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Periodic Binding of Troponin C·I and Troponin I to Tropomyosin-Actin Filaments

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We investigated the distribution of troponin C·I and troponin I along tropomyosin-actin filaments by immunoelectron microscopy and found that anti-troponin I antibody formed transverse striations at 38 nm intervals along the bundle of filaments of both troponin C·I–tropomyosin–actin and troponin I-tropomyosin-actin. Since the length of 38 nm corresponds to the repeating period of filamentous tropomyosin along actin double strands, the present study indicates that troponin I is located at a specific region of each tropomyosin, suggesting that a specific interaction between troponin I and tropomyosin is involved in determining the periodic distribution of troponin I along tropomyosin-actin filaments.

Key words: actin, calcium regulation, tropomyosin, troponin C, troponin I.

Troponin is a Ca^{2+}–receptive protein necessary for the Ca^{2+} regulation of the physiological contraction-relaxation cycle in vertebrate striated muscle, distributed along the thin filament at regular intervals of 38 nm (*1, 2*). In the thin filament, two head-to-tail filaments of fibrous tropomyosin molecules of about 40 nm in length wind along the grooves of actin double strands almost in register. Troponin binds to a specific region of each tropomyosin and hence forms the 38-nm periodicity.

This protein consists of three components: a Ca^{2+}-binding component (troponin C), an inhibitory component (troponin I) and a tropomyosin-binding component (troponin T) (*3*). All three components of troponin are required for the Ca^{2+} regulation of contraction. The essential processes of Ca^{2+} regulation are the inhibition of the contractile interaction between myosin and actin-tropomyosin by the inhibitory action of troponin I and the reversal of this inhibition by troponin I through the binding of Ca^{2+} to troponin C.

To explore the detailed mechanisms of the inhibitory activity of troponin I, we examined the binding location of troponin I along the tropomyosin-actin filament. An immunoelectron microscopic study showed that troponin I or troponin I combined with troponin C was distributed along the tropomyosin-actin filament with a 38 nm periodicity. This indicates that troponin I exerts its inhibitory activity through binding axially to a site at a specific region of each tropomyosin along the tropomyosin-actin filament.

EXPERIMENTAL PROCEDURES

Preparation of Proteins—Troponin and its three subunits were prepared from chicken breast muscle as described previously (*4, 5*). Tropomyosin was prepared from rabbit skeletal muscle (*6*) and porcine aorta and chicken gizzard (*7*). Actin was prepared from the acetone powder made from minced rabbit skeletal muscle after the extraction of native tropomyosin (*6*), and it was then purified by the procedure of Spudich and Watt (*8*).

Preparation of Anti-Troponin I Antibody—Rabbits were immunized with troponin I prepared from chicken breast muscle, and the gamma globulin fraction was separated from the anti-serum as described previously (*9, 10*).

Preparation of Troponin C·I-Tropomyosin-Actin Filaments—A reaction mixture (8 ml) composed of 60 mM KCl, 20 mM MOPS (pH 7.0), 2.0 mM $MgCl_2$, 1.0 mM EGTA, 0.1 μg/ml pepstatin A, 15 mM 2-mercaptoethanol, 0.25 mg of F-actin, 0.06 mg of tropomyosin, and 0.12 mg of troponin C·I was incubated for 30 min, then centrifuged at 100,000 ×*g* for 120 min at 25°C. The resulting pellet (troponin C·I-tropomyosin-actin filament) was suspended in a small amount of salt solution with the same composition as the reaction mixture.

Preparation of Troponin I-Tropomyosin-Actin Filaments—A reaction mixture (8 ml) composed of 30 mM KCl, 5 mM CDTA, 20 mM Tris, 0.1 μg/ml pepstatin A, 15 mM 2-mercaptoethanol, 1.0 mg of F-actin, 0.25 mg of tropomyosin, and 0.5 mg of troponin C·I (pH 7.2) was incubated for 30 min, then centrifuged at 100,000 ×*g* for 120 min at 25°C. The resulting sediment (troponin I-tropomyosin-actin) was suspended in a small amount of a solution containing 50 mM KCl and 1 mM $NaHCO_3$.

SDS-Gel Electrophoresis—Sodium dodecyl sulfate (SDS) gel electrophoresis was carried out according to the procedure of Laemmli at an acrylamide concentration of 12% (*11*).

