Effect of Tropomyosin on Formin-Bound Actin Filaments

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ABSTRACT Formins are conservative proteins with important roles in the regulation of the microfilament system in eukaryotic cells. Previous studies showed that the binding of formins to actin made the structure of actin filaments more flexible. Here, the effects of tropomyosin on formin-induced changes in actin filaments were investigated using fluorescence spectroscopic methods. The temperature dependence of the Förster-type resonance energy transfer showed that the formin-induced increase of flexibility of actin filaments was diminished by the binding of tropomyosin to actin. Fluorescence anisotropy decay measurements also revealed that the structure of flexible formin-bound actin filaments was stabilized by the binding of tropomyosin. The stabilizing effect reached its maximum when all binding sites on actin were occupied by tropomyosin. The effect of tropomyosin on actin filaments was independent of ionic strength, but became stronger as the magnesium concentration increased. Based on these observations, we propose that in cells there is a molecular mechanism in which tropomyosin binding to actin plays an important role in forming mechanically stable actin filaments, even in the case of formin-induced rapid filament assembly.

INTRODUCTION

Actin filaments are crucial in the development of cell shape, polarity, cell division, and transportation of intracellular vesicles within eukaryotic cells (1,2). These processes take place under the control of various actin-binding proteins. Formins are actin-binding proteins that play important roles in the arrangement and realignment of the cytoskeleton. Formins are evolutionarily conserved multidomain proteins that can be found in a wide range of animal and plant species (1). The first member of the formin family was identified as a product of a mutated gene that caused limb deformities (3). The formin homology (FH) domains were defined by Castrillon and Wasserman (4). Most formins have FH1 and FH2 domains. The FH2 domain is the most conservative, and shows no sequence similarity to any other domain or polypeptide. It binds to the barbed end of the actin filaments, and provides protection against capping proteins (5). The FH2 domain is necessary and sufficient for actin nucleation and dimer stabilization (5). Formins exert their effects on the actin cytoskeleton by stimulating the polymerization of unbranched filaments (6,7). The conformational properties of actin and actin-binding proteins were studied previously, using a wide range of spectroscopic methods (8–15). It was shown recently by fluorescence spectroscopy that formin fragments have the ability to increase the flexibility of actin filaments through long-range allosteric interactions, after binding to the barbed end of filaments (16,17). The biological function attributed to these dynamic changes is not properly understood.

Tropomyosins are abundant proteins in nonmuscle cells, and the regulation of muscle contraction is attributed to them (18,19). Despite numerous observations regarding their function in muscle regulation, the biological function of tropomyosins in nonmuscle cells has not been fully described. A recent study showed that tropomyosins and formins can express their effects on actin filaments simultaneously, and together they can protect these filaments from severing (20).

The observation that tropomyosin colocalizes with formin-induced actin filament structures in living cells (21–25) suggests that tropomyosin binding plays a role in the adjustment of conformational properties of flexible actin filaments polymerized by the assistance of formins. To test this possibility, we characterized the effects of tropomyosin on the conformational properties of formin-bound actin filaments by using temperature-dependent fluorescence resonance energy transfer and time-dependent fluorescence anisotropy decay measurements. The results indicated that flexible formin-bound filaments were stabilized by the binding of tropomyosin, and the stabilizing effect reached its maximum under conditions where all binding sites on actin were occupied by tropomyosin. The stabilization effect was strongly dependent on magnesium concentration, but showed little dependence on ionic strength.

MATERIALS AND METHODS

Materials

The KCl, $MgCl_2$, $CaCl_2$, Tris, glycogen, *N*-(iodoacetaminoethyl)-1-naphtylamine-5-sulfonic acid (IAEDANS), 5-(iodoacetamido)fluorescein (IAF), EGTA, NaN_3, ammonium sulfate, ATP, and β-mercaptoethanol were obtained from Sigma-Aldrich (Budapest, Hungary). Dimethylsulfoxide was obtained from Fluka (Buchs, Switzerland).

Protein preparation and purification

Acetone-dried powder of rabbit skeletal muscle was obtained as described previously (26). Rabbit skeletal-muscle actin was prepared according to the method of Spudich and Watt (27), and stored in a buffer containing

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4 mM Tris-HCl (pH 7.3), 0.2 mM ATP, 0.1 mM $CaCl_2$, and 0.5 mM DTT (buffer A). The concentration of G-actin was determined spectrophotometrically, with an absorption coefficient of 0.63 mg mL^{-1} cm^{-1} at 290 nm (28). A molecular weight of 42,300 was used for the G-actin (29).

The FH2 fragments of mammalian formin mDia1 (mDia1-FH2) were prepared as described previously (17,30). The mDia1-FH2 fragments were expressed as glutathione transferase fusion proteins in the *Escherichia coli* BL21 (DE3)pLysS strain. The concentration of protein was determined photometrically, with an absorption coefficient of $A_{280} = 21{,}680\ M^{-1}\ cm^{-1}$ at 280 nm (ProtParam, http://us.expasy.org/tools/). The purified protein was frozen in liquid nitrogen and stored at −80°C. Formin concentrations are given as mDia1-FH2 monomer concentrations throughout this study.

