Role of Actin C-Terminus in Regulation of Striated Muscle Thin Filament

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ABSTRACT In striated muscle, regulation of actin-myosin interactions depends on a series of conformational changes within the thin filament that result in a shifting of the tropomyosin-troponin complex between distinct locations on actin. The major factors activating the filament are Ca^{2+} and strongly bound myosin heads. Many lines of evidence also point to an active role of actin in the regulation. Involvement of the actin C-terminus in binding of tropomyosin-troponin in different activation states and the regulation of actin-myosin interactions were examined using actin modified by proteolytic removal of three C-terminal amino acids. Actin C-terminal modification has no effect on the binding of tropomyosin or tropomyosin-troponin + Ca^{2+}, but it reduces tropomyosin-troponin affinity in the absence of Ca^{2+}. In contrast, myosin S1 induces binding of tropomyosin to truncated actin more readily than to native actin. The rate of actin-activated myosin S1 ATPase activity is reduced by actin truncation both in the absence and presence of tropomyosin. The Ca^{2+}-dependent regulation of the ATPase activity is preserved. Without Ca^{2+} the ATPase activity is fully inhibited, but in the presence of Ca^{2+} the activation does not reach the level observed for native actin. The results suggest that through long-range allosteric interactions the actin C-terminus participates in the thin filament regulation.

INTRODUCTION

Muscle contraction is driven by interactions of myosin cross-bridges with the actin thin filament. Myosin binding to actin is regulated by a complex of the thin-filament-associated proteins tropomyosin (TM) and troponin (Tn). TM is a coiled-coil dimer that binds along seven actin subunits. Neighboring TM molecules form long strands on both sides of the filament via end-to-end interactions. Each TM binds one Tn, a complex of three subunits that confers calcium sensitivity to the actomyosin regulation. At low Ca^{2+} concentrations, the myosin-actin interactions are strongly inhibited. Binding of Ca^{2+} to Tn triggers a series of events leading to conformational rearrangements within the thin filament followed by cross-bridge activation (1,2). Based on kinetic measurements of myosin binding, McKillop and Geeves (3) proposed that the thin filament is in equilibrium among three activation states referred to as B-state (blocked), C-state (closed or calcium-induced), and M-state (myosin-induced or open) (for a recent review see Boussouf and Geeves (4)). The thin filament activation states correspond to structural states observed by electron microscopy and three-dimensional reconstructions of the thin filament. As calcium binds to Tn, TM is azimuthally shifted from a position on the filament outer domain predominantly occupied in the blocked state toward an actin inner domain. This uncovers weak myosin-binding sites on actin. Binding of myosin heads induces an additional TM shift, which uncovers all myosin-binding sites on actin and allows full cross-bridge activity (5–8).

Repositioning of TM strands on actin explains the actomyosin regulation mechanism in terms of steric blocking hypothesis. However, the thin filament is also regarded as an allosteric/cooperative system (4,9,10, and references therein). Defining the interfaces among actin, TM, Tn, and myosin in different regulatory states as well as mapping actin conformational changes that accompany the events important for the actomyosin regulation are crucial to our understanding of the regulation mechanisms. Although many direct and indirect interactions among all the components of the regulatory system have been characterized (11–16), more data are required for full description of the thin filament regulation.

In this work, we examined the role of actin C-terminus in the interactions with TM and Tn and in the regulation of actin-myosin interactions. Selection of actin C-terminus for examination of actin involvement in the regulatory processes was dictated by the fact that the position of this region seems to be central for protein-protein interactions and transmission of conformational changes within the filament.

