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# Restoration of Defective Mechanochemical Properties of Cleaved Actins by Native Tropomyosin: Involvement of the 40-50 Loop in Subdomain 2 of Actin in Interaction with Myosin and Tropomyosin

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**Native tropomyosin activated sliding movement *in vitro* of F-actin with ATP by 30 %. Actin cleaved at the 40-50 loop by subtilisin or proteinase K slid on HMM much slower than intact actin, but native tropomyosin strikingly recovered this defective motility of cleaved actin by 2 to 3 times. On the other hand, with ATP analogues of CTP and ITP, sliding movements of cleaved actin and particularly intact actin were inhibited by native tropomyosin, indicating that native tropomyosin augmented specificity of the myosin substrate of NTP. These results suggested that the 40-50 loop in the small domain 2 of actin interacted directly or indirectly with tropomyosin and play a significant role in cross talk between myosin and native tropomyosin.** 

Contraction of muscle is regulated by native tropomyosin through $Ca^{2+}$ binding to troponin C (1). In the relaxed state, myosin binding site of actin is sterically blocked by tropomyosin (2, 3). In addition to steric blocking by tropomyosin, the structural changes in the actin molecule have been reported to be associated with activation of muscle by X-ray diffraction studies (4-7). Thus, the entire structural basis on regulatory mechanism of muscle contraction is still controversial.

We reported previously that sliding velocity of the actin filament on myosin was specifically determined by the life time of the intermediate state at the rate limiting step of myosin ATPase cycle, by using cleaved actins and ATP analogues (8). In this work, from the functional view point of actin, we investigated the effect of modification of cleaved actin with native tropomyosin on its motility. Native tropomyosin greatly recovered the defective motility of cleaved actin and also augmented the substrate specificity of myosin, indicating that the significant role of the 40-50 loop in actin-myosin interaction and cross talk of myosin directly or indirectly with native tropomyosin.

## MATERIALS AND METHODS

*Muscle proteins.* Actin and myosin were prepared from rabbit skeletal muscle, and heavy meromyosin (HMM) was prepared by chymotryptic digestion of purified myosin (8). Subfragment 1 (S1) was prepared by digestion of purified myosin with $\alpha$-chymotrypsin according to Weeds and Taylor (9). Native tropomyosin was purified according to the method of Ebashi et al. (10). Tropomyosin was purified from native tropomyosin with an ion exchange column in the presence of 6 M urea (11). G-actin was cleaved by plasmin, subtilisin and proteinase K as previously described (8, 12).

*In vitro motility assay.* *In vitro* motility was assayed by using a flow chamber with a collodion coated coverslip where HMM was attached (13). The movement of F-actin labelled with rhodamin-phalloidin was observed with an epifluorescent microscope (Olympus BH2) at 23°C in M-buffer (1 mM ATP or an ATP analogue, 2 mM $MgCl_2$, 40 mM KCl and 50 mM Hepes at pH 7.6) containing 20 mM DTT and the oxygen scavenger enzymatic system. For motility assay of regulated actin, 0.2 mM EGTA or 0.1 mM $CaCl_2$ was added. In order to change the assay condition, the medium more than 5 volumes of the chamber was perfused. Motilities of various actins were assayed on the restricted area of the surface by washing out the prior actin species with M-buffer containing 0.15 M KCl, as described (8).

Regulated actin was freshly prepared prior to motility assay by adding native tropomyosin to F-actin in M-buffer at a weight ratio of more than 10 to 1, and incubated for about 1 hour on ice before use. As regulated actin filaments tended to form bundles and the bundles formed gradually larger aggregates with time in the absence of $Ca^{2+}$, usually the motility assay was performed within a few hours after incubation. Tropomyosin was mixed with F-actin in M-buffer at a weight ratio of **4** to 1, and incubated for about 1 hr on ice before use.

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Abbreviations: HMM, heavy meromyosin; S1, subfragment 1; DTT, ditiothreitol; NTP, nucleotide triphosphate; Pla-actin, actin cleaved by plasmin; Sub-actin, actin cleaved by subtilisin; Pro-actin, actin cleaved by proteinase K.



