The DNase-I Binding Loop of Actin May Play a Role in the Regulation of Actin-Myosin Interaction by Tropomyosin/Troponin*

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Joanna Moraczewska‡, Joanna Gruszczynska-Biegała‡, Maria J. Redowicz‡, Sofia Yu. Khaitilina‡, and Hanna Strzelecka-Golaszewska‡

From the ‡Department of Muscle Biochemistry, Nencki Institute of Experimental Biology, 3 Pasteur Street, 02-093 Warsaw, Poland, the ‡Kazimierz Wielki University of Bydgoszcz, Institute of Biology and Environmental Protection, 85-064 Bydgoszcz, Poland, and the ‡Department of Cell Culture, Institute of Cytology, 194064 St Petersburg, Russia

Various lines of evidence suggest that communication between tropomyosin and myosin in the regulation of vertebrate-striated muscle contraction involves yet unknown changes in actin conformation. Possible participation of loop 38–52 in this communication has recently been questioned based on unimpaired Ca\(^{2+}\) regulation of myosin interaction, in the presence of the tropomyosin-troponin complex, with actin cleaved by subtilisin between Met\(^{41}\) and Gly\(^{46}\). We have compared the effects of actin cleavage by subtilisin and by protease ECP32, between Gly\(^{42}\) and Val\(^{43}\), on its interaction with myosin S1 in the presence and absence of tropomyosin or tropomyosin-troponin. Both individual modifications reduced activation of S1 ATPase by actin to a similar extent. The effect of ECP cleavage, but not of subtilisin cleavage, was partially reversed by stabilization of interprotomer contacts with phalloidin, indicating different pathways of signal transmission from the N- and C-terminal parts of loop 38–52 to myosin binding sites. ECP cleavage diminished the affinity to tropomyosin and reduced its inhibition of acto-S1 ATPase at low S1 concentrations, but increased the tropomyosin-mediated cooperative enhancement of the ATPase by S1 binding to actin. These effects were reversed by phalloidin. Subtilisin-cleaved actin more closely resembled unmodified actin than the ECP-modified actin. Limited proteolysis of the modified and unmodified F-actins revealed an allosteric effect of ECP cleavage on the conformation of the actin subdomain 4 region that is presumably involved in tropomyosin binding. Our results point to a possible role of the N-terminal part of loop 38–52 of actin in communication between tropomyosin and myosin through changes in actin structure.

It is now generally accepted that the regulation of vertebrate striated muscle contraction involves cooperative and allosteric interactions between actin, myosin, tropomyosin (Tm),\(^1\) and troponin (Tn). Positive cooperativity of actin-myosin interactions in the presence of regulatory proteins is indicated by sigmoidal profiles of both the binding of the myosin heads to the thin filament (1) and the actin-activated ATPase plotted as a function of myosin concentration (2). This behavior, for which the binding of Tm to actin is a necessary and sufficient requirement, has led to a modification of the classic steric blocking hypothesis of the regulation of striated muscle contraction. Based on equilibrium binding studies in solution, Hill et al. (3) suggested that seven actin monomers together with one molecule of Tm and one of Tn form a functional unit that exists in equilibrium between two states, inactive and activated; the latter having higher binding affinity for Ca\(^{2+}\) and for myosin or its subfragment 1 (S1). Subsequent kinetic studies have led McKillop and Geeves to a three-state model (4) in which the kinetics of S1 binding to the regulated actin filament is closely coupled to the two-step mechanism of S1 binding to actin alone (5, 6). In this model, removal of calcium converts the actin-Tm-Tn units to a state unable to bind S1 (blocked state). Actin-Tm as well as actin-Tm-Tn with Ca\(^{2+}\) bound to troponin are in a state that permits weak binding of S1 (closed state, no acceleration of ATPase rate) in equilibrium with a third, fully active state (open state) that allows isomerization of the weakly bound S1 to the strongly bound, rigor-like state. The ratio of open/closed thin filament cooperative units depends on the fraction of actin sites occupied by S1, because occupancy of the actin filament with strongly bound S1 stabilizes the open state. These three biochemical states can be related to three distinct positions of the Tm-Tn complex on the actin filament (7–9). One should emphasize that states analogous to the blocked, closed, and open state are also present in the Hill model as substates of the two major states, but the incapability of thin filament to bind S1 in the absence of Ca\(^{2+}\) postulated in the McKillop and Geeves model is a matter of dispute (10–13).

Both models assume that the cooperativity of Tm and S1 binding to actin depend on the strength of end-to-end interactions between adjacent Tm molecules. Strong end-to-end contacts between Tm molecules are also the basic requirement in the more recent view that points to the role of flexibility of Tm strands in determination of the apparent cooperative unit size (14–16). However, Tm with impaired end-to-end interactions still bind cooperatively to actin, suggesting that the cooperativity involves tropomyosin-promoted conformational changes within the actin polymer (16–21). A putative myosin- and Tm-induced conformational change in actin has been incorporated in the more recent models of cooperative myosin binding and activation of muscle contraction (22, 23).