The actin C-terminus is located in subdomain 1 in the outer domain of the filament, and it is involved in extensive intermolecular interactions. It was proposed to form intermonomer interfaces among three subunits together with DNase I-binding loop and a hydrophobic loop Phe^{262}-Ile^{274} (17,18). There is some evidence for contacts between C-terminal residues 368–375 and helix 223–230 in subdomain 3 of the neighboring subunit across the filament axis (19). The C-terminus is also coupled intramolecularly with DNase I-binding loop and the nucleotide-binding site located in a cleft between actin domains (20,21). Interactions between

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actin subdomain 1 and TM in relaxing conditions were suggested by electron microscopy reconstructions of the regulated thin filaments (22,23). In activating conditions TM strands are shifted away from this actin region, but a possibility of allosteric coupling between TM and an actin C-terminal region was suggested by the Lorenz et al. (24) atomic model of the actin-TM complex. Involvement of the actin C-terminus in TM binding was observed with actin devoid of two C-terminal amino acids (25); however, the study on actin with three C-terminal residues removed did not confirm this interaction (26). Another important contact is formed between the actin C-terminal segment and Tn. The thin filament three-dimensional reconstructions in relaxing, low Ca^{2+} concentration revealed a bridge of density extending from the Tn core domain to actin subdomain 1 (23). Extensive electrostatic interactions between the mobile domain, ascribed to the C-terminal domain of TnI, and the actin C-terminus were also observed by Murakami et al. (27). Finally, the actin C-terminal region is modeled to be part of the actin-myosin interface in a strong myosin binding state (19,28).

Our results, obtained with actin modified by proteolytic removal of three amino acids from C-terminus ($actin_{-3}$), demonstrate involvement of the C-terminal segment in the binding of TM in blocked and open states and in activation of myosin S1 ATPase. The results are discussed in terms of direct or long-range interactions between actin and the regulatory proteins.

MATERIALS AND METHODS

Protein preparations

Actin was isolated from chicken pectoral muscle acetone powder according to standard methods (29).

Three C-terminal amino acids (Lys^{373}, Cys^{374}, and Phe^{375}) were removed using trypsinolysis of G-actin in which strongly bound Ca^{2+} was exchanged for Mg^{2+} before proteolysis, as described earlier (30).

TM was obtained by use of recombinant DNA methods. The cDNA encoding rat skeletal α-TM cloned into pET11d plasmid (Novagene, Houston, TX) was used for *E. coli* expression strain BL21(DE3) cell transformation. The protein was expressed and purified as described (31). The bacterially expressed TM is unacetylated at the N-terminus (32). The cDNA clone was a gift of Dr. Sarah Hitchcock-DeGregori.

Myosin S1 was prepared by papain digestion of chicken pectoral muscle myosin according to the standard method (33). Tn was isolated from chicken pectoral muscle with the method described by Potter (34) with modifications as by Moraczewska et al. (31).

Actin concentration was determined spectrophotometrically using an extinction coefficient at 290 nm for 0.1% actin of 0.63 and molecular weight 42,000. For myosin S1 and Tn concentrations, extinction coefficients of 0.83 and 0.45 at 280 nm (0.1%) and proteins with molecular weight 130,000 and 76,000 were used. The concentration of recombinant TM was determined by differential absorption spectra of tyrosine (31). The concentration of C-terminally truncated actin was determined by densitometry of actin bands separated on SDS gels. Native actin was used as a standard curve. The concentration determination from the extinction coefficient, conventionally used for intact actin, was not suitable for truncated actin because this produced values that were 6 ± 1.6% higher than concentrations determined densitometrically.