Tropomyosin was purified from rabbit skeletal muscle according to the procedure of Eisenberg and Kielley (31) and Smillie (32). The first steps of the preparation involved three isoelectric precipitations where the pH was brought down to 4.6 by using 0.1 M HCl each time, followed by centrifugation (6000 × *g* for 10 min; Beckman Coulter (Fullerton, CA) Optima MAX Ultracentrifuge), and then the pH of the dissolved pellet was adjusted to 8.0 with 1 N KOH. Ammonium sulfate precipitations were produced (31.2 g $(NH_4)_2SO_4$/100 mL buffer) twice at the end of the procedure. The concentration of the protein solution was determined photometrically, using an absorption coefficient of $A_{278} = 0.29$ mL mg^{-1} cm^{-1} at 278 nm (31).

Fluorescent labeling of actin

Actin was labeled fluorescently with IAEDANS or IAF dyes at Cys^{374}, according to the method of Miki et al. (33). We incubated 2 mg/mL F-actin (in buffer A without DTT, supplemented with 100 mM KCl and 2 mM $MgCl_2$) with a 10-fold molar excess of IAEDANS at room temperature for 1 h. The label was first dissolved in a small amount of (~50 μL) dimethylsulfoxide, and then DTT free buffer A was added to the solution (drop by drop, to a range of 800–1000 μL) before being added to the protein. Labeling was terminated with 2 mM β-mercaptoethanol. After ultracentrifugation of the sample for 45 min at 328,000 × *g*, the pellet was incubated in buffer A for 2 h, and then gently homogenized with a homogenizer. The homogenized sample was dialyzed overnight against buffer A at 4°C. The concentration of fluorescent dye in the protein solution was determined by using an absorption coefficient of 6100 M^{-1} cm^{-1} at 336 nm for actin-bound IAEDANS (34). The extent of labeling was 0.8–0.9 mol/mol of actin monomer.

Labeling Cys^{374} with IAF was performed according to standard procedures (35,36). The monomeric actin (46 μM) was labeled in DTT-free buffer A with a 15-fold molar excess of IAF, which was dissolved in 0.1 N NaOH and added to the actin solution drop by drop at room temperature while the pH was kept constant. Afterward, the sample was incubated at 4°C for 24 h. After this incubation, the actin was polymerized for 2 h at room temperature, and then centrifuged at 328,000 × *g* for 45 min at 4°C. The pellet was treated in a way similar to that described in the case of labeling with IAEDANS. The concentration of the probe was determined using an absorption coefficient 60,000 M^{-1} cm^{-1} at 495 nm. The molar ratio of bound probe to actin concentration was 0.6–0.7.

Sample preparation

Preparations of samples were made in the same way for both steady-state and anisotropy decay measurements each time. Buffer A and labeled G-actin were added to quartz cuvettes, and 0.2 mM EGTA and 0.05 mM $MgCl_2$ were added (final concentrations) to initiate the exchange of actin-bound calcium for magnesium. Five minutes or 10 min later, we added formin or formin and tropomyosin, and the final concentration of $MgCl_2$ and KCl was adjusted to 1 mM and 50 mM, respectively, to initiate the polymerization of actin. The total amount of sample was always 1 mL. Samples were incubated overnight at 4°C in the dark, and before the measurements, they were kept at room temperature for at least 30 min.

Steady-state fluorescence experiments

Steady-state fluorescence measurements were performed with Horiba Jobin Yvon (Longjumea cedex, France) Fluorolog-3 and PerkinElmer (Walthom, MA) LS50B Luminescence Spectrometers, both equipped with a thermostated sample holder. To calculate FRET efficiency, the fluorescence intensities of the donor (IAEDANS) were recorded in the presence and absence of the acceptor (IAF). The excitation wavelength for IAEDANS was 350 nm. The optical slit was set to 5 nm in the excitation light path, and 5 nm on the emission side. During measurements, the emission spectra of the donor were recorded between 370–550 nm, and the integral was used for calculations. Fluorescence intensities were corrected for inner filter effect. The corrected fluorescence intensity of IAEDANS was integrated between 440–460 nm. The FRET efficiency (E) was calculated as:

$$E = 1 - (F_{DA}/F_D), \tag{1}$$

where F_{DA} and F_D are the integrated fluorescence intensities of the donor molecule in the presence and absence of acceptors, respectively. The value of E was determined at different temperatures between 6–34°C, and a special FRET parameter, the normalized FRET efficiency (f'), was calculated using:

$$f' = E/F_{DA}. \tag{2}$$

The temperature dependence of f' can be informative regarding the flexibility of the investigated protein (37,38). For the interpretation of FRET results, the temperature dependence of the relative f', defined as the value of f' at the given temperature divided by the value at the lowest temperature (6°C), is presented. The larger temperature-induced changes in the value of normalized FRET efficiency are indicative of a more flexible protein matrix (37,38). Relative f' was measured at 10 μM actin (1:9 = donor/acceptor) in the presence of 500 nM formin. The experiments were performed in 4 mM Tris-HCl (pH 7.3), 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM DTT, 0.2 mM EGTA, 1 mM $MgCl_2$, and 50 mM KCl.