Several lines of evidence point to a possible role of subdomain 2 of actin in transmission of cooperative changes between actin protomers in thin filaments. This subdomain, and in particular its DNase-I binding loop (residues 38–52), are the most dynamic elements of the actin filament structure. Their confor-
natorial transitions strongly influence the interprotomer interactions among the filament (24, 25), consistent with participation of loop 38–52 in these interactions (Refs. 26 and 27 and references therein). Inhibition of actin motion over myosin in the in vitro motility assays by interprotomer cross-linking of actin filaments between various residues revealed the importance of conformational freedom of the subdomain 2/subdomain 1 interface for generation of motion and force with myosin (28, 29). In vitro motility of actin filaments is also inhibited by subtilisin cleavage within loop 38–52 (30). Mutations D56A/E57A within subdomain 2 of yeast actin were shown to alter the affinity of this actin for both Tm and myosin S1 and impair thin filament regulation by Ca$^{2+}$ without tethering Tm in position blocking the myosin binding sites in subdomain 1 (31). X-ray diffraction patterns of oriented F-actin gels revealed a small radial shift of loop 38–52 on Tm binding to actin (32), and possible contribution of subdomain 2 movements within actin protofilaments to changes in x-ray diffraction patterns from muscle in response to Ca$^{2+}$ activation of thin filament has been discussed (33). On the other hand, fluorescence studies on both muscle actin and yeast actin mutants with probes on residues 41 or 51 indicated that transitions among the blocked, closed, and open regulatory states involve no significant subdomain 2 movement (34). A most recent study of effects of the regulatory proteins on the affinity for myosin S1 and in vitro motility of subtilisin-cleaved actin has also led to the conclusion that subdomain 2 conformation does not play an active role in the regulation of actomyosin interactions (35).

We have previously shown that proteolytic cleavage of the DNase-I binding loop of actin between Gly$^{42}$ and Val$^{43}$ with protease ECP32 from Escherichia coli A2 strain has a profound effect on the interprotomer interactions in F-actin, whereas subtilisin cleavage between Met$^{47}$ and Gly$^{48}$ has not (36). This prompted us to compare the effects of these modifications on the Tm-mediated cooperativity of myosin S1-actin interactions. The results of this investigation support the conclusion that modification of the C-terminal portion of loop 38–52 by subtilisin cleavage does not substantially influence the regulation of actin-myosin interaction. In contrast, modification of the N-terminal part of this loop generated substantial changes in Tm binding to actin, its inhibitory effect on acto-S1 ATPase at low S1 concentrations, and the cooperative, Tm-mediated potentiation of the ATPase by the initial S1 binding. This is the first indication that changes at the intermolecular interface of the DNase-I binding loop may play a role in the regulation of muscle contraction. A preliminary report of this work has been presented (37).

Materials and Methods

Reagents—The protease ECP32 from E. coli A2 strain was kindly provided by Dr. A. Morozova (Institute of Cytology, St. Petersburg, Russia). Subtilisin (Carlsberg, from Bacillus licheniformis), ATP, HEPES, MOFS, diithiothreitol, were from Sigma, N-$\gamma$-(1-pyrenyl)iodoacetamidocadaverine from Molecular Probes (Eugene, OR). Sephadex G-100 was from Amersham Biosciences, and SP-Trisacryl M was from Biosepra (Sergy-Saint-Christophe, France).

Protein Preparations—Actin was prepared from rabbit skeletal muscles and purified according to Ref. 38. It was stored in the form of G-actin in buffer containing 2 mM Hepes, pH 7.6, 0.2 mM ATP, 0.1 mM CaCl$_2$, 0.2 mM diithiothreitol, and 0.02% NaN$_3$ (buffer G). Labeling of actin with N-$\gamma$-(1-pyrenyl)iodoacetamide at Cys$^{74}$ was performed as described previously (39) with an additional step of gel filtration of labeled G-actin on a Sephadex G-100 column. Actin cleaved between residues 42 and 43 was obtained by a 1–2 h cleavage with partially purified ECP32 at 25 °C (41). Precise conditions of the reaction (quantity of the enzyme, time of digestion) were established for each batch of the enzyme by examining the extent of actin cleavage with the use of SDS-PAGE. The cleaved actin was used within 10–12 h. The G-actins were transformed into Mg-bound form by 3–5 min incubations with 0.2 mM EGTA, 0.1 mM MgCl$_2$ at 25 °C and polymerized with 0.1 mM NaCl directly before measurements.

Myosin from rabbit fast skeletal muscle longissimus dorsi and its S1 were prepared as described in Refs. 42. S1 fractionation into S1(A1) and S1(A2) isoenzymes was performed by ion-exchange chromatography on SP-Trisacryl M (43). Before use, samples of S1 were clarified by centrifugation at 300,000 × g for 30 min.

Rabbit skeletal muscle a,b-tropomyosin and troponin were prepared as described in Refs. 44 and 45, respectively. Labeling of Tm with $\gamma$-[32P]ATP and Py(1) was performed as described by Ishii and Lehrer (46). The labeling ratio of pyrene to Tm in preparations used throughout this work was in the range of 1.3–1.5, indicating that a significant fraction of the molecules was double-labeled. Purity of all protein preparations was controlled by SDS-PAGE according to Ref. 47.