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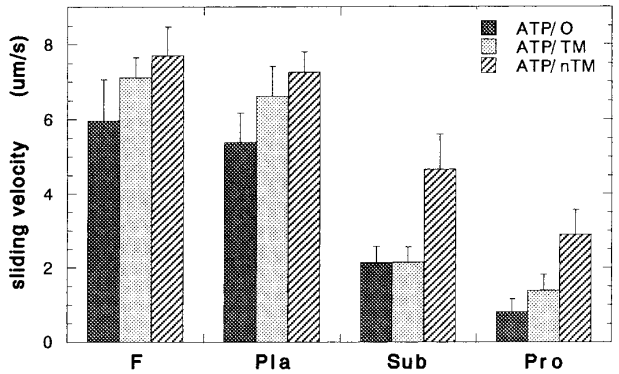


**FIG. 1.** Effects of tropomyosin and native tropomyosin on motility of intact and cleaved actins. Motility was activated by tropomyosin and much more by native tropomyosin. F, intact actin; Pla, plasmin cleaved actin; Sub, subtilisin cleaved actin; Pro, proteinase K cleaved actin. Black, gray and shaded columns show pure actin, actin with tropomyosin, and with native tropomyosin, respectively. Bar indicates s.d.

*NTPase assay.* NTPase activity of HMM or S1 and their actin activated NTPase activities were assayed in M-buffer containing 1 mM DTT by the method as described (8). In case of regulated actin, $Ca^{2+}$ was added at pCa = 5 in the assay medium. F-actins were used without modification by phalloidin for ATPase assay, except proteinase K-cleaved actin (Pro-actin) which could polymerize only in the presence of phalloidin (12).

## RESULTS

### *Effect of Native Tropomyosin on Motility of Cleaved Actins*

Tropomyosin activated sliding movement of intact actin by 20 % as previously reported (11). It also activated the movement of cleaved actins. Particularly, the sliding velocity of Pro-actin increased by 70 %, though that of subtilisin cleaved actin (Sub-actin) did not increase in the presence of tropomyosin (Fig. 1 gray columns).

When intact or cleaved actin was regulated by native tropomyosin, it moved only in the presence of $Ca^{2+}$. The sliding velocity of intact actin increased by 30 % when regulated, as shown in Fig. 1. Upon perfusion of M-buffer containing EGTA into the assay chamber, actin filaments stopped moving immediately and remained being attached stationary to HMM on the glass surface (Fig. 2 a to c). Immediately after perfusion of the medium containing $Ca^{2+}$, all stationary filaments began sliding (Fig. 2 c to h). And a few seconds later, the bundles attached on the surface, which had been formed in the assay solution without $Ca^{2+}$, started dispersing into single filaments through sliding movement (Fig. 2). During the repetitive change of the medium with and without $Ca^{2+}$, actin filaments on the surface repeated moving and cessation, respectively, without dissociation from the surface. Some filaments occasion-