Fluorescence lifetime and emission anisotropy decay measurements

Time-dependent fluorescence measurements were performed with an ISS K2 multifrequency phase fluorometer (ISS Fluorescence Instrumentation, Champaign, IL), using the frequency cross-correlation method. The excitation light was provided by a 300-W Xe-arc lamp, and was modulated with a double-crystal Pockel cell. The excitation wavelength was set at 350 nm, and the emission was monitored through a 385FG03-25 high-pass filter. The modulation frequency was changed in 10 steps (linearly distributed on a logarithmic scale) from 5 to 80 MHz during fluorescence-lifetime measurements, and in 15 steps from 2 to 100 MHz when anisotropy decay was measured. Freshly prepared glycogen solution was used as a reference (lifetime = 0 ns). The fluorescence lifetimes of the fluorophore were determined by the use of nonlinear least-square analysis. Data were analyzed with ISS187 and Vinci 1.6 decay analysis software (ISS, Champaign, IL). In fluorescence-lifetime measurements, all data were fit to double-exponential decay curves, assuming a constant, frequency-independent error in both phase angle (±0.200°) and modulation ratio (±0.004). The goodness of fit was determined from the value of the reduced χ^2 probe (39). Average fluorescence lifetimes (τ_{aver}) were calculated from the results of the analysis, assuming discrete lifetime distribution:

$$\tau_{aver} = (\tau_1\alpha_1 + \tau_2\alpha_2)/(\alpha_1 + \alpha_2), \tag{3}$$

where α_i and τ_i are the individual amplitudes and lifetimes, respectively (39). The anisotropy is expected to decay as a sum of exponentials (40). The experimentally obtained data were fitted to a double exponential function:

$$r(t) = r_1\exp(-t/\varphi_1) + r_2\exp(-t/\varphi_2), \tag{4}$$

where ϕ_1 and ϕ_2 are rotational correlation times, with amplitudes r_1 and r_2.

The concentration of actin was 30 μM (1.3 mg/mL) during fluorescence anisotropy decay measurements, except as noted otherwise. The

experiments were performed in the presence of 1 mM $MgCl_2$ and 50 mM KCl, unless otherwise stated.

RESULTS AND DISCUSSION

In this study, we applied steady-state and time-dependent fluorescence methods to describe the effects of tropomyosin on formin-bound actin filaments. To exclude the possibility that formin binding weakens the affinity of tropomyosin for actin, cosedimentation experiments were performed (Fig. 1). Samples of tropomyosin (3 μM) and actin filaments (5 μM) in the absence or presence of formin (500 nM) were centrifuged (for 30 min at 386,000 $\times$ g and at 20°C), and the pellets were analyzed using SDS-PAGE. The concentration of tropomyosin in pellets was independent of the presence of formin, indicating that mDia1-FH2 did not substantially modify the affinity of tropomyosin for actin (Fig. 1). On the other hand, the appearance of the formin band in pellets of tropomyosin containing samples showed that tropomyosin did not displace the formins from actin, in agreement with the observations of Wawro et al. (20). The gels also indicated no contaminating proteins in these protein samples.

It was shown previously, using a temperature-dependent FRET method (37), that mDia1-FH2 formin dimers bound to the barbed end of actin filaments have the ability to increase the flexibility of filaments (17). Here we used the same FRET method to characterize the effects of tropomyosin on the dynamic properties of formin-bound actin filaments. The temperature dependence of FRET efficiency was determined between 6–34°C, using donor and acceptor probes attached to the Cys^{374} of actin protomers. One protomer bound only one fluorophore, and FRET occurred between the probes on neighboring protomers. With this arrangement, the inter-protomer flexibility of actin filament can be characterized. In these experiments, a steeper temperature dependence of FRET efficiency indicates a more flexible protein matrix between the donor and acceptor probes (37,38). In control experiments, the normalized FRET efficiency, i.e., the relative f' (Eq. 2), was determined with 10 μM actin in the absence of tropomyosin and formin. The results showed ~120% increase of relative f' over a temperature range of 6–34°C (Fig. 2). In the presence of 500 nM mDia1-FH2, the temperature profile of relative f' became steeper (increasing to ~330%), indicating that the binding of formin fragments changed the conformation of actin filaments by making them more flexible (Fig. 2). These formin-induced changes were in agreement with our previous observations (17). To describe the effect of tropomyosin, tropomyosin was added to formin-bound actin filaments at a 2 μM concentration. One tropomyosin binds to seven actin protomers in the filaments. Considering that the affinity of tropomyosin for actin filaments is ~0.5 μM (46) under these conditions, the 10 μM actin and 2 μM tropomyosin concentrations provided appropriate conditions to saturate the tropomyosin-binding sites on actin. The results obtained in the presence of both formin and tropomyosin showed that the value of relative f' increased to 150% over the investigated temperature range (Fig. 2). This tendency was similar to that observed in the absence of formin and tropomyosin, or in the presence only of tropomyosin (Fig. 2). These observations indicate that the binding of tropomyosin stabilized the formin-bound actin filaments, and their conformation