The concentrations of proteins were determined spectrophotometrically by using the following absorption coefficients and molecular mass:

- $\varepsilon_{280}$ (Mg$_2$) $\varepsilon_{280}$ (actin) $\varepsilon_{280}$ (Tm) $\varepsilon_{280}$ (EGTA) $\varepsilon_{280}$ (Ca$^{2+}$) $\varepsilon_{280}$ (Mg$^{2+}$) $\varepsilon_{280}$ (Ca$^{2+}$, Mg$^{2+}$) $\varepsilon_{280}$ (EGTA, Ca$^{2+}$, Mg$^{2+}$) $\varepsilon_{280}$ (EGTA, Ca$^{2+}$, Mg$^{2+}$)

The concentration of pyrene-labeled Tm was determined by the method of Bradford (48).

Determination of Critical Concentration for Polymerization (C$\text{c}$) of the Modified Actins—In view of the enhancement of actin C, by both ECP and subtilisin cleavage (39, 40), it was necessary to determine the C$\text{c}$ values for these actins under the ionic conditions of our experiments.

About 70 μM Ca-G-actins containing 10% of the respective pyrenyl-labeled actin (in 10 mM Hepes, pH 7.6, 0.5 mM ATP, 0.1 mM CaCl$_2$, 0.5 mM dithiothreitol, and 0.02% NaN$_3$) were transformed into Mg-G-actins and polymerized with 20 mM NaCl, 2 mM MgCl$_2$ for 45 min. The F-actin solutions were twice diluted with a solution of 10 mM Hepes, pH 7.6, 0.5 mM ATP, 20 mM NaCl, and 2 mM MgCl$_2$ without and, in a parallel sample, with tropomyosin added at a 1:4 molar ratio to actin. After 30 min, the solutions were diluted with the same buffer-salt solution to the concentration of 0.3 μM actin and the intensity of pyrenyl fluorescence was measured after 2 h and again after 24 h, at 25 °C. From plots of the difference in the fluorescence intensities between the F-actin and G-actin samples diluted to the same final concentrations versus actin concentration, the C$\text{c}$ values were calculated to be 0.5 μM for subtilisin-cleaved and 4 μM for ECP-cleaved actin both in the presence and absence of Tm.

ATPase Activity Measurements—The MgATPase activity of S1 and acto-S1 was measured in 10 mM Hepes, pH 7.6, 0.5 mM dithiothreitol, 0.1 mM CaCl$_2$, 0.5 mM dithiothreitol, and 0.02% NaN$_3$ and terminated with 2.5% SDS. The released phosphate was determined according to Ref. 49.

Spectroscopic Measurements—Fluorescence and light-scattering measurements were carried out in a Spex Fluorolog 3 spectrofluorometer in the ratio mode. Fluorescence emission spectra of pyrene-Tm were registered after excitation at 340 nm, and the excimer fluorescence was measured at 489 nm after excitation at 394 nm. Intensity of light scattered at 90° was measured at 350 nm.

Probing the Conformation of F-actins by Limited Digestion with Subtilisin—F-actins, obtained by polymerization of Mg-G-actins with 0.1 mM KCl, were digested with subtilisin at an enzyme/protein mass ratio of 1:20 at 25 °C. At different time intervals, aliquots of the solution were withdrawn, and the reaction was stopped by adding 1% EDTA and 0.5 mM ethylmercaptosulfon fluoride. The digests were analyzed by SDS-PAGE on 12% (w/v) polyacrylamide gel slabs according to Ref. 47. The apparent molecular masses of actin fragments were calculated from standard curves obtained with Bio-Rad SDS-PAGE standards.

Results

Effects of Proteolytic Modifications of Actin on F-actin-Tm Interaction—Possible changes in F-actin-Tm binding induced by proteolytic modifications of actin could not be examined by standard methods of analyzing the binding of protein ligands to F-actin, such as sedimentation, because the instability of ECP-cleaved F-actin results in disruption of its filaments by ultracentrifugal forces (39). It is a common practice to overcome problems arising from instability of F-actin, e.g., in experiments

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that require low F-actin concentrations, by taking the measurements in the presence of phalloidin, a toxin known to decrease $C_v$ of actin and stabilize F-actin filaments. This procedure is inapplicable to ECP-cleaved actin because, as will be shown below, phalloidin binding produces substantial changes not only in the filament stability but also in other properties of such modified F-actin. Therefore, to evaluate the effects of the proteolytic modifications of actin on F-actin interaction with Tm, we resorted to measuring F-actin-induced changes in the fluorescence of pyrene-Tm. As shown in Fig. 1, both subtilisin-cleaved and ECP-cleaved F-actin retain the ability of intact actin to increase the intensity of monomer fluorescence (two peaks centered at 385 and 405 nm) and decrease the intensity of excimer fluorescence (maximum at 485 nm) of N-(1-pyrenyl)acetamide label attached to Cys$^{190}$ of Tm (46). Fluorescence titrations of pyrene-Tm with F-actins showed that the final change in the excimer fluorescence of double-labeled Tm was similar for the three actins (about 40% quenching). However, as shown in Fig. 2, saturation of the fluorescence change with ECP-cleaved actin required about 3-fold higher F-actin concentration than the saturation with intact actin that was reached at about 7 mol per mol of Tm, indicating that cleavage at Gly$^{42}$ weakens Tm to F-actin binding. The titration curve for subtilisin-cleaved actin closely followed that for intact actin.