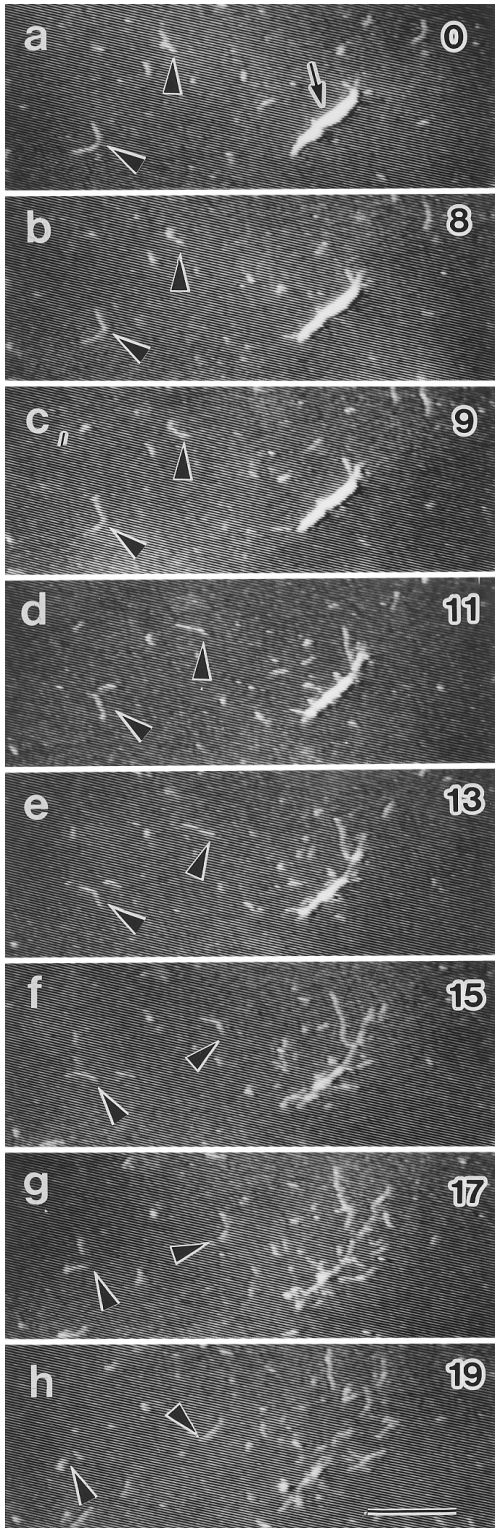


**FIG. 2.** Serial photorgaphs of movements of regulated actin filaments upon perfusion of $Ca^{2+}$ containing medium. Arrowheads point to a single filament moving, and an arrow points to the bundle composed of regulated actin filaments. All filaments attached were stationary from a to c in the $Ca^{2+}$ free medium, and started sliding at the time between c to d, when $Ca^{2+}$ containing medium was reached the observing field. Number at upper right corner shows the time in second. Bar indicates 20 $\mu$m.

ally dissociated at 40 mM KCl when $Ca^{2+}$ free medium was perfused, but they did not at 0 mM KCl.

Plasmin-cleaved actin (Pla-actin) behaved in a manner very similar to that of intact F-actin and native tropomyosin activated its movement by 30 % (Fig. 1). Sub-actin and Pro-actin slid much slower than intact actin. When they were regulated, strikingly their sliding velocities were increased in the presence of $Ca^{2+}$ by 2 and 3 times, respectively (Fig. 1 shaded columns). Regulated Sub-actin slid at 4.7 ± 0.9 $\mu$m/s, which indicated the recovery of motility near the level of intact actin. These striking increases of the sliding velocities of cleaved actins indicated the functional recovery of cleaved actins by native tropomyosin.

### *Sliding Movement of Regulated Actin with ATP Analogues*

As described in the previous section, regulated intact and cleaved actins slid faster than unregulated actins with ATP (Figs. 1 and 3A). On the other hand, with ATP analogues, sliding movements of intact actin were inhibited by native tropomyosin, in particular with ITP as shown in Figs. 3 B and C. Similarly, motility of Pla-actin was inhibited by native tropomyosin with ATP analogues. In contrast to inhibition of motility of intact actin with ATP analogues by native tropomyosin, Sub-actin and Pro-actins moved at very similar velocities irrespective of the presence or absence of native tropomyosin with CTP or ITP.

As intact and cleaved actins slid very slowly with ITP, to confirm strong inhibition of motility of regulated intact actin with ITP, we assayed motility with particular care by selecting a restricted area where the surface condition was the best (8). Relative values of the sliding velocities of intact actin with ATP, CTP and ITP were 1.00, 0.33 and 0.06 when unregulated, respectively, and 1.00, 0.17 and 0.003 when regulated, respectively. Inhibitory effect of native tropomyosin with ATP analogues on sliding movements of intact F-actin showed that native tropomyosin gave the ability for actomyosin to sense the true substrate more specifically and cleavage of the loop of actin weakened this sensitivity to the substrate analogue bound with myosin.

### *HMM ATPase Activation by Regulated Actin*

Since *in vitro* motilities of cleaved actins were recovered greatly by adding native tropomyosin with ATP as described above, the acto-HMM ATPase activities were investigated. HMM ATPase was highly activated by unregulated intact actin, but by Sub- or Pro-actin it was activated much less. Their activities decreased almost in parallel with their sliding velocities. The ratios of the activities of F (intact actin) : Sub-actin : Pro-actin in the sliding velocity and in the actin activated HMM ATPase activity were 100 : 37 : 11 and 100 : 24 : 11, respectively, when actins were unregulated. When these actins were regulated, they activated HMM ATPase higher than respective unregulated actins did. Thus, increment of the cleaved-actin activated HMM ATPase activities by native tropomyosin was in parallel with increase of sliding velocities of regulated cleaved actins (Fig. 3 A). When respective actin species were regulated, the ratios of the activities of F : Sub-actin : Pro-actin in the sliding velocity and in HMM ATPase activation were 100 : 61 : 34 and 100 : 47 : 49, respectively. In case of Pro-actin, for instance, sliding velocity and the actin activated HMM ATPase activity increased by 3.1 and 4.5 times, respectively, when regulated.