TM binding assay

Binding assays were performed by TM cosedimentation with F-actin essentially as described earlier (31), with the following modifications. The tightly bound Ca^{2+} in actin was replaced with Mg^{2+} before polymerization by 10 min of incubation of G-actin at room temperature with 0.2 mM EGTA and 0.1 mM $MgCl_2$. The resulting G-Mg-actin was polymerized for 0.5 h at room temperature. Depending on the ionic strength and the presence of Tn and Ca^{2+}, TM binds to actin with a wide range of affinities. To make a comparison of TM binding to native and truncated actins in different activation states possible, salt concentrations were selected separately for each state, so that the binding constants were within the resolution of this assay. Binding of TM alone was assayed in 5 mM imidazole, pH 7.0, 1 mM DTT, 2 mM $MgCl_2$, and 100 mM NaCl. In the presence of Tn and Ca^{2+}, $CaCl_2$ was added to 0.2 mM, and NaCl concentration was increased to 150 mM to prevent unbound TM-Tn polymerization, which would interfere with the assay (35). TM binding with Tn in the absence of Ca^{2+} was conducted in the buffer solution as above, supplemented with 0.2 mM EGTA and NaCl concentration increased to 300 mM. Protein concentrations were as follows: F-Mg-actin 5 μM, α-TM increasing from 0 to up to 20 μM, and Tn, when present, a 1.2 molar excess over TM concentration. After mixing of all assay components, samples were incubated at room temperature for ~0.5 h to ensure attainment of the steady-state. Protein mixtures were ultracentrifuged for 1 h at 40,000 rpm in Beckman rotor 42.2. The composition of the proteins in pellets and the amount of free TM left in the supernatant were examined on 10% SDS-PAGE. The gels stained with Coomassie Blue were scanned with Plustek OpticPro ST12 and quantified using EasyDens (Cortex Nova, Bydgoszcz, Poland) software. Intensities of SDS-gel bands of pelleted native and truncated actins were similar, indicating that differences in critical concentrations reported before (30) were not detected under conditions of our experiment. TM bound to actin was calculated as TM/actin band intensity ratio normalized to the maximum ratio reached at saturation. The concentration of unbound TM was calculated from band intensities of TM left in the supernatant. The apparent association constant (K_{app}) and cooperativity coefficient (α^H) were calculated by fitting the collected experimental data to the Hill equation (Eq. 1) in SigmaPlot version 9.0 (Systat Software, San Jose, CA):

$$v = n[\mathrm{TM}]^{\alpha H} K_{app} / (1 + [\mathrm{TM}]^{\alpha H} K_{app}^{\alpha H}), \quad (1)$$

where v = fraction maximal TM binding to actin, n = maximal TM bound, [TM] = free [TM], and α^H = cooperativity coefficient.

Independent-samples t-test was used for comparing the K_{app} mean values obtained for native and truncated actin. The test was performed in SigmaPlot. The differences were considered as significant at $p < 0.05$.

Myosin S1-induced binding of TM to F-actin

Myosin S1-induced TM binding to actin was analyzed by the cosedimentation method as described (31), except 5 μM F-Mg-actin and 1 μM TM were used. Initial TM binding (without S1) was lowered by selection of the following conditions: 5 mM imidazole, pH 7.0, 30 mM NaCl, 0.5 mM $MgCl_2$, 0.5 mM DTT. Actin saturation with TM and S1 was calculated from densitometric analysis of pellets separated on 12% SDS gels. Saturation curves were plotted as TM/actin and S1/actin ratios, normalized to the values reached at maximal binding versus the S1/actin molar ratio. The number of S1 per actin required for half-maximal saturation of actin filament with TM was calculated by fitting the modified Hill equation (Eq. 2) to the experimental data:

$$v = (n[\mathrm{X}]^{\alpha H} K_{app}^{\alpha H} / 1 + [\mathrm{X}]^{\alpha H} K_{app}^{\alpha H}) + \mathrm{C}, \quad (2)$$

where v = TM:actin density ratio, [X] = S1/actin molar ratio, and C = TM/actin density ratio at 0 S1 concentration. S1/actin molar ratio required for half maximal saturation of actin with TM was calculated as 1/K. The significance of the observed differences in $1/K$ was evaluated as described above.

Actomyosin MgATPase

The dependence of myosin S1 ATPase activity on F-actin concentration was measured in 5 mM imidazole, pH 7.0, 30 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT at 0.38 μM S1 with actin concentrations varying between 5 and 20 μM. TM, when present, was added to actin stock solution at 2 molar excess to ensure actin filament saturation.

Ca^{2+}-dependent regulation was measured in the buffer given above supplemented with either 0.2 mM $CaCl_2$ or 1 mM EGTA. Actin, S1, and Tn concentrations were kept constant at 5 μM, 1 μM, and 1 μM, respectively. The TM concentration was increased from 0 to 1.0 μM.

The reaction was initiated by adding MgATP to 5 mM and stopped after 20 min with 3.3% SDS and 30 mM EDTA. The amount of liberated phosphate was determined colorimetrically (36).