Assuming that the 7:1 stoichiometry of actin to Tm binding is preserved, and the fluorescence change is proportional to the fraction of maximal number of actins bound per Tm molecule, the free F-actin concentrations in any F-actin to Tm ratio can be obtained as a difference between concentration of total F-actin and F-actin bound calculated from the fractional fluorescence change versus free F-actin concentration (Fig. 2, inset), estimates of the apparent association constants ($K_{app}$) were obtained by fitting the data to a Hill equation in the form shown in Equation 1,

$$v = n[A]^{dil}K_{app}^{dil}(1 + [A]^{dil}K_{app}^{dil})$$

(Eq. 1)

where $v$ = fraction of maximal actin binding to Tm = fractional fluorescence change, $n$ = number of actin binding sites on Tm = 7, [A] = free F-actin concentration, and $a_H$ is the Hill coefficient. The best fit for ECP-cleaved actin was obtained with $K_{app} = 1.5 \times 10^5$ M$^{-1}$ and $a_H$ of 1.2. The $K_{app}$ value of $5 \times 10^6$ M$^{-1}$ obtained for intact actin (with $a_H = 1.0$) from cumulated data points from four experiments is only an approximation because the tight binding of the two proteins precludes accurate calculation of free F-actin concentrations below the saturating F-actin concentration. Nevertheless, this value is in good agreement with the values obtained in other laboratories using different methods (17, 18, 50–52). It is to be noted that the cooperativity of Tm to actin binding, observed when actin is in excess (common approach), is not experimentally detectable (the Hill coefficient equal to or close to 1.0) in our type of measurements (Tm in excess).

Thin Filament Cooperativity Probed by Acto-S1 ATPase Measurements—Fig. 3 shows S1 concentration dependence of actin-activated S1 ATPase at a constant F-actin concentration. The enhancement of actin critical concentration for polymerization by subtilisin and ECP cleavage (see “Materials and Methods”) was compensated for by correspondingly increasing the total concentration of the modified actins. One can see that at the low F-actin and S1 concentrations used in these measurements (activity far from $V_{max}$), the dependence of acto-S1 ATPase on [S1] in the absence of regulatory proteins was linear with both intact and the modified actins. In agreement with an

![Fig. 1. Fluorescence emission spectra of pyrene-labeled Tm alone (solid lines) and in the presence of saturating concentrations of F-actin (dashed lines) or F-actin and S1 (dotted lines). The spectra were registered after excitation at 340 nm, at 25 °C. Double-labeled Tm (1 μM) in 10 mM Heps, pH 7.6, 1 mM dithiothreitol, 32 mM NaCl, and 3 mM MgCl$_2$ was first titrated with nonmodified (A), subtilisin-cleaved (B), or ECP-cleaved F-actin (C). After saturation of changes in the excimer fluorescence of the pyrene label, the mixtures were supplemented with hexokinase (1 unit/ml) and 0.15 mM glucose to convert ATP introduced with actin into ADP, and the solutions were titrated with S1. The spectra are corrected for dilution.](http://www.jbc.org/doi/abs/10.1074/jbc.M200858200)

![Fig. 2. Binding of Tm to F-actin monitored by a change in the excimer fluorescence of double-labeled pyrene-Tm. Conditions: 0.7 μM pyrene-Tm, 10 mM Heps, pH 7.6, 1 mM dithiothreitol, 15–50 μM ATP, 32 mM NaCl, 3 mM MgCl$_2$, and unmodified (○), subtilisin-cleaved (□), or ECP-cleaved F-actin (△) at the concentrations indicated on the abscissa. Representative data from one of four experiments are shown. The concentrations of ECP-cleaved and subtilisin-cleaved F-actins are corrected for the presence of the monomeric species with an assumption that F-actin diluted into Tm solution, with Tm in excess over actin, is protected from depolymerization by fast Tm binding. The fluorescence intensity after each addition of actin was measured at 489 nm after excitation at 340 nm, at 25 °C. The fluorescence intensities in the absence of actin and at saturation with F-actin were normalized to 1.0 and to 0, respectively. Insert, fluorescence fractional change versus concentration of Tm-free F-actin calculated as described in the text. The curves fitting the data were calculated using the Hill equation (see “Results”).](http://www.jbc.org/doi/abs/10.1074/jbc.M200858200)
enhancement of $K_{app}$ for this reaction upon subtilisin cleavage of actin (40), activation of S1 by subtilisin-cleaved actin was an order of magnitude lower relative to intact actin. A similar decrease in actin-activated ATPase was produced by ECP cleavage. Effects of the two modifications of actin on the S1 concentration dependence of acto-S1 ATPase in the presence of the regulatory proteins were however different. Subtilisin cleavage had no significant influence on the pattern of changes in the ATPase activity with increasing S1 concentrations. With ECP-modified actin, the transition from inhibition of the ATPase relative to that with F-actin alone at low S1 to activation at high S1 concentrations (Ref. 2 and references therein) took place at severalfold lower [S1] than with intact and subtilisin-cleaved actin. This shift could not be caused by possible increases in the concentration of ECP-modified F-actin with increasing S1 concentrations arising from S1-induced polymerization of the monomeric species present in the assay mixtures. If that were the case, a departure of the data from the linear dependence on [S1] would be also observed with actin alone. Moreover, no significant activation of S1 ATPase by ECP-cleaved actin was observed at its concentrations below 4 $\mu M$ (see Fig. 5), the critical concentration for polymerization of this actin under ionic conditions of our experiments. Thus, the data in Fig. 3 indicate greater cooperativity of the filaments of ECP-modified actin relative to those of intact or subtilisin-cleaved actin.