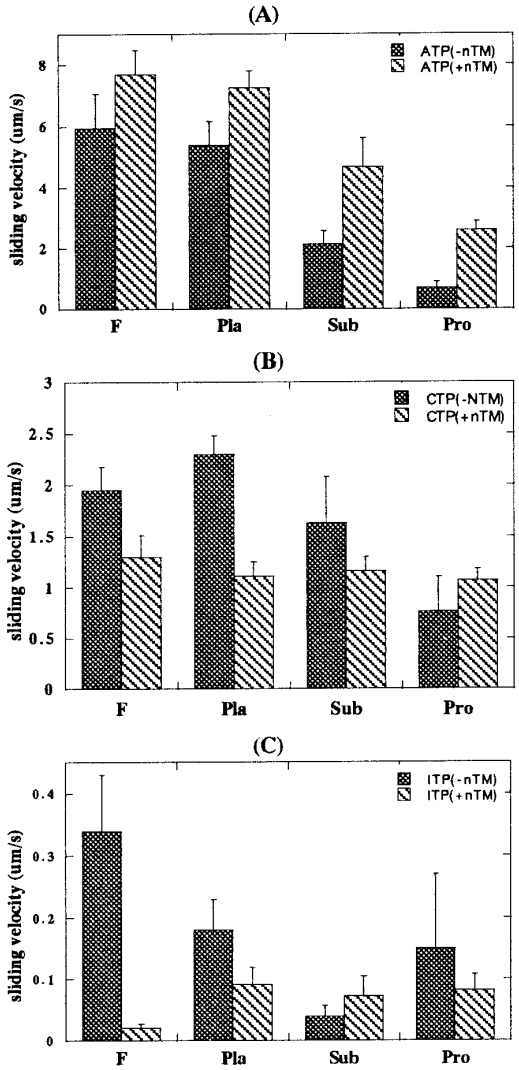


**FIG. 3.** Effect of native tropomyosin on sliding movement of cleaved actins with ATP analogues. (A) with ATP, all intact and cleaved actins were activated in their motility when regulated. With ATP analogues, (B) CTP and (C) ITP, native tropomyosin affected negatively on actin movement. Gray column, without native tropomyosin; shaded column, with native tropomyosin. Bar indicats s.d.

*NTPase Activated by Regulated Actin*

As we reported previously, the most important factor of the determinant of the sliding velocity was associated with NTPase of myosin itself, but not with actomyosin NTPase (8). Actually, the absolute value of actin activated S1 NTPase activity did not correlate to the sliding velocity with NTP (Figs. 4 A and B). In contrast, the reciprocal of S1 NTPase activity was in proportional to the sliding velocity of intact and cleaved actins with NTP (Fig. 4C black columns). The ratio of acto-S1 NTPase activity to that of S1 alone also correlated roughly with the sliding velocity with NTP in both cases of regulated and unregulated actins (Fig. 4 C), though the relative NTPase activities with CTP and ITP were still high, if compared with the NTP profile of sliding velocities. Thus, motility change of intact or cleaved actin upon modification by native tropomyosin was supported by the increase of actomyosin NTPase activity upon addition of native tropomyosin.

## DISCUSSION

Native tropomyosin in the presence of $Ca^{2+}$ activated the sliding movement of F-actin more than tropomyosin did. This suggested the possibility that native tropomyosin play a role not only in switching the contraction by displacing the position of tropomyosin bound to F-actin, but also modifying actomyosin interaction more efficiently.

Cleavage of actin at the site of the 40-50 loop by subtilisin or proteinase K markedly reduced its mechanochemical activities. Despite this damage, native tropomyosin strikingly restored its motility and HMM ATPase activation. Native tropomyosin increased the sliding velocity of Sub-actin and Pro-actin by 2 and 3 times, respectively and HMM ATPase activation by 3 and 4 times, respectively, indicating that the 40-50 loop interacted directly or indirectly with tropomyosin and was also directly or indirectly involved in interaction among actin, native tropomyosin and myosin.

Pla-actin truncated 2 residues from the C-terminus, showed behavior very similar to intact actin. F-actin consisting of actin truncated 2 residues from the C-terminus by tryptic digestion had the structure different from the intact F-actin, but phalloidin stabilized the intact conformation of truncated actin (14, 15). Modification of F-actin with rhodamine-phalloidin for motility assay might be the reason of the absence of significant difference in motility between Pla-actin and intact actin.

Conformational change in subdomain 2 during activation of muscle was observed by the recent extensive X-ray diffraction studies (4-7). The conformational change of subdomain 2 upon binding of myosin was observed by fluorescent energy transfer (16), and suggested by cooperative binding of HMM to F-actin (17).