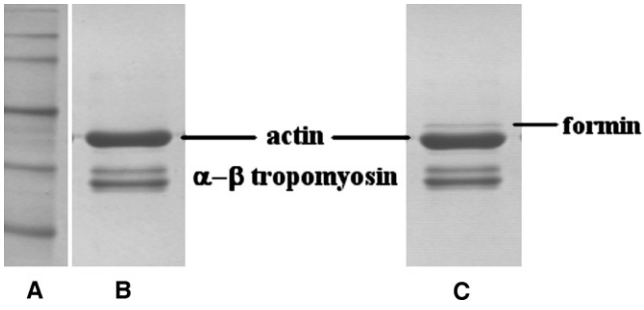


FIGURE 1 Tropomyosin binds to mDia1-FH2 formin-bound actin filaments. SDS-PAGE gels of pellets of actin, formin, and tropomyosin samples from cosedimentation assays. (*A*) Calibration proteins with molecular weights of 150, 100, 75, 50, 35, and 25 kDa (*from top*). (*B*) SDS-PAGE results obtained with 5 μM actin and 3 μM tropomyosin. (*C*) Gel for 5 μM actin, 0.5 μM formin, and 3 μM tropomyosin. Observed protein bands are indicated. Experiments were performed in 4 mM Tris-HCl (pH 7.3), 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM DTT, 0.2 mM EGTA, 1 mM $MgCl_2$, and 50 mM KCl.

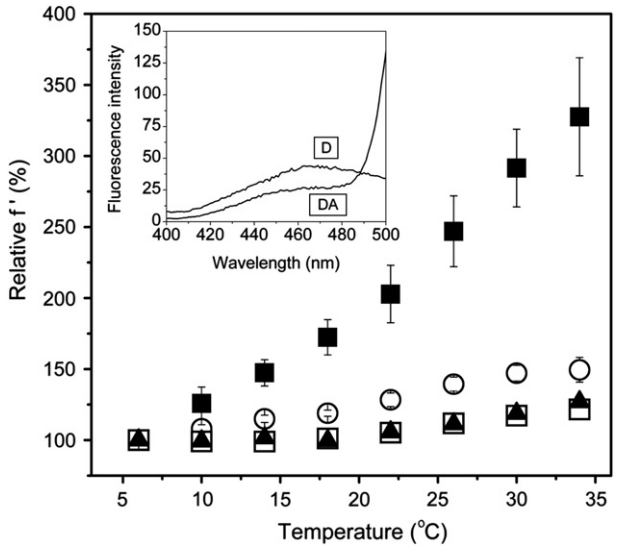


FIGURE 2 Tropomyosin decreases flexibility of formin-bound actin filaments. Temperature dependence is shown of normalized FRET efficiency (relative f') for actin filaments. Experiments were performed with 10 μM actin in absence of actin-binding proteins (*open squares*), or in presence of mDia1-FH2 (500 nM; *solid squares*). Data obtained in presence of tropomyosin (2 μM) and in absence (*solid triangles*) or presence (*open circles*) of 500 nM formin are shown. Errors presented are standard errors from at least three independent experiments. (*Inset*) Fluorescence intensity of donor measured in absence of actin-binding proteins. Data were obtained in absence (D) or presence (DA) of acceptor (as indicated). Buffer conditions are as in Fig. 1.

became similar to that observed in the absence of formin and tropomyosin.

For the interpretation and confirmation of FRET results, anisotropy decay measurements were performed with IAE-DANS-labeled actin filaments. This method was successfully used previously to describe the dynamic properties of actin (17,41,42). Recent studies showed that the affinity of binding of mDia1-FH2 to the barbed end of actin filaments falls into a range of 20–50 nM (43,44). Therefore, the formin concentration we applied here (500 nM mDia1-FH2) sufficed to saturate the binding sites at the ends of filaments. The analysis of data resolved two rotational correlation times. The value of the shorter rotational correlation time was between 1–4 ns, and showed no formin or tropomyosin concentration dependence. In our interpretation, the shorter rotational correlation time can be related to the motion of the probe relative to the protein, which did not show significant formin-induced or tropomyosin-induced changes. In a previous work (16), the longer rotational correlation time indicated formin concentration dependence. In this study, the longer rotational correlation time was ~700–800 ns in the absence of formins, and decreased to ~250 ns in the presence of 500 nM mDia1-Fh2 (Fig. 3). This observation is in agreement with our previous results (16), and indicates that formin-binding made the actin filaments more flexible. We repeated the anisotropy decay experiments with formin-bound actin filaments in the presence of tropomyosin at various concentrations (Fig. 3, *A* and *B*).

