Fattoum, A., J. H. Hartwig, and T. P. Stossel. 1983. Isolation and some structural and functional properties of macrophage tropomyosin. *Biochemistry* 22:1187–1193.
17. Flicker, P. F., G. N. Phillips, and C. Cohen. 1982. Troponin and its interaction with tropomyosin. An electron microscope study. *J. Mol. Biol.* 162:495–501.
18. Fowler, V. M. 1986. New views of the red cell network. *Nature (Lond.)* 322:777–778.
19. 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.
20. Fowler, V. M., and V. Bennett. 1984. Erythrocyte membrane tropomyosin. Purification and properties. *J. Biol. Chem.* 259:5978–5989.
21. Fowler, V. M., and V. Bennett. 1984. Tropomyosin: a new component of the erythrocyte membrane skeleton. In *Erythrocyte Membranes: Recent Clinical and Experimental Advances*. Vol. 3. Alan R. Liss., New York.

- 57-71.
22. Fowler, V. M., J. P. Davis, and V. Bennett. 1985. Human erythrocyte myosin. Identification and purification. *J. Cell Biol.* 100:47-55.
  23. Fraser, R. D. B., and T. P. MacRae. 1973. *Conformation in Fibrous Proteins*. Academic Press, Inc., New York. 628 pp.
  24. Fujii, T., T. Ozawa, Y. Ogama, and Y. Kondo. 1988. Interaction between chicken gizzard caldesmon and tropomyosin. *J. Biochem. (Tokyo)*. 104:734-737.
  25. Gardner, K., and V. Bennett. 1986. A new erythrocyte membrane-associated protein with calmodulin binding activity. Identification and purification. *J. Biol. Chem.* 261:1339-1348.
  26. Geiger, B., A. H. Dutton, K. T. Tokuyasu, and S. G. Singer. 1981. Immunoelectron microscope studies of membrane microfilament interactions: distributions of  $\alpha$ -actinin, tropomyosin, and vinculin in intestinal epithelial brush border and chicken gizzard smooth muscle cells. *J. Cell Biol.* 91:614-628.
  27. Graceffa, P. 1987. Evidence for interaction between smooth muscle tropomyosin and caldesmon. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 218:139-142.
  28. Heald, R. W., and S. E. Hitchcock-Degregori. 1988. The structure of the amino terminus of tropomyosin is critical for binding to actin in the absence and presence of troponin. *J. Biol. Chem.* 263:5254-5259.
  29. Hill, A. V. 1910. The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. *J. Physiol. (Lond.)*. 40:4-8.
  30. 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.
  31. Koury, S. T., M. J. Koury, and M. C. Bondurant. 1989. Cytoskeletal distribution and function during the maturation and nucleation of mammalian erythroblasts. *J. Cell Biol.* 109:3005-3013.
  32. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
  33. Lazarides, E. 1975. Tropomyosin antibody: the specific localization of tropomyosin in non-muscle cells. *J. Cell Biol.* 65:544-561.
  34. Lazarides, E. 1976. Actin,  $\alpha$ -actinin, and tropomyosin interaction in the structural organization of actin filaments in nonmuscle cells. *J. Cell Biol.* 68:202-219.
  35. Lazarides, E., and K. Burridge. 1975.  $\alpha$ -Actinin: immunofluorescent localization of a muscle structural protein in non-muscle cells. *Cell*. 6:289-298.
  36. Leavis, P. C., and J. Gergely. 1984. Thin filament proteins and thin filament-linked regulation of vertebrate muscle contraction. *CRC Crit. Rev. Biochem.* 16:235-305.
  37. Liu, H., and A. Bretscher. 1989. Disruption of the single tropomyosin gene in yeast results in the disappearance of actin cables from the cytoskeleton. *Cell*. 57:233-242.
  38. Liu, Y., C. A. Carothers Carraway, and K. L. Carraway. 1986. Nonmuscle tropomyosin from ascites tumor cell microvilli. *J. Biol. Chem.* 261:4568-4573.
  39. Lowry, O. H., N. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
  - 39a. Lyon, M. K., and P. N. T. Unwin. 1988. Two-dimensional structure of the light harvesting chlorophyll a/b complex by cryoelectron microscopy. *J. Cell Biol.* 106:1515-1523.
  40. MacLeod, A. R. 1987. Genetic origin of diversity of human cytoskeletal tropomyosins. *Bioessays*. 6:208-212.
  41. Mak, A. S., G. Roseborough, 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.
  42. Marchesi, V. T. 1985. Stabilizing infrastructure of cell membranes. *Annu. Rev. Cell Biol.* 1:531-561.
  43. Marston, S. B., and C. W. J. Smith. 1985. The thin filaments of smooth muscles. *J. Muscle Res. Cell Motil.* 6:669-708.