Anti-troponin I Staining—Anti-troponin I staining was carried out on the preparations of troponin I·C-tropomyosin-actin and troponin I-tropomyosin-actin as described previously (*9, 10*). The suspension of troponin I·C-tropomyosin-actin or troponin I-tropomyosin-actin was mixed with anti-troponin I gamma globulin solution. This suspension was then repeatedly sucked into and pulled out of a Pas-

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Abbreviation: CDTA, trans-1,2-cyclohexanediamine-*N,N,N′,N′*-tetra-acetic acid.

teur pipette, then observed under an electron microscope.

Electron Microscopy—Specimens were stained negatively with 1% uranyl acetate and observed in a JEOL 100CX-II electron microscope with an accelerating voltage of 80 kV.

RESULTS

Binding of Troponin C·I to the Tropomyosin-Actin Filament—Troponin I is a basic peptide that precipitates in physiological conditions of low ionic strength (*5*). This insolubility of troponin I has made it rather difficult to examine its binding to other muscle structural proteins (*3, 5, 12*). To overcome this problem, we solubilized troponin I at low ionic strength by combining it with troponin C.

The binding of troponin C·I complex to tropomyosin-actin filaments was first examined by a cosedimentation assay. Figure 1 shows the SDS-gel electrophoretic pattern of pellets obtained after centrifugation of the mixture of troponin C·I and actin in the presence and absence of tropomyosin and of EGTA. In the absence of EGTA, no significant binding of troponin C·I to either actin or tropomyosin-actin was observed (Fig. 1, b and d). When the free Ca^{2+} concentration was decreased in the presence of 1 mM EGTA, however, a definite amount of troponin C·I bound to tropomyosin-actin, while troponin C·I bound only slightly to actin in the absence of tropomyosin (Fig. 1, a and c), indicating that the composite filament of troponin C·I-tropomyosin-actin was formed only in the presence of EGTA (in the absence of Ca^{2+}) (*13, 14*).

Figure 2 shows electron micrographs of the filament bundles of troponin C·I-tropomyosin-actin after treatment with anti–troponin I antibody. The anti-troponin I antibody formed transverse striations at regular intervals of approximately 38 nm along the bundle of troponin C·I-tropomyosin-actin filaments (Fig. 2b), whereas it did not stain the bundle of tropomyosin-actin filaments prepared from the mixture of troponin C·I-tropomyosin-actin in the absence of EGTA (Fig. 2a).

Binding of Troponin I to the Tropomyosin-Actin Filament—Troponin I is known to form a stable complex with troponin C (*13, 14*). The interaction between troponin C and troponin I has been shown to be weakened by removing both Ca^{2+} and Mg^{2+} from troponin C with strong divalent cation chelators such as EDTA or CDTA (*15, 16*). Under the present experimental conditions (5 mM CDTA in the presence of 30 mM KCl at pH 7.2), troponin I formed a complex with troponin C and hence did not significantly precipitate with actin after centrifugation (Fig. 3b). The affinity of troponin C·I complex to actin increased after

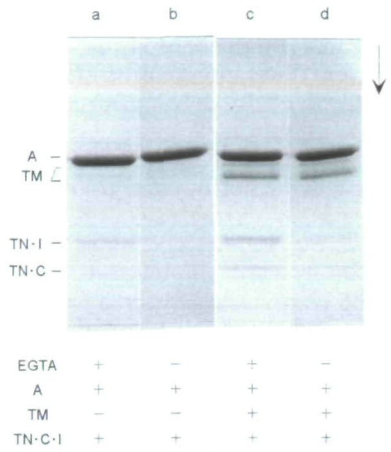

Fig. 1. **Binding of troponin C·I to actin and tropomyosin-actin.** (A) Actin in the absence of Ca^{2+} (1 mM EGTA). (B) Actin in the presence of Ca^{2+} (without EGTA). (C) Tropomyosin-actin in the absence of Ca^{2+}. (D) Tropomyosin-actin in the presence of Ca^{2+}. The compositions of the main constituents in the reaction mixture are illustrated in the lower portion of the figure. All other details are described under "EXPERIMENTAL PROCEDURES." Abbreviations: A, actin; TM, tropomyosin; TN·I, troponin I; TN·C, troponin C.