In complementary experiments, the inhibition of acto-S1 ATPase by Tm at low ionic strength (10 mM NaCl, MgCl$_2$ in a 2 mM excess over ATP) and low S1 to actin molar ratio of 1:6 was measured as a function of Tm concentration. In agreement with earlier reports (53, 54), with intact actin the maximal inhibition, reached at the stoichiometric ratio of one Tm per seven actin monomers, amounted to about 70% of the activity of unregulated acto-S1. Similar results were obtained with subtilisin-cleaved actin. With ECP-cleaved actin, the maximal inhibitory effect of Tm was reduced to only about 20% of the ATPase with actin alone (data not shown).

The greater cooperativity of actin-myosin interaction is also reflected in the higher degree of the regulation of ECP-modified F-actin by Cu$^{2+}$ in the presence of Tm-Tn at S1 concentrations exceeding the concentration of actin. From data in Fig. 3, e.g. at 10 $\mu M$ S1, the percentage of regulation defined as shown in Equation 2,

$$
\frac{(Ca^{2+}-activated ATPase)}{(Ca^{2+}-activated ATPase)} \times 100\% \quad (Eq. 2)
$$

was calculated to be 65.7 $\pm$ 4.2% for intact actin, 71.8 $\pm$ 5.6% for subtilisin-cleaved actin, and 83.7 $\pm$ 7.9% for ECP-cleaved actin (mean values $\pm$ S.D. for $n=3$). The $p$ values obtained according to the Student’s $t$ test for independent samples show that the difference between ECP-cleaved and intact actin is statistically significant ($p = 0.025$), whereas that between subtilisin-cleaved and intact actin is not ($p = 0.20$).

Effects of Phalloidin on the Interaction of Actin with Tm and with S1—Fig. 4 shows the effects of phalloidin, added at a 1.5 molar excess over actin, on the actin-activated ATPase of S1 as a function of S1 concentration at a constant concentration of actin. The conditions were the same as in Fig. 3 except for ECP-cleaved actin, which was used in two different concentrations: 4 $\mu M$, i.e. equal to the $C_c$ for this actin under ionic conditions of these experiments, and 8 $\mu M$, which in the absence of phalloidin should yield 4 $\mu M$ F-actin, the concentration equal to that in samples with intact and subtilisin-cleaved actin. As it was predicted, ECP-cleaved actin at the total concentration of 4 $\mu M$ did not activate the S1 ATPase unless phalloidin was present. However, the effects of phalloidin on such modified actin were much larger than might be expected from enhancement by this toxin of the polymer concentration. With 4 $\mu M$ ECP-cleaved actin.
phalloidin-stabilized F-actin, the acto-S1 ATPase activities were about 4-fold higher than with 8 μM total actin in the absence of phalloidin (i.e. with 4 μM F-actin untreated with phalloidin), and phalloidin treatment of 8 μM ECP-cleaved actin resulted in a 9-fold enhancement of acto-S1 ATPase activities. No such effect of phalloidin was observed with either intact or subtilisin-cleaved actin.

Fig. 5 compares the effects of Tm on activation of S1 ATPase by ECP-cleaved actin in the presence and absence of phalloidin. One can see that the Tm-dependent transition from an inhibition to potentiation of the ATPase with increasing concentrations of S1, observed in the absence of phalloidin, changed into an inhibition in the whole range of S1 concentrations used when actin was treated with phalloidin. It is clear that phalloidin binding reverses the effects of ECP cleavage of actin on its interaction with myosin in both the absence and presence of Tm. As shown in Fig. 6, the effects of phalloidin on intact and on subtilisin-cleaved actin were to diminish rather than increase the inhibition by Tm of acto-S1 ATPase at low S1 to actin ratios and to lower the S1 concentration required for the shift from inhibition to potentiation. These results call for caution in the interpretation of experimental data on phalloidin-stabilized F-actin that has been frequently used in studies on actin-myosin interaction.

The specific effects of phalloidin on Tm-mediated changes in the interaction of ECP-cleaved actin with S1 stimulated us to evaluate possible influence of this toxin on the interaction of such modified actin with Tm. This was done by measuring phalloidin-induced changes in the fluorescence spectra of pyrene-Tm combined with actin at a 1:10 Tm to actin molar ratio. In agreement with data in Fig. 2, at this ratio of the two proteins, the actin-induced decrease in the intensity of excimer fluorescence of pyrene-Tm was fully saturated with intact and only half-saturated with the cleaved actin. As one can see in Fig. 7, subsequent addition of phalloidin (in a 1.5-fold molar excess over actin) to Tm complexed with ECP-cleaved actin resulted in a drop of the fluorescence intensity to its level in the Tm complex with intact actin. Phalloidin addition to the latter complex did not produce any further change. These measurements show that the changes in actin-Tm interaction produced by ECP cleavage of actin are entirely reversed by phalloidin.