RESULTS

The effect of actin C-terminal truncation on binding of skeletal α-TM in the blocked and closed state

To gain insight into the contribution of actin C-terminal region in stabilization of the actin-TM interactions in different activation states, we compared the binding of α-TM to native F-actin and F-actin$_{-3}$. Because of the high TM affinity for actin in the blocked state (in the presence of Tn without Ca^{2+}), we used recombinant TM, which, because of the lack of N-terminal acetylation, has lower affinity to actin than its muscle counterpart (37). This allowed us to measure TM binding to both actins in a direct cosedimentation assay. Additionally, salt concentrations were adjusted as described in Materials and Methods.

Actin filaments with TM-Tn complex bound at low Ca^{2+} concentration are mostly in blocked state. The binding curves shown in Fig. 1 *A* demonstrate that the removal of three amino acids reduced α-TM affinity for actin ~1.3-fold. The binding constant (K_{app}) decreased from $7.69 \pm 0.38 \times 10^6$ M^{-1}, observed for native F-actin, to $5.85 \pm 0.14 \times 10^6$ M^{-1} for F-actin$_{-3}$ (Table 1). This is a statistically significant change.

Actin filament is thought to be predominantly in the closed state when associated with TM alone or with α-TM-Tn+Ca^{2+} (3,9). Therefore, both types of filaments were examined to evaluate the involvement of actin C-terminal region in forming the closed state. The affinity of α-TM to actin alone was low, as expected for recombinant unacetylated α-TM. The C-terminal truncation of actin did not affect the binding (Fig. 1 *B*). In the presence of Tn and Ca^{2+}, the affinities of α-TM to F-actin and F-actin$_{-3}$ were higher than in the absence of Tn. Again, no difference in TM binding between native and truncated actin was detected. The binding constants are summarized in Table 1.

As judged from the slope of the binding curves, in the blocked state, F-actin$_{-3}$ tended to bind α-TM with higher cooperativity than unmodified actin (Table 1). The Hill coefficient (α^H) obtained from binding curves of α-TM complexed with Tn in the absence of Ca^{2+} was ~1.7-fold higher for F-actin$_{-3}$ than for native F-actin. In the closed state, the cooperativity with which α-TM alone and with Tn+Ca^{2+} bind to truncated actin also appears to be higher, but, because of the error, this difference is not significant.

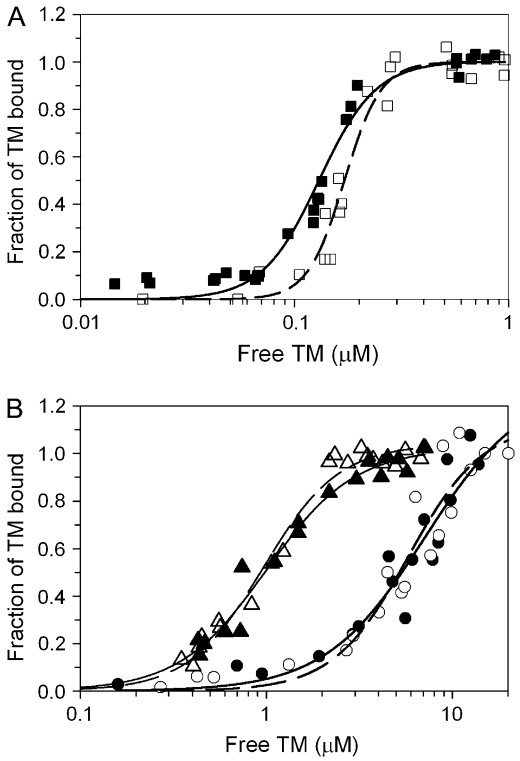

FIGURE 1 Binding of α-TM to native and C-terminally truncated actin alone and in the presence of Tn. Binding of α-TM to native F-actin (*solid symbols*, *solid line*) and F-actin$_{-3}$ (*open symbols*, *dashed line*) were assayed as described in Materials and Methods. (*A*) α-TM binding in the presence of Tn and EGTA. Conditions: 5 mM imidazole, pH 7.0, 1 mM DTT, 2 mM $MgCl_2$, 0.2 mM EGTA, 300 mM NaCl. (*B*) α-TM binding in the absence of Tn (*circles*) and in the presence of Tn and Ca^{2+} (*triangles*). Conditions: 5 mM imidazole, pH 7.0, 1 mM DTT, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 100 mM NaCl (in the absence of Tn) or 150 mM NaCl (with Tn). Symbols represent collected experimental points from three experiments. Binding curves were obtained by fitting the experimental data to the Hill equation (Eq.1).