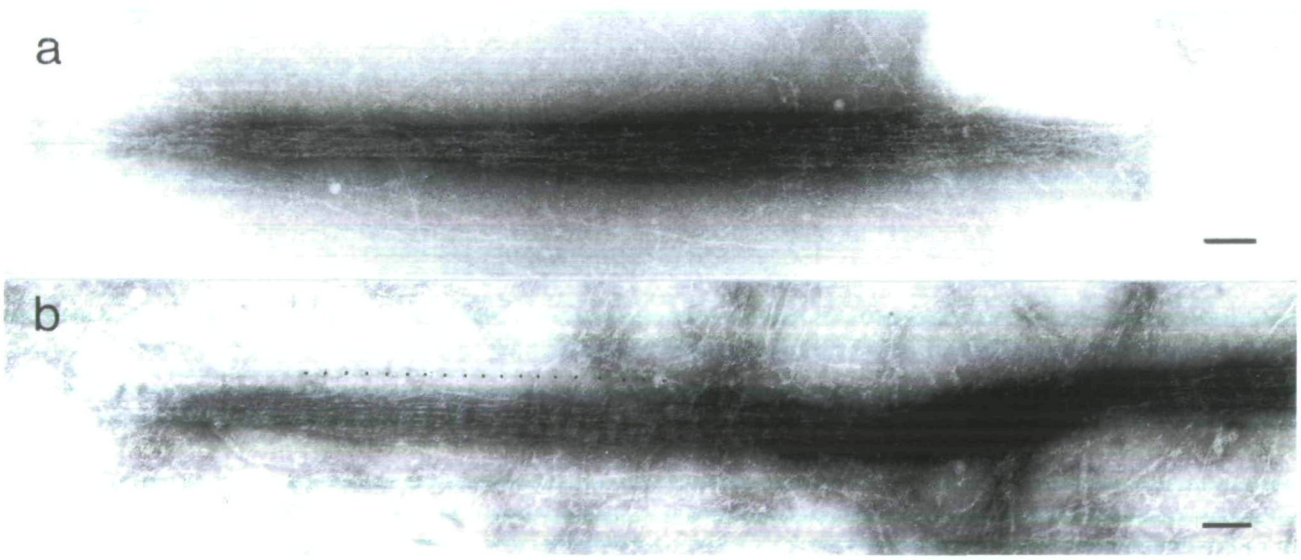

Fig. 2. **Anti-troponin I staining on troponin C·I-tropomyosin-actin filament bundles.** (A) Tropomyosin-actin filaments prepared from the mixture of troponin C·I-tropomyosin-actin in the presence of Ca^{2+}. (B) Troponin C·I-tropomyosin-actin filaments prepared in the absence of Ca^{2+}. The position of regular striations formed by anti-troponin I is indicated by dots (·). Magnification, ×65,000. Scale bars indicate 0.1 μm.

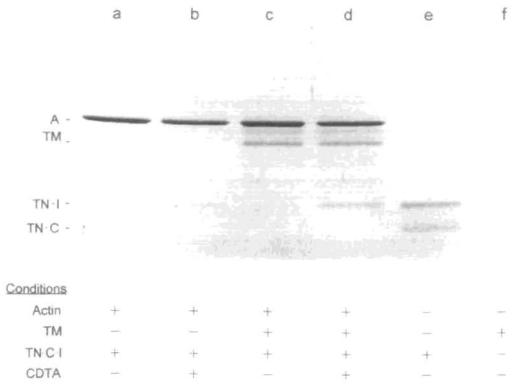

Fig. 3. **Troponin I binding to actin and tropomyosin-actin.** The compositions of the main constituents in the reaction mixture are illustrated in the lower portion of the figure. Other experimental details are described under "EXPERIMENTAL PROCEDURES." Abbreviations: A, actin; TM, tropomyosin; TN·I, troponin I; TN·C, troponin C.

addition of tropomyosin (Figs. 2c and 3d). However, troponin C was dissociated from troponin I when troponin C·I complex bound to tropomyosin-actin, because troponin I interacted with troponin C very weakly in the presence of CDTA (Fig. 3d). The composite filament of troponin I-tropomyosin-actin thus formed was precipitated by centrifugation.