Cooperative Effects of S1 Binding to Actin Probed by Changes in the Fluorescence of Actin-bound Pyrene-Tm—Earlier studies on regulated actin have led to a suggestion that the S1-dependent increase in the excimer fluorescence of actin-bound Tm double-labeled with N-(1-pyrenyl)iodoacetamide reports a change in the geometric relationship between Tm and actin that is associated with a cooperative transition from the closed to open state of the regulated actin filament. Binding of S1 has been shown to cause a 2.2-fold increase in the excimer fluorescence and a slight increase in the monomer fluorescence of the fluorophore (46). Similar effects were seen here with Tm bound to the proteolytically modified actins (Fig. 1). Our S1 titrations of pyrene-Tm-complexes with intact, subtilisin-cleaved, and ECP-cleaved F-actin in the absence of ATP (Fig. 8) showed a maximum increase in the excimer fluorescence by a factor of 2.37 ± 0.06, 2.00 ± 0.06, and 1.81 ± 0.07 (mean values from 9–14 experiments ± S.E.), respectively. In agreement with an earlier finding (46), comparison of the fluorescence profiles obtained with intact actin with simultaneously measured changes in light-scattering intensity reporting S1 binding to actin showed that the fluorescence change saturated when about three S1s were bound to seven actin protomers. With the modified actins, the fluorescence profiles were shifted to even lower S1/actin ratios in the order: ECP-cleaved actin — subtilisin-cleaved actin — intact actin.
titrated with myosin S1 in the presence of 10 mM Hepes, pH 7.6, 32 mM NaCl, 3 mM MgCl₂, hexokinase (1 unit/ml), and 0.15 mM glucose. Excimer fluorescence at 489 nm after excitation at 340 nm and light scattering intensity at 350 nm were measured after each addition of S1, at a molar ratio of S1 to actin.

Fig. 8. Effects of myosin S1 binding to nonmodified and proteolytically modified actins complexed with pyrene-Tm on excimer fluorescence of pyrene. Conditions: 7 μM nonmodified (A) or subtilisin-cleaved F-actin (B), and 18 μM ECP-cleaved F-actin (C) were combined with 1 μM double-labeled pyrene-Tm. The complexes were titrated with myosin S1 in the presence of 10 mM Hepes, pH 7.6, 32 mM NaCl, 3 mM MgCl₂, hexokinase (1 unit/ml), and 0.15 mM glucose. Excimer fluorescence at 489 nm after excitation at 340 nm and light scattering intensity at 350 nm were measured after each addition of S1, at 25 °C. Insets show the increase in the light scattering intensity as a function of S1 to actin molar ratio.

subtilisin-cleaved actin < intact actin. No quantitative evaluation of these effects of actin modifications was however possible because of difficulties with interpretation of the light-scattering changes during S1 titration of the modified actins, in particular of ECP-cleaved actin that has to be added in a large excess over Tm to saturate the initial quenching of excimer fluorescence of pyrene-Tm (see Fig. 2). Therefore, the changes in light-scattering intensity on subsequent titration of actin-Tm complex with S1 measure S1 binding to both actin-Tm and free actin, which is reflected in a further increase of the intensity of scattered light at S1 concentrations exceeding the concentration of the actin-Tm complex. Another difficulty with ECP-cleaved actin is the instability of its filaments under the conditions of this experiment because of the enhanced rate of monomer dissociation from the filament ends (36). In the absence of free ATP, the monomeric ADP-form of this actin probably undergoes fast denaturation and aggregation, which is seen as a development of turbidity in the course of the long lasting titration. Turbidity development was also frequently observed during S1 titration of subtilisin-cleaved actin. This effect may contribute to the diminished maximum fluorescence change as compared with unmodified actin. On the other hand, the filaments of ECP-cleaved actin are largely stabilized by Tm binding, and this effect is cooperative (55). Moreover, Tm is known to largely increase S1 affinity to actin. One can therefore expect a preferential binding of S1 to the ECP-actin filaments or their fragments saturated with Tm. Thus, the shift of the saturation of the fluorescence change to lower S1:actin ratios upon the proteolytic modifications of actin can be taken as an indication of increased cooperativity of the S1-induced change in the state of Tm on the filaments of ECP-cleaved and, to a lesser degree, of subtilisin-cleaved actin.