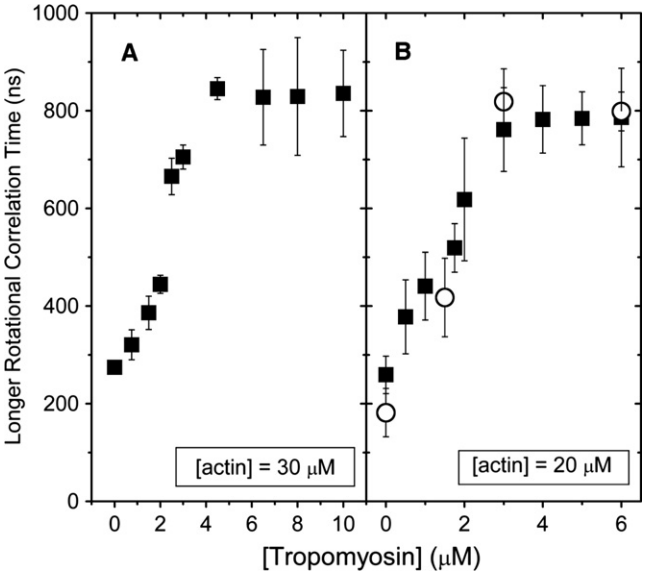


FIGURE 3 Tropomyosin affects anisotropy decay of formin-bound IAE-DANS-actin filaments. Tropomyosin concentration dependence is shown for longer rotational correlation times obtained for actin filaments. (*A*) Actin concentration was 30 μM, and sample also contained 1.25 μM mDia1-FH2. (*B*) Tropomyosin concentration dependence of longer rotational correlation times, measured with 20 μM actin and either 500 nM mDia1-FH2 (*solid squares*) or 500 nM mDia1-FH1FH2 (*open circles*). Errors presented are standard errors from at least three independent experiments. Buffer conditions are as in Fig. 1.

The value of the longer rotational correlation time increased with increasing tropomyosin concentrations from its value in the absence of tropomyosin (250 ns) to ~800 ns. When the actin concentration was 30 μM, the longer rotational correlation time reached its maximum at ~4 μM tropomyosin (Fig. 3 *A*). At 20 μM actin, the breaking point appeared at ~3 μM tropomyosin (Fig. 3 *B*). These tropomyosin concentrations correlate well with the [tropomyosin]/[actin] = 1:7 binding stoichiometry, indicating that the maximal tropomyosin effect was achieved when all tropomyosin-binding sites were occupied on the actin filaments. Under saturating conditions, the longer rotational correlation times of formin-bound actin filaments remained constant. These results indicate that the relatively flexible formin-bound actin filaments were stabilized by the binding of tropomyosin, in agreement with our conclusions from the FRET results (Fig. 2).

It is well-established that formin-domain FH1 also plays an important role in the biological function of formins (45). In another set of experiments, we investigated whether the presence of the FH1 domain could modify the effect of the FH2 domain on the conformation of actin filaments. The experiments were performed at 20 μM actin and at various mDia1-FH1FH2 concentrations. The longer rotational correlation time observed in the presence of mDia1-FH1FH2 and in the absence of tropomyosin was ~200 ns, similar to that observed with mDia1-FH2, indicating that the binding of mDia1-FH1FH2 increased the flexibility of actin filaments. The longer rotational correlation time increased when tropomyosin was added to the mDia1-FH1FH2-bound actin filaments. The values were identical to those measured with mDia1-FH2 at the corresponding tropomyosin concentrations (Fig. 3 *B*). This observation suggests that the FH1 domain does not modify the effect of the FH2 domain on the structure of actin filaments.

The effect of formins on actin filaments was shown to be ionic strength-dependent in previous studies (16,17). We measured the salt concentration dependence of the effect of tropomyosin on mDia1-FH2-bound actin filaments. The results showed that the effect of tropomyosin on actin filaments was independent of the KCl concentration between 10–30 mM KCl (Fig. 4 *A*).

These experiments were performed at 1 mM $MgCl_2$. Previous results showed that the interaction between tropomyosin and actin is magnesium-dependent. The affinity of tropomyosin for actin, and the corresponding association and dissociation rates, were dependent on magnesium concentration (46). The affinity is tighter at higher $MgCl_2$ concentrations (K_D = 2.4 μM at 0.5 mM $MgCl_2$, and K_D = 0.5 μM at 2.5 mM $MgCl_2$). These observations indicated that magnesium has an influence on the interaction between actin and tropomyosin. We tested the effect of magnesium on the interaction of tropomyosin and formin-bound actin filaments by using fluorescence anisotropy decay experiments. In these experiments, the actin concentration was 30 μM,