These results show that distortion of the thin filament structure caused by removing the three C-terminal residues destabilizes the blocked state but has no effect on the filament in the closed state.

Binding of α-TM to C-terminally truncated actin in the open state

Myosin heads strongly bound to actin are required to shift the filament toward a fully active open state (3), but the presence of myosin S1 increases TM affinity for actin (38). Strong TM binding to actin saturated with myosin heads makes it difficult to analyze the effect of actin C-terminal truncation on TM affinity in this state. To overcome this difficulty, TM binding to the open state was analyzed using the S1-induced TM binding assay in which actin saturation with

TABLE 1 Binding of rat skeletal α-TM to native F-actin and F-actin$_{-3}$

	Native F-actin		F-actin$_{-3}$	
	$K_{app} \times 10^6$ M^{-1}	α^H	$K_{app} \times 10^6$ M^{-1}	α^H
α-TM/Tn + EGTA*	7.69 ± 0.38	2.87 ± 0.33	5.85 ± 0.14	5.07 ± 0.64
α-TM/Tn + Ca^{2+}	0.99 ± 0.07	1.84 ± 0.21	1.04 ± 0.06	2.15 ± 0.22
α-TM	0.14 ± 0.04	1.62 ± 0.44	0.17 ± 0.03	2.15 ± 0.59

Data obtained from the parameters of the Hill equation (Eq. 1) fit to points collected from three independent experiments shown in Fig. 1; errors are mean ± SE. *Significance of the difference in K_{app} ($p = 0.005$) and α^H ($p = 0.012$). Conditions as in the legend to Fig. 1.

TM under conditions unfavorable for TM binding alone was followed as a function of increasing S1 concentration (31,38).

As illustrated in Fig. 2, myosin S1 induced α-TM binding to both types of actin, but the numbers of myosin heads required for saturation of truncated and native actins with TM were different. For F-actin$_{-3}$ half-maximal saturation with TM (calculated from Eq. 2) was obtained at 0.21 ± 0.01 S1/actin molar ratio. This number was lower than that for native F-actin, which half-saturated with TM at 0.32 ± 0.02 S1/actin. This means that binding of 1.5 myosin heads per segment of seven actin subunits was required to induce binding of one TM molecule to truncated actin, whereas binding of one TM to native actin required 2.2 myosin heads per seven actins. Actin C-terminal modification did not alter rigor binding of myosin S1. As shown in Fig. 2 (*inset*), S1 saturated modified and nonmodified actin at a 1:1 molar ratio. Therefore, saturation of the F-actin$_{-3}$ with TM at lower occupancy of S1 suggests that truncation of actin induces structural changes in the filament that increase actin cooperativity with S1.

Taken together, the above results show that actin truncation affects TM binding to actin in each of the three activation states differently. Because switching the thin filament among the activation states is a key element of actomyosin regulation, the effect of the actin modification on the dynamics of the cross-bridge cycle in the absence and presence of the regulatory proteins was analyzed.