We next examined by electron microscopy the localization of troponin I along the troponin I-tropomyosin-actin filament prepared as described above. After staining with anti-troponin I antibody, transverse striations at regular intervals of about 38 nm were formed along the filament bundles (Figs. 4 and 5). Regions thickened by the antibody were distributed along each filament at intervals of about 38 nm (Fig. 5; indicated by arrowheads). Periodic striations were also observed by anti-troponin I staining when tropomyosin from porcine aorta or chicken gizzard was used instead of rabbit skeletal muscle tropomyosin (data not shown). No regular striations were formed by the antibody to troponin I along the bundle of filaments prepared from a

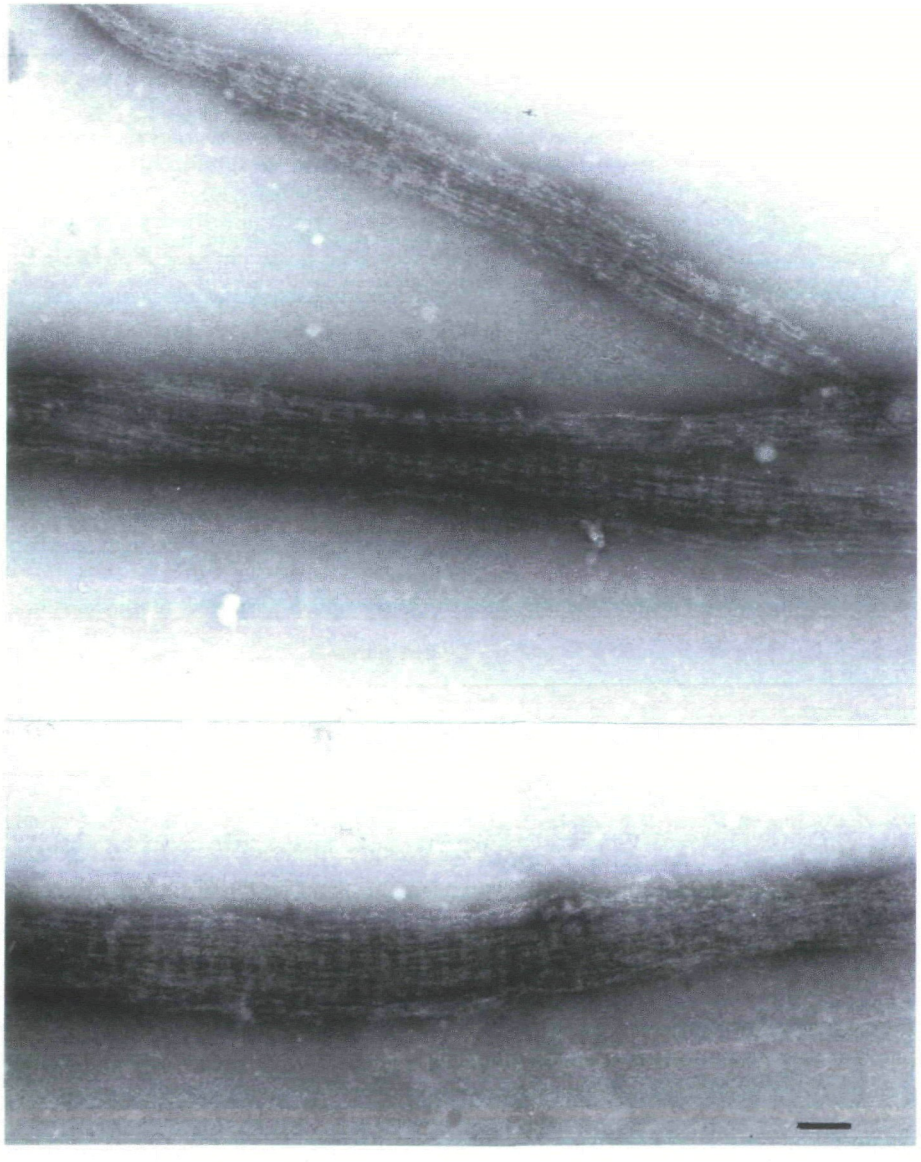

Fig. 4. **Anti-troponin I staining on the bundles of troponin I-tropomyosin-actin filaments.** Periodic transverse striations of the antibody with regular intervals of about 38 nm are observed along the bundles of troponin I-tropomyosin-actin filaments. Magnification, ×63,000. Scale bar indicates 0.1 μm.