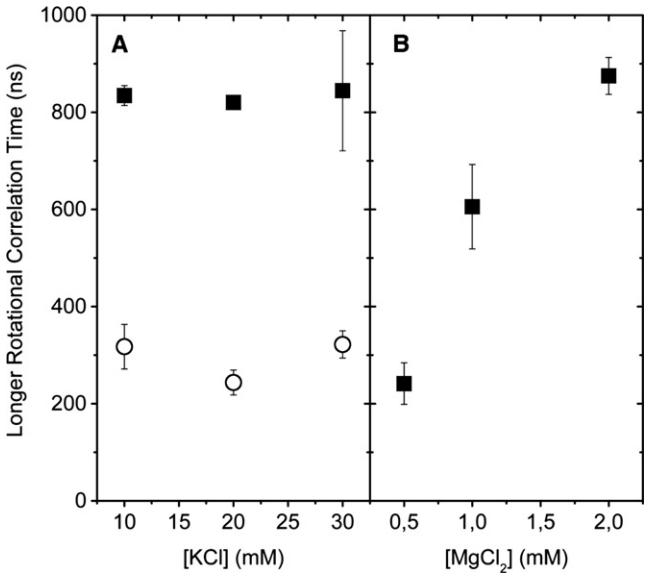
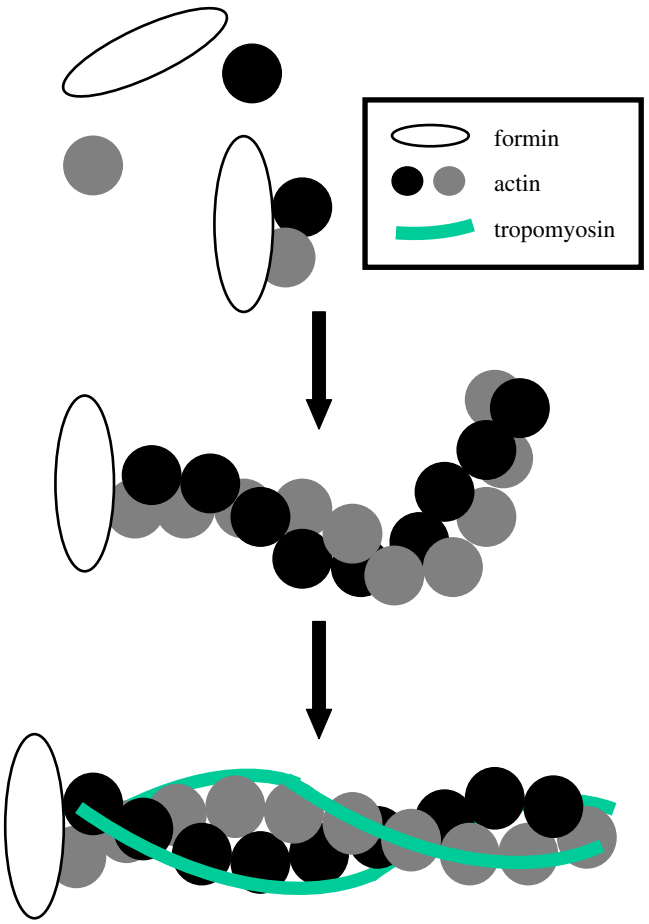


FIGURE 4 KCl and $MgCl_2$ dependence of effect of tropomyosin on flexibility of formin-bound actin filaments. (*A*) Results obtained at fixed $MgCl_2$ concentration (1 mM) with 30 μM actin and 1.25 μM formin in either absence (*open circles*) or presence (*solid squares*) of tropomyosin (8 μM). Experiments were performed at various KCl concentrations in 4 mM Tris-HCl (pH 7.3), 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM DTT, 0.2 mM EGTA, and 1 mM $MgCl_2$. (*B*) results of magnesium-dependent experiments at fixed KCl concentration (30 mM) with 30 μM actin, 1.25 μM formin, and 4 μM tropomyosin. Other buffer conditions are as in Fig. 1.

and the tropomyosin and formin concentrations were 4 μM and 1.25 μM, respectively. Considering the affinity of tropomyosin for actin (46), under these conditions most of the tropomyosin-binding sites on the actin filaments were occupied by tropomyosin, regardless of the magnesium concentration. Thus, these experiments reflected the alterations of the interaction between actin and tropomyosin, and not the level of saturation of tropomyosin-binding sites on actin. The fluorescence data showed that the longer rotational correlation time increased from ~250 ns at 0.5 mM $MgCl_2$ to ~850 ns at 2 mM $MgCl_2$ (Fig. 4 *B*). The strong magnesium concentration dependence of the tropomyosin effect was in accordance with previous observations (46,47), and indicated that the tropomyosin-induced stabilization effect was modified by magnesium.

CONCLUSIONS

The observation that formins can make actin filaments more flexible (16,17) can be understood in two ways. It is possible that the change in actin filament dynamics is only the consequence of the rapid and distinct process of polymerization in the presence of formins, and the increase in flexibility by itself does not have a biological function. Alternatively, the generation of flexible actin filaments can serve a well-defined biological aim, as manifested under special intracellular conditions for particular functions. One may speculate that the latter reason is less probable, because the functions (as attributed to actin filaments so far) require the mechanical stability of these filaments. If the first explanation is valid, then it is reasonable to assume that a cellular mechanism regulates the dynamic properties of actin filaments, and provides the opportunity for formin-induced filaments to become similar to those polymerized in the absence of formins. Tropomyosin appears to be the right candidate to play a role in this mechanism, because tropomyosin was associated with formin-mediated actin filament structures in cells (20,21). In our study, we tested whether tropomyosin can stabilize the conformation of actin filaments, and found that tropomyosin is able to reverse formin-induced conformational changes. This observation provides support for the contention that the increased flexibility of formin-induced actin filaments is only the consequence of the special process of their polymerization, and to adapt these filaments to their biological function, a subsequent tropomyosin-based mechanism exists in cells. The stabilization by tropomyosin is probably manifested structurally by the binding of contact points between appropriate surfaces on actin and tropomyosin, which implements



SCHEME 1 Model for sequence of events during formin-induced actin polymerization and subsequent tropomyosin binding. First step is elongation, after rapid formin-induced actin nucleation. Second step is binding of tropomyosin, which stabilizes structure of actin filaments from formin-mediated polymerization.

molecular strains and forces to restore the conformational state of the actin filaments, similar to that established during polymerization in the absence of formins. Based on this consideration, one can envisage that other actin-binding proteins possessing binding sites on neighboring actin protomers can also function as molecular clamps in cells, adjusting and maintaining the conformation of actin filaments. We suggest that an alternative candidate could exist in the myosin superfamily. Experiments testing this hypothesis are in progress.