  44. Matsumura, F., and S. Yamashiro-Matsumura. 1986. Modulation of actin-bundling activity of 55-kDa protein by multiple isoforms of tropomyosin. *J. Biol. Chem.* 261:4655-4659.
  45. Mooseker, M. S. 1985. Organization, chemistry, and assembly of the cytoskeletal apparatus of the intestinal brush border. *Annu. Rev. Cell Biol.* 1:209-241.
  46. 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.
  47. Nomura, M., K. Yoshikawa, T. Tanaka, K. Sobue, and K. Maruyama. 1987. The role of tropomyosin in the interactions of F-actin with caldesmon and actin-binding protein (or filamin). *Eur. J. Biochem.* 163:467-471.
  48. Pan, K.-M., D. L. Roelke, and M. L. Greaser. 1986. Isolation and characterization of a new 40-kilodalton protein from bovine cardiac muscle. *J. Biol. Chem.* 261:9922-9928.
  49. Payne, M. R., and S. E. Rudnick. 1985. Tropomyosin: Structural and functional diversity. In *Cell and Muscle Motility*. J. W. Shay, editor. Vol. 6. Plenum Publishing Corp., New York. 141-184.
  50. Pearlstone, J. R., and L. B. Smillie. 1983. Effects of troponin-I plus -C on the binding of troponin -T and its fragments to  $\alpha$ -tropomyosin. *J. Biol. Chem.* 258:2534-2542.
  51. Potter, J. D. 1982. Preparation of troponin and its subunits. *Methods Enzymol.* 85:241-263.
  52. Prulière, G., A. d'Albis, and E. der Terrossian. 1986. Effect of tropomyosin on the interactions of actin with actin-binding proteins isolated from pig platelets. *Eur. J. Biochem.* 159:535-547.
  53. Prulière, G., S. D. Fuller, A. G. Weeds, A. d'Albis, and E. der Terrossian. 1986. Pig platelet tropomyosin: interactions with the other thin-filament proteins. *J. Mol. Biol.* 192:815-830.
  54. Sanger, J. W., J. M. Sanger, and B. M. Jockusch. 1983. Differences in the stress fibers between fibroblasts and epithelial cells. *J. Cell Biol.* 96:961-969.
  55. Smith, C. W. J., K. Pritchard, and S. B. Marston. 1987. The mechanism of  $Ca^{2+}$  regulation of vascular smooth muscle thin filaments by caldesmon and calmodulin. *J. Biol. Chem.* 262:116-122.
  56. Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the TM-TN complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866-4871.
  57. Velaz, L., M. E. Hemric, C. E. Benson, and J. M. Chalovich. 1989. The binding of caldesmon to actin and its effect on the ATPase activity of soluble myosin subfragments in the presence and absence of tropomyosin. *J. Biol. Chem.* 264:9602-9610.
  58. Wegner, A. 1979. Equilibrium of the actin-tropomyosin interaction. *J. Mol. Biol.* 131:839-853.
  59. Wegner, A. 1982. Kinetic analysis of actin assembly suggests that tropomyosin inhibits spontaneous fragmentation of actin filaments. *J. Mol. Biol.* 161:217-227.
  60. Wilkinson, J. M., and R. J. A. Grand. 1978. Comparison of amino acid sequence of troponin I from different striated muscle. *Nature (Lond.)*. 271:31-35.
  61. Wong, A. J., D. P. Kiehart, and T. D. Pollard. 1985. Myosin from human erythrocytes. *J. Biol. Chem.* 260:46-49.
  62. Yamamoto, K., and K. Maruyama. 1973. Interaction of troponin I and tropomyosin. *J. Biochem. (Tokyo)*. 73:1111-1114.
  63. Yamashiro-Matsumura, S., and F. Matsumura. 1988. Characterization of 83-kilodalton non-muscle caldesmon from cultured rat cells. Stimulation of actin binding of non-muscle tropomyosin and periodic localization along microfilaments like tropomyosin. *J. Cell Biol.* 106:1973-1983.
  64. Yang, Y.-Z., E. D. Korn, and E. Eisenberg. 1979. Cooperative binding of tropomyosin to muscle and Acanthamoeba actin. *J. Biol. Chem.* 254:7137-7140.
  65. Zeece, M. G., R. M. Robson, and P. J. Bechtel. 1979. Interaction of  $\alpha$ -actinin, filamin and tropomyosin with F-actin. *Biochim. Biophys. Acta*. 581:365-370.
  66. 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.