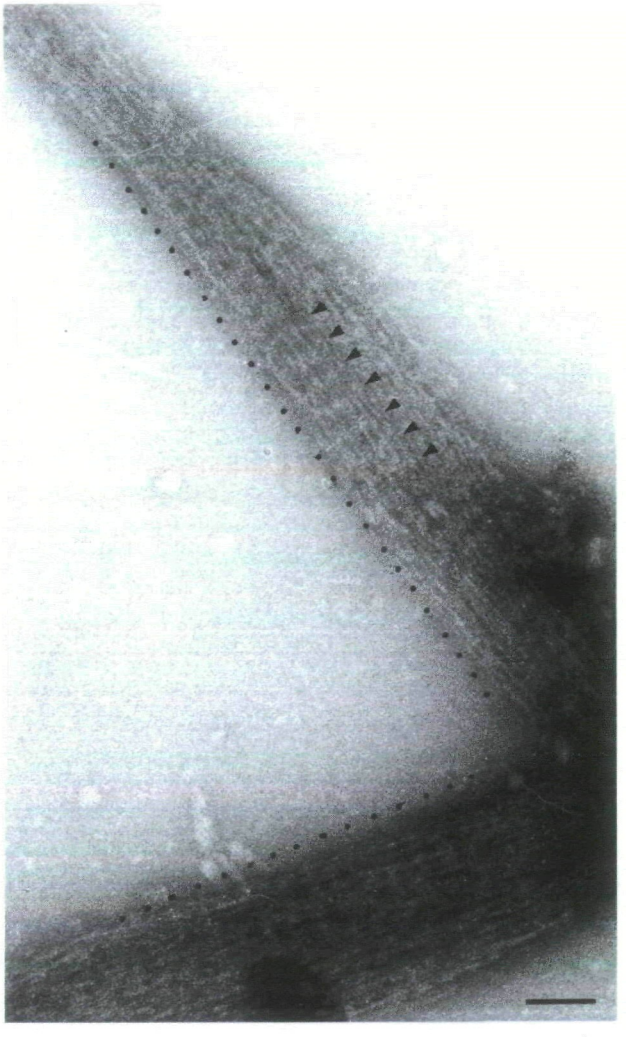

Fig. 5. **Anti-troponin I staining on troponin I-tropomyosin-actin filaments.** The position of regular striations formed by anti-troponin I is indicated by dots (·). The position of periodic thickening along single filaments by the antibody is indicated by arrowheads. Magnification, ×89,500. Scale bar indicates 0.1 μm.

mixture of troponin C·I and actin (data not shown).

DISCUSSION

The inhibitory activity of troponin I on actomyosin-tropomyosin is considered to represent the suppression of contractile interaction of myosin-actin by troponin-tropomyosin in the absence of Ca^{2+}. In the absence of tropomyosin, the inhibitory action of troponin I on actomyosin is known to be very weak, but most ATPase activity as well as superprecipitation of actomyosin is inhibited by troponin I in the presence of tropomyosin (*3, 12–14*). It has been shown that the binding of troponin I to actin filaments is enhanced by tropomyosin (*13, 17*) (Fig. 3). The present study demonstrated that the anti-troponin I antibody formed transverse striations with 38 nm periodicity along the bundle of reconstituted tropomyosin-actin filaments, indicating that troponin I is distributed at regular intervals of 38 nm along each filament (Figs. 4 and 5). Since the length of 38 nm corresponds to the repeating period of coiled-coil tropomyosin molecules running along the grooves of actin double strands (*3*), the result of the present study indicates that troponin I is located at a specific region of each tropomyosin along the tropomyosin-actin filament. The formation of transverse narrow antibody striations would reflect a structure in which two molecules of troponin I, and hence tropomyosin, in each 38 nm period along actin double strands are aligned almost in register. Two tropomyosin molecules in each period by themselves would bind cooperatively to the grooves of actin double strands.

Biochemical investigations have shown that troponin I binds to actin (*13, 17*). The interaction of troponin I with tropomyosin has been also indicated under different experimental conditions (*18–20*). This study presented structural evidence suggesting the interaction between troponin I and a specific region of tropomyosin in troponin I (troponin C·I)-tropomyosin-actin filaments. Zhou *et al.* demonstrated, using pyrene-labeled tropomyosin, that troponin I and troponin C·I interacted stoichiometrically with the region near Cys190 of tropomyosin with the troponin I (troponin C·I)-tropomyosin-actin complex (*21*). These findings strongly