Long Range Conformational Effects of the Proteolytic Modifications of the DNase-I Binding Loop—It has earlier been shown that proteolytic cleavage of the DNase-I binding loop with ECP renders F-actin susceptible to digestion with trypsin at two sites in subdomain 2, Arg⁵² and Lys⁶⁸, that are easily cleaved in G-actin but become inaccessible in KCl-polymerized F-actin. F-actin modified by subtilisin cleavage of the DNase-I binding loop more closely resembles intact F-actin in its resistance to trypsin (36). Here we used limited digestion with subtilisin to further probe conformational effects of the proteolytic modifications of the DNase-I binding loop. As can be seen in Fig. 9A, in unmodified F-actin the major site of subtilisin cleavage between Met⁴⁷ and Gly⁴⁸, yielding a 35-kDa C-terminal product (residues 48–375), is strongly protected. Therefore, a slow degradation of the alanine- and serine-rich segment 227–235 in subdomain 4 (56, 57) results in fragmentation of the F-actin subunits into the N-terminal 30-kDa peptide (residues 1–227) and 16-kDa C-terminal peptide (residues 235–375). The faint 33-kDa band is a product of subtilisin cleavage between Leu⁶⁷ and Lys⁶⁸ (57). Digestion of subtilisin-modified F-actin (Fig. 9B), that had already been cleaved between Met⁴⁷ and Gly⁴⁸, yielded a 25-kDa fragment (residues 48–227) instead of the N-terminal 30-kDa fragment. No other changes in the digestion pattern with respect to unmodified F-actin were seen, and the rates of appearance of the main digestion products were also similar. This contrasts with the digestion pattern of ECP-modified F-actin (Fig. 9C) showing much faster degradation of segment 227–235, as judged from the faster appearance of the 16- and 25-kDa products, and acceleration of the cleavage between Leu⁶⁷ and Lys⁶⁸ yielding the C-terminal 33-kDa product and its 19-kDa fragment encompassing residues 68–227 (57). These differences could not simply be caused by the high critical concentration of ECP-modified actin because at subtilisin concentrations used in this experiment G-actin is very quickly degraded to small peptides (not shown). The degradation of monomers coexisting with the polymer (about 17% of total actin) is however expected to drive depolymerization of F-actin, and this seems to account for the gradual decrease in intensity of the initially accumulated products of cleavage at specific sites as can be seen in Fig. 9C.

These results confirm the structural similarity of subtilisin-modified F-actin to intact F-actin and show that modification of the DNase-I binding loop by its cleavage with ECP leads to extensive changes in F-actin structure.

DISCUSSION

Docking the atomic structure of S1 to the model of the F-actin structure and extensive biochemical studies have led to a consensus view that the main myosin binding sites in actin are located in its subdomain 1 and at the junction of subdomains 1 and 3 (60, 61). Additional involvement of residues 40–42 of the DNase-I binding loop in the weak binding of myosin, suggested by the modeling of the acto-S1 structure (60), might explain the reported S1-induced changes in the fluorescence of a dansyl Loop 38–52 of Actin and Thin Filament Regulation

http://www.jbc.org/content/198/5/31202.full.pdf
probe attached to Gln41 of actin and in the interaction of this actin with antidanay IgG (62, 63). However, the insensitivity of S1 interaction with dansyl-labeled actin to antidanay IgG binding has led to interpretation of these data in terms of allosteric effects of S1 binding to the other sites in actin (63). Evidence for intramolecular signal transmission in the reverse direction, from loop 38–52 to subdomain 1, has been provided by studies on G-actin (64, 65). An intra- or intermolecular conformational coupling between loop 38–52 and myosin binding sites in subdomain 1 of actin is also a likely explanation for the suppression of acto-S1 ATPase activity when this loop is disrupted by subtilisin or ECP cleavage.

An intermolecular pathway of communication between the DNase-I binding loop and myosin binding sites is consistent with the fact that activation of myosin ATPase requires actin in its polymer form, with a prediction from F-actin models that this loop interacts with subdomains 1 and 3 of an adjacent F-actin subunit along the same long pitch helical strand (26, 66, 67), and with biochemical evidence for its intermolecular interaction with the C terminus in subdomain 1 of actin (27, 68, 69). However, the dramatic enhancement of the turnover rate of F-actin subunits in ECP-cleaved F-actin, contrasting with a small effect of subtilisin cleavage (36), argue for participation of only the N-terminal part of the DNase-I binding loop, encompassing ECP cleavage but not subtilisin cleavage site, in the intersubunit interactions as predicted in the F-actin model of Holmes et al. (26). One can suppose that the effect of subtilisin cleavage is transmitted to an adjacent F-actin subunit indirectly, through its influence on the conformation of the N-terminal part of the loop. This is however difficult to reconcile with the observation that, in the absence of regulatory proteins, the inhibitory effect of ECP cleavage on acto-S1 ATPase is largely reduced by phalloidin binding to actin, whereas the inhibition resulting from subtilisin cleavage is not.

The effect of phalloidin on the activation of S1 by ECP-cleaved actin is consistent with an intermolecular pathway of communication between the N-terminal portion of loop 38–52 and myosin binding sites because this toxin is known to stabilize this loop and strengthen its contacts with an adjacent subunit along the filament (66). Stabilization by phalloidin of intersubunit interactions in ECP-modified F-actin has also been demonstrated (36). On the other hand, with limited digestion by trypsin (36) and subtilisin as a probe (this work) it has been shown that ECP cleavage leads to a significant change in the conformation of segment 61–69 of subdomain 2 in F-actin. This effect too is reversed by phalloidin binding to the modified F-actin (36). These data point to a possible role of segment 61–69 in an alternative, intramolecular pathway of communication between the DNase-I binding loop and myosin binding sites in subdomain 1. A recent comparison of the crystal structures of the ADP and ATP form of monomeric actin shows how a rearrangement of the loop encompassing residues 70–78, next to segment 61–69, triggers the change in conformation of the DNase-I binding loop initiated by release of the nucleotide γ-phosphate from the site of its interaction with residues in subdomain 1 (70). A study of β-actin mutants, showing that replacements of residues 60–69 with alanine abolish the ability of actin to bind DNase I (71), provides more evidence for conformational coupling between this stretch of residues and 38–52 loop on the top of subdomain 2.