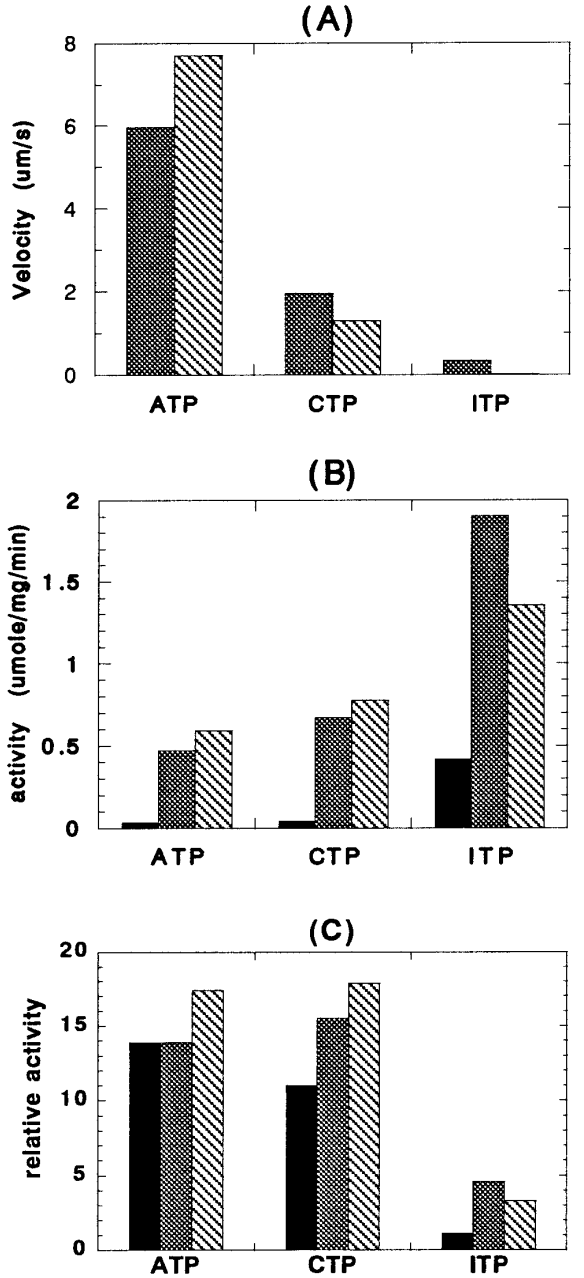


**FIG. 4.** S1 NTPase activated by regulated actin. (A) Sliding velocity, (B) corresponding S1 NTPase activated by actin and (C) actin activated S1 NTPase activity relative to the respective S1 NTPase activity, and the reciprocal of S1 NTPase. The concentrations of actin, S1 and native tropomyosin used for assay were 0.04, 0.2 and 0.05 mg/ml, respectively. Black column in (B) shows NTPase activity of S1 alone and that in (C) shows 1/NTPase activity of S1. Gray column, unregulated actin; shaded column, regulated actin.

These findings were consistent with the results obtained in this work. Restoration of motility of cleaved actins by native tropomyosin strongly suggested that $Ca^{2+}$ activation of thin filament and/or binding of myosin to F-actin involved the conformational change of the 40-50 loop associated with the global change in

subdomain 2. Actually, the small positional change of the 40-50 loop occurred in contracting muscle (7).

The augment of substrate specificity of myosin by native tropomyosin was observed particularly with ITP in motility of intact actin (Fig. 3 C). This observation on the motility change with the ATP analogue by native tropomyosin, is a new fact and suggests that myosin and tropomyosin intercommunicate with each other through actin. The inhibitory effect of native tropomyosin in intact actin with CTP or ITP was stronger than those in cleaved actins (Fig. 3). Subtle change of conformation in myosin which bound the substrate analogue, such as CTP or ITP, would induce subtle misfitting between myosin and actin, possibly including the loop. Residues $His^{40}$ to $Gly^{43}$ in the loop of actin subunit below in an F-actin were directly in contact with residues $Asn^{552}$ to $His^{556}$ of the myosin head (18). Therefore, this direct contact of the loop of actin with the myosin head could be one of the pathways of cross talk between myosin and native tropomyosin. Communication between myosin and tropomyosin could also occur directly because tropomyosin displaced further upon binding with myosin (19).

Taken together, our results indicate that the 40-50 loop plays a significant role in interaction of actin with myosin, and also native tropomyosin can modulate dynamic actin-myosin interaction through interacting with the loop directly or indirectly, in addition to ON/OFF switching of the thin filament.

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