Based on our observations, a simple model for the time sequence of events is presented in Scheme 1. According to this model, formins nucleate actin and initiate the elongation of actin filaments. This polymerization results in flexible actin filaments that require further adjustments for their biological function. The subsequent step is the binding of tropomyosin and the formation of formin-actin-tropomyosin complexes. The structure of actin filaments is stabilized in these complexes, and their dynamic properties are more appropriate for expressing force or fulfilling other mechanical tasks. Regardless of whether this model is complete and valid or needs adjustment in later studies, our results here provide experimental support for a molecular mechanism where the effect of tropomyosin plays a central role in forming mechanically stable actin filaments, even in the case of formin-induced rapid filament assembly.

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REFERENCES

1. Evangelista, M., S. Zigmond, and C. Boone. 2003. Formins: signaling effectors for assembly and polarization of actin filaments. *J. Cell Sci.* 116:2603–2611.
2. Higgs, H. N., and T. D. Pollard. 1999. Regulation of actin polymerization by Arp2/3 complex and WASp/Scar proteins. *J. Biol. Chem.* 274:32531–32534.
3. Trumpp, A., P. A. Blundell, J. L. de la Pompa, and R. Zeller. 1992. The chicken limb deformity gene encodes nuclear proteins expressed in specific cell types during morphogenesis. *Genes Dev.* 6:14–28.
4. Castrillon, D. H., and S. A. Wasserman. 1994. Diaphanous is required for cytokinesis in *Drosophila* and shares domains of similarity with the products of the limb deformity gene. *Development.* 120:3367–3377.
5. Zigmond, S. H. 2004. Formin-induced nucleation of actin filaments. *Curr. Opin. Cell Biol.* 16:99–105.
6. Pruyne, D., M. Evangelista, C. Yang, E. Bi, S. Zigmond, et al. 2002. Role of formins in actin assembly: nucleation and barbed-end association. *Science.* 297:612–615.
7. Robinson, N. G., L. Guo, J. Imai, E. A. Toh, Y. Matsui, et al. 1999. Rho3 of *Saccharomyces cerevisiae*, which regulates the actin cytoskeleton and exocytosis, is a GTPase which interacts with Myo2 and Exo70. *Mol. Cell. Biol.* 19:3580–3587.
8. Hild, G., M. Nyitrai, R. Gharavi, B. Somogyi, and J. Belagyi. 1996. Fluorescence quenching of the tryptophan emission from the F- and G-forms of actin. *J. Photochem. Photobiol. B.* 35:175–179.
9. Lőrinczy, D., and J. Belagyi. 2007. Intermediate states of myosin head during ATP hydrolysis cycle in psoas muscle fibres by EPR and DSC (a review). *J. Therm. Anal. Calorim.* 90:611–621.
10. Lőrinczy, D., F. Könczöl, B. Gaszner, and J. Belágyi. 1998. Structural stability of actin as studied by DSC and EPR. *Thermochim. Acta.* 322:95–100.
11. Nyitrai, M., G. Hild, E. Bodis, A. Lukacs, and B. Somogyi. 2000. Flexibility of myosin-subfragment-1 in its complex with actin as revealed by fluorescence resonance energy transfer. *Eur. J. Biochem.* 267:4334–4338.
12. Orban, J., K. Pozsonyi, K. Szarka, S. Barkó, E. Bodis, et al. 2006. Thermal characterization of actin filaments prepared from ADP-actin monomers. *J. Therm. Anal. Calorim.* 84:619–623.
13. Romero, S., D. Didry, E. Larquet, N. Boisset, D. Pantaloni, et al. 2007. How ATP hydrolysis controls filament assembly from profilin-actin: implication for formin processivity. *J. Biol. Chem.* 282:8435–8445.
14. Paul, A., and T. Pollard. 2008. The role of the FH1 domain and profilin in formin-mediated actin-filament elongation and nucleation. *Curr. Biol.* 18:9–19.
15. Baumann, B. A., H. Liang, K. Sale, B. D. Hambly, and P. G. Fajer. 2004. Myosin regulatory domain orientation in skeletal muscle fibers: application of novel electron paramagnetic resonance spectral decomposition and molecular modeling methods. *Biophys. J.* 86:3030–3041.
16. Papp, G., B. Bugyi, Z. Ujfalusi, S. Barko, G. Hild, et al. 2006. Conformational changes in actin filaments induced by formin binding to the barbed end. *Biophys. J.* 91:2564–2572.
17. Bugyi, B., G. Papp, G. Hild, D. Lorinczy, E. M. Nevalainen, et al. 2006. Formins regulate actin filament flexibility through long range allosteric interactions. *J. Biol. Chem.* 281:10727–10736.
18. Yasui, B., F. Fuchs, and F. N. Briggs. 1968. The role of the sulfhydryl groups of tropomyosin and troponin in the calcium control of actomyosin contractility. *J. Biol. Chem.* 243:735–742.
19. Mizuno, H., A. Hamada, K. Shimada, and H. Honda. 2007. Tropomyosin as a regulator of the sliding movement of actin filaments. *Biosystems.* 90:449–455.
20. Wawro, B., N. J. Greenfield, M. A. Wear, J. A. Cooper, H. N. Higgs, et al. 2007. Tropomyosin regulates elongation by formin at the fast-growing end of the actin filament. *Biochemistry.* 46:8146–8155.
21. Evangelista, M., D. Pruyne, D. C. Amberg, C. Boone, and A. Bretscher. 2002. Formins direct Arp2/3-independent actin filament assembly to polarize cell growth in yeast. *Nat. Cell Biol.* 4:260–269.
22. Wu, J. Q., J. R. Kuhn, D. R. Kovar, and T. D. Pollard. 2003. Spatial and temporal pathway for assembly and constriction of the contractile ring in fission yeast cytokinesis. *Dev. Cell.* 5:723–734.
23. Chang, F., D. Drubin, and P. Nurse. 1997. cdc12p, a protein required for cytokinesis in fission yeast, is a component of the cell division ring and interacts with profilin. *J. Cell Biol.* 137:169–182.
24. Pruyne, D., and A. Bretscher. 2000. Polarization of cell growth in yeast. I. Establishment and maintenance of polarity states. *J. Cell Sci.* 113:365–375.
25. Pruyne, D., and A. Bretscher. 2000. Polarization of cell growth in yeast. *J. Cell Sci.* 113:571–585.
26. Feuer, G., F. Molnár, E. Pettkó, and F. B. Straub. 1948. Studies on the composition and polymerisation of actin. *Hung. Acta Physiol.* 1: 150–163.
27. Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.
28. Houk, T. W., Jr., and K. Ue. 1974. The measurement of actin concentration in solution: a comparison of methods. *Anal. Biochem.* 62:66–74.
29. Elzinga, M., J. H. Collins, W. M. Kuehl, and R. S. Adelstein. 1973. Complete amino-acid sequence of actin of rabbit skeletal muscle. *Proc. Natl. Acad. Sci. USA.* 70:2687–2691.