Regulation of actomyosin S1 activity by TM and Tn

The actomyosin ATPase is a measure of the interactions between actin filament and myosin heads. To examine the effect of actin C-terminal modification on the regulation of actomyosin interactions, activation of myosin S1 ATPase was analyzed as a function of increasing concentrations of F-actin$_{-3}$ and native F-actin, both in the presence and absence of saturating concentrations of α-TM (Fig. 3). In our conditions, removal of three amino acids from the actin C-terminus reduced the maximal rate (V_{max}) of actomyosin S1 ATPase by 27%, a value slightly higher than the difference observed for F-actin devoid of two residues (25). At

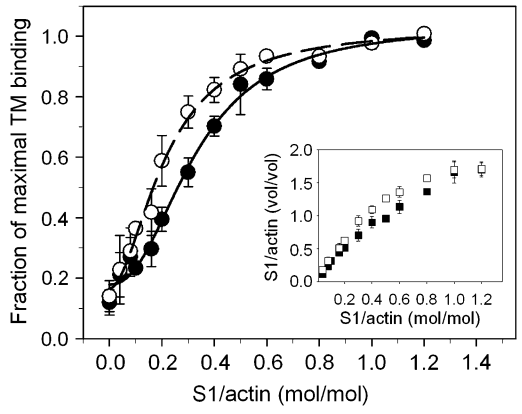

FIGURE 2 Effect of actin truncation on S1-induced α-TM binding. Saturation of F-actin (*solid circles*) and F-actin$_{-3}$ (*open circles*) with α-TM as a function of increasing S1 concentration was assayed in 5 mM imidazole, pH 7.0, 30 mM NaCl, 0.5 mM $MgCl_2$, 0.5 mM DTT. Binding curves were generated by fitting the experimental data for native F-actin (*solid line*) and F-actin$_{-3}$ (*dashed line*) to Eq. 2. The data are averages from five experiments. Vertical bars represent standard errors. Significance of the difference between F-actin and F-actin$_{-3}$: $p = 0.008$. (*Inset*) Binding of the myosin S1 to native F-actin (*solid squares*) and F-actin$_{-3}$ (*open squares*).

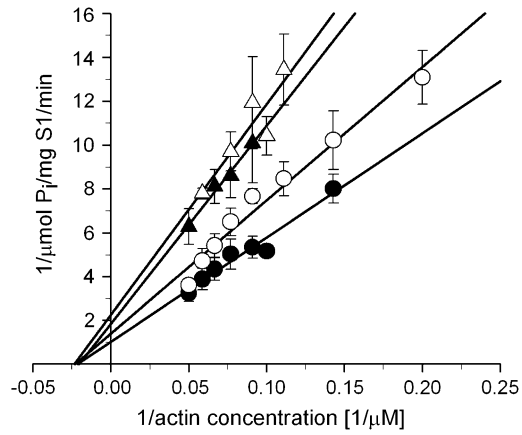

FIGURE 3 Lineweaver-Burk plot for the activation of S1 ATPase activity by actin (*circles*) and actin-TM (*triangles*). The activation in the presence of native F-actin (*solid symbols*) and F-actin$_{-3}$ (*open symbols*) was measured at 22°C in 5 mM imidazole, pH 7.0, 30 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT at 0.38 μM S1. The data are averaged from five experiments, and vertical bars indicate standard errors. Lines are linear regressions calculated for each set of averaged points.

saturation with α-TM, V_{max} was reduced in the presence of both actins. With F-actin$_{-3}$ the ATPase seemed more inhibited; however, the substantial error involved in calculation of the V_{max} values from double-reciprocal plots at subsaturating actin concentrations did not allow us to determine the degree of differences among the actins (Table 2). The Michaelis-Menten binding constant (K_M) was not significantly changed by the C-terminal modification of actin (Table 2), showing that the apparent affinity for active cross-bridges was not affected by the modification of actin.

Ca-dependent regulation was examined by following changes in the ATPase activation as a function of TM-Tn complex binding to actin in the presence or absence of Ca^{2+}. As illustrated in Fig. 4, in the absence of Ca^{2+} at subsaturating TM-Tn concentrations, truncated actin filaments were more inhibited than native actin. When saturated with the regulatory complex, the difference in activity between the two actins diminished, and the ATPase reached similar minimal levels. This shows that under relaxing conditions the TM-Tn complex restores the normal function of the truncated actin filament. In the presence of Ca^{2+}, binding of the TM-Tn complex increased the acto-S1 ATPase activity; however, as compared with native F-actin, the activation with F-actin$_{-3}$ was lower within the whole range of TM concentrations tested. This may indicate that, in activating conditions, the TM-Tn complex is not able to repair the functional defect in actin caused by the C-terminal truncation.