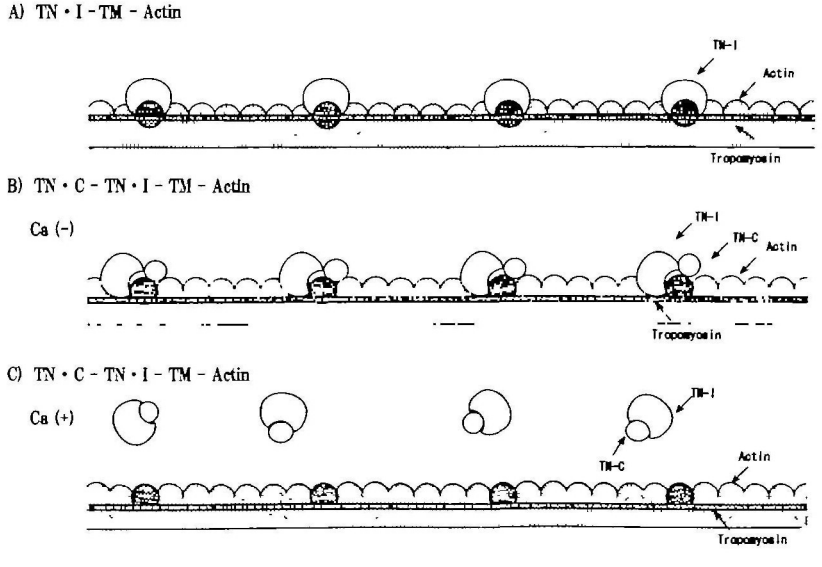

Fig. 6. **Schematic illustration of the periodic distribution of troponin I along the tropomyosin-actin filament.** (A) Distribution of troponin I along the tropomyosin-actin filament. (B) Distribution of troponin C·I along the tropomyosin-actin filament in the absence of Ca^{2+}. (C) Distribution of troponin C·I along the tropomyosin-actin filament in the presence of Ca^{2+}. For the sake of simplicity, only one of two strands of actin and tropomyosin is illustrated. Troponin I forms a complex with the specific actin molecule(s) (shaded in the figure) and/or the specific localized region of tropomyosin, thus showing the periodic distribution along the tropomyosin-actin filament with 38-nm intervals (A). A single molecule of actin in each period is assumed to bind to troponin I in the figure for simplicity, though we cannot exclude the possibility that troponin I binds to two or more actin molecules along either a single actin strand or two strands. Troponin C·I complex binds to tropomyosin-actin in the absence of Ca^{2+} (B), but troponin C·I is dissociated from tropomyosin-actin in the presence of Ca^{2+}, due to the enhanced affinity of troponin I to troponin C (C).

suggest that troponin I forms a complex with both actin and tropomyosin at a specific region of tropomyosin.

Although the inhibition of actomyosin-tropomyosin by troponin I is neutralized by troponin C regardless of Ca^{2+}-concentration (*14*), the binary complex of troponin C-troponin I has been shown to bind to tropomyosin-actin in the absence of Ca^{2+} but not in the presence of Ca^{2+} (*13*) (Fig. 1). In the present study, troponin C·I complex was also shown to be distributed at regular 38 nm intervals along the tropomyosin-actin filament in the absence of Ca^{2+} (Fig. 2b). The broad anti-troponin I striations occupied nearly a half of the period length, wider than the antibody striations along the troponin I-tropomyosin-actin filament (Fig. 2b). This might be related to the difference in the axial size between a sole troponin I molecule and troponin I combined with troponin C.

A previous immunoelectron microscopic study demonstrated that troponin C·I·T complex was distributed along the reconstituted troponin C·I·T-tropomyosin-actin filaments with 38 nm periodicity (*9*). Troponin C·I·T is considered to be distributed along the tropomyosin-actin filament through the binding of troponin T to tropomyosin. In the present study, however, troponin I alone or combined with troponin C, even in the absence of troponin T, was demonstrated to show a specific structural relation to tropomyosin along the axial length of the tropomyosin-actin filament. In the native thin filament, troponin I is located in a region about two-thirds of the molecular length of tropomyosin away from the amino-terminal end (*3*). The fluorescence-label study also showed that both troponin I and troponin C·I interacted with tropomyosin-actin in a region located near Cys 190 of tropomyosin with 284 residues, i.e., about two thirds of the length away from the N-terminus (*20*), suggesting that the axial relation of troponin I to tropomyosin along the reconstituted troponin I-tropomyosin-actin filament is essentially the same as that along the native thin filament. The molecular arrangement of troponin I, tropomyosin and actin discussed above is schematically illustrated in Fig. 6.

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