30. Shimada, A., M. Nyitrai, I. R. Vetter, D. Kuhlmann, B. Bugyi, et al. 2004. The core FH2 domain of diaphanous-related formins is an elongated actin binding protein that inhibits polymerization. *Mol. Cell.* 13:511–522.

31. Eisenberg, E., and W. W. Kielley. 1974. Troponin-tropomyosin complex. Column chromatographic separation and activity of the three, active troponin components with and without tropomyosin present. *J. Biol. Chem.* 249:4742–4748.

32. Smillie, L. B. 1982. Preparation and identification of α- and β-tropomyosins. *Methods Enzymol.* 85:234–241.

33. Miki, M., C. G. dos Remedios, and J. A. Barden. 1987. Spatial relationship between the nucleotide-binding site, Lys-61 and Cys-374 in actin and a conformational change induced by myosin subfragment-1 binding. *Eur. J. Biochem.* 168:339–345.

34. Hudson, E. N., and G. Weber. 1973. Synthesis and characterization of two fluorescent sulfhydryl reagents. *Biochemistry.* 12:4154–4161.

35. Hild, G., M. Nyitrai, and B. Somogyi. 2002. Intermonomer flexibility of Ca- and Mg-actin filaments at different pH values. *Eur. J. Biochem.* 269:842–849.

36. Miki, M. 1990. Resonance energy transfer between points in a reconstituted skeletal muscle thin filament. A conformational change of the thin filament in response to a change in Ca^{2+} concentration. *Eur. J. Biochem.* 187:155–162.

37. Somogyi, B., J. Matko, S. Papp, J. Hevessy, G. R. Welch, et al. 1984. Forster-type energy transfer as a probe for changes in local fluctuations of the protein matrix. *Biochemistry.* 23:3403–3411.

38. Somogyi, B., Z. Lakos, A. Szarka, and M. Nyitrai. 2000. Protein flexibility as revealed by fluorescence resonance energy transfer: an extension of the method for systems with multiple labels. *J. Photochem. Photobiol. B.* 59:26–32.

39. Lakowicz, J. R. 1983. Principles of Fluorescence Spectroscopy. Plenum Press, New York., 75–81 p.

40. Belford, G. G., R. L. Belford, and G. Weber. 1972. Dynamics of fluorescence polarization in macromolecules. *Proc. Natl. Acad. Sci. USA.* 69:1392–1393.

41. Miki, M., P. Wahl, and J. C. Auchet. 1