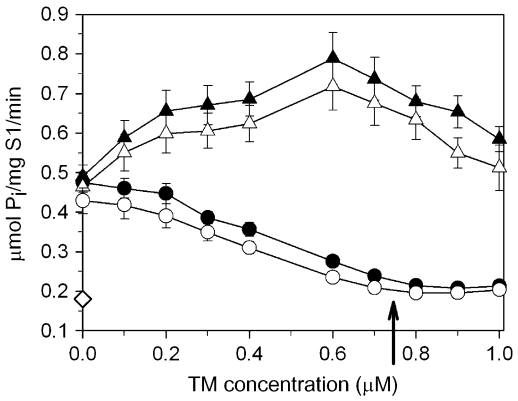

FIGURE 4 Regulation of the actomyosin S1 ATPase activity with Tn and Ca^{2+}. A concentration of 1 μM myosin S1 was incubated with either 5 μM F-actin (*solid symbols*) or F-actin$_{-3}$ (*open symbols*), 1 μM Tn, and TM at concentrations increasing from 0 to 1 μM in the presence of 0.2 mM $CaCl_2$ (*triangles*) or 1 mM EGTA (*circles*). Conditions: 5 mM imidazole, pH 7.0, 30 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT. The arrow points to the α-TM:actin stoichiometric concentration. ATPase activity of myosin S1 alone (*diamond symbols*).

DISCUSSION

Regulation of interactions between thin filament and myosin heads require binding of regulatory proteins, TM, and Tn to different actin regions and transmission of conformational changes within the filament. Site-directed mutagenesis and limited proteolysis helped to localize some regions of the actin molecule that are directly and allosterically involved in the mechanisms of regulation (11–16,25,26,39,40). In this study, we analyzed the role of actin C-terminal segment in the thin filament regulation using actin modified by proteolytic removal of three C-terminal residues.

Electron microscopy studies of the regulated thin filament suggest a different TM-actin interface in three activation states (5). In the blocked state, strands of skeletal TM cover the inner edge of the outer domain, where direct contacts between TM and actin C-terminal segment are possible.

TABLE 2 Actin-activated myosin S1 ATPase activity

	K_M (μM)	V_{max} (μmol P_i/mg S1/min)
F-actin	47.2 ± 11.3	0.99 ± 0.38
F-actin + α-TM	49.2 ± 13.8	0.54 ± 0.16
F-actin$_{-3}$	43.7 ± 9.1	0.72 ± 0.23
F-actin$_{-3}$ + α-TM	43.0 ± 11.2	0.46 ± 0.27

Values of the parameters were calculated from linear regressions shown in Fig. 3; errors are mean ± SE.

Our results show that indeed the C-terminal residues are required for TM binding with the affinity characteristic of native actin. However, direct contact between TM and actin C-terminus was not conclusively proven in this experiment. Earlier studies suggested that in relaxing conditions C-terminal truncation affects actin interactions with TM-Tn complex through reduced binding of Tn inhibitory subunit (TnI) (26). Our unpublished data, showing that isolated TnI binds weakly to truncated actin both in the absence and presence of TM, confirm this notion. This might indicate that direct binding of TnI to the actin C-terminus is involved in the mechanism of TM binding to actin in the blocked state. However, the fact that the actin C-terminus is intramolecularly coupled to distant regions of the molecule suggests that not only C-terminus but also other actin segments might be involved in these interactions. This is supported by the observations that actin truncation slightly changes absorption coefficient (this work) and tryptophan fluorescence (J. Moraczewska, unpublished data), which together suggest alterations in the tryptophan microenvironment. Because all tryptophan residues are located in the actin subdomain 1, modification of the C-terminus evidently induces conformational changes in this region. This probably is responsible for the weaker binding of TnI. In the presence of whole Tn complex and Ca^{2+} (closed state), the contacts with the C-terminus and/or a wider area of subdomain 1 are released, and, as shown in this work, TM binding is no longer sensitive to actin C-terminal truncation. No effect of actin truncation on TM binding in the closed state was observed for binding of α-TM without Tn. This result is at odds with data reported previously, which demonstrated that actin devoid of two C-terminal residues bound skeletal TM more weakly than native actin (25). Such a discrepancy might be a result of

using different methods of analysis of TM-actin interactions. In a previous study (25), the authors used AEDANS-labeled TM, which might alter TM location on actin and make it sensitive to actin C-terminal modification. The direct cosedimentation method used in our assay allows us to analyze TM-actin binding without additional TM modification.