In contrast to cleavage with ECP, the effect of subtilisin cleavage on the conformation of segment 61–69 in F-actin subunits is small (Ref. 36 and this work), supporting the conclusion that the N- and C-terminal parts of the DNase-I binding loop communicate with the sites of myosin binding in different ways.

It has recently been shown that, despite impaired interaction with myosin in the absence of regulatory proteins, subtilisin-cleaved actin does not significantly differ from uncleaved actin in the Ca2+ regulation and the extent of potentiation of its in vitro motility in the presence of Tm-Tn (35). The data reported here clearly show that the consequences of proteolytic modifications of the actin loop 38–52 for the regulation of actin-myosin interaction depend on the location of the cleavage site within the loop. Cleavage between residues 42 and 43 greatly reduced the inhibitory effect of Tm (or Tm-Tn in the absence of Ca2+) on acto-S1 ATPase activity at low S1 concentrations, and enhanced the Tm-mediated potentiation of the ATPase by S1 binding to actin. The attenuation of the inhibitory effect of Tm seems to be related to its diminished affinity to ECP-modified F-actin because phallolidin treatment of this actin resulted in a restoration of both the Tm binding properties of intact actin and of the strong inhibitory effect of Tm on acto-S1 ATPase at low S1 concentrations. In contrast, actin cleaved with subtilisin between residues 47 and 48 more closely resembled unmodified actin than the ECP-modified actin. These results suggest that the intersubunit contacts of the N-terminal part of loop 38–52, which are weakened by ECP cleavage but little influenced by subtilisin cleavage (36), play a role in both Tm- and myosin-dependent transitions between different functional states of the thin filament.

A comparison of x-ray fiber diffraction diagrams of oriented pure F-actin gels and F-actin-Tm gels revealed subtle Tm-dependent changes in actin structure, including a change in orientation of the hydrophobic loop Phe362 to Ile374, of a helix from Phe223 to Ala230 in subdomain 4 of actin, and of the DNase-I binding loop (32). It is plausible that changes in the arrangement of these structural elements are mutually related. The N-terminal segment of the DNase-I binding loop and Phe362 to Ile374 loop, along with the C terminus of actin, form a common interface between three adjacent F-actin subunits (26, 27, 72). Conformational coupling between the N-terminal segment of the DNase-I binding loop and the Phe223 to Ala230 helix has been demonstrated by our limited digestion experiments showing an exposure of proteolytic cleavage sites within segment 227–235 in ECP-modified F-actin. This coupling may be important for the regulation of actin-myosin interaction because biochemical (58), structural (32), and mutational studies (59) indicate that helix 223–230 and the following loop 230–238 participate in Tm binding in the closed state of the thin filament. One can speculate that a rearrangement of these structural elements, triggered by modification of the N-terminal
part of loop 38–52, facilitates the shift of the Tm strand toward the F-actin inner domain which is required for strong binding of the myosin heads (7–9). This is one of possible explanations of the attenuation of the inhibitory effect of Tm on acto-S1 ATPase observed with ECP-cleaved actin. Signal transmission in the opposite direction, from Tm binding site(s) in subdomain 4 to the DNase-I binding loop and, through it, to myosin binding sites in subdomain 1 might contribute to the Tm-dependent cooperative enhancement (potentiation) of actomyosin ATPase.

As it was briefly reported elsewhere (55), Tm binding nearly completely suppresses the enhanced subunit exchange in ECP-cleaved F-actin, indicating stabilization of the interprotomer contacts that are perturbed by this modification, and this stabilizing effect of Tm is cooperative. These observations are in line with the conclusion that changes in the interprotomer interactions of the DNase-I binding loop may contribute to the regulation of the actomyosin ATPase. Earlier spectroscopic studies on fluorescently labeled yeast actin mutant Q41C indicated that transitions between the three regulatory states of the thin filament involve no significant movement of loop 38–52 (34). It does not seem to exclude the possibility of subtle changes at the intermolecular interface of this loop that, through other structural elements, are intra- and/or intermolecularly propagated to Tm and myosin binding sites. In this work we concentrated on the transition from the closed to open state of the thin filament. Our measurements of the actin-activated ATPase of S1 in the presence of the Tm-Tn complex provide an indication that the Ca2+-dependent changes in the equilibrium between the blocked and closed state may also be influenced by the conformation of the N-terminal segment of actin loop 38–52. A more detailed investigation of this aspect of thin filament regulation will be pursued in future studies.

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The DNase-I Binding Loop of Actin May Play a Role in the Regulation of Actin-Myosin Interaction by Tropomyosin/Troponin
Joanna Moraczewska, Joanna Gruszczynska-Biegala, Maria J. Redowicz, Sofia Yu. Khaitlina and Hanna Strzelecka-Golaszewska

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