A movement of skeletal TM strands toward the inner aspect of the actin inner domain in association with strong binding of myosin heads was observed by electron microscopy (7). Because in this state TM runs through subdomains 3 and 4, away from the actin C-terminus, one could expect that binding of TM in the myosin-induced open state is independent of actin truncation. However, as demonstrated here, this is not the case. Myosin S1 induced binding of TM to modified actin more efficiently than to native actin. Because strong binding of S1 was not affected by removal of three actin residues, this effect must be caused by a conformational change in an actin region involved in open-state TM binding, which apparently is coupled to changes in the conformation of the C-terminus. Conformational coupling between subdomain 4 and the C-terminus was shown previously. Removal of three C-terminal amino acids caused a conformational change that rendered segment 227–235 less susceptible to proteolysis (41).

Thus, our analyses strongly support the idea that C-terminal segment is part of a pathway transmitting signals along the filament. Actin subunits communicate through the interprotomer contacts their C-termini make with the N-terminal segment of DNase I-binding loop along the long-pitch helix and with hydrophobic loop Phe^{262}–Ile^{274} across the filament axis (17,18). We observed increased cooperativity of TM binding to truncated actin in the blocked state and increased S1-induced cooperativity of TM binding to actin in the open state suggesting that C-terminal modification altered the pathway of communication within the filament. Because distortion of actin structure in the N-terminal segment of DNase I-binding loop, a region known to form contacts with the C-terminus along the filament axis, also increased S1-induced cooperativity of TM binding to actin (40), the intersubunit contact formed by both actin regions seems responsible for the integration of the whole filament structure.

The acto-S1 ATPase activity measurements demonstrate that the truncation lowers the effectiveness of actin in activating myosin S1 ATPase by reducing maximal rate of the ATPase activity. This finding agrees with the data obtained for actin devoid of two C-terminal residues (25) and shows that F-actin$_{-3}$ is somehow defective in supporting the normal cycle of myosin cross-bridges. This defect is not caused by distortion of myosin binding sites because the apparent affinity of myosin for actin remains unchanged, as indicated by the same K_M. It is possible that the enhanced flexibility of the filaments assembled from C-terminally truncated actin observed in electron micrographs (30) is the major factor responsible for the lower actomyosin ATPase. However, the presence of TM, which is known to stabilize the filament, does not fully compensate the C-terminal modification. When saturated with TM and TM-Tn complex in the presence of Ca^{2+}, F-actin$_{-3}$ still tends to activate the ATPase less efficiently than native actin. The only function that is fully restored by actin saturation with the regulatory proteins is the acto-S1 ATPase inhibition at low Ca^{2+}. Earlier observations on actin proteolytically modified within the DNase I binding loop have also shown that binding of TM-Tn does not restore the normal level of myosin S1 ATPase activity despite retained Ca-sensitivity (40).

In summary, the results obtained with C-terminally truncated actin support the view that, depending on the state of the thin filament activation, skeletal α-TM binds to discrete sites on actin. The involvement of actin C-terminal region in TM binding is different in each state. In the blocked state, C-terminal residues contribute to the affinity for TM most probably through binding of TnI. However, the actin C-terminus does not participate in forming the TM-actin interface in the closed state, whereas TM binding in the open, myosin-induced state can be modulated allosterically by the conformations of the C-terminal region. Regulation of the actin-activated myosin ATPase is not a direct function of TM (and TM-Tn) actin affinity. In terms of myosin ATPase activation, the integrity of actin C-terminal segment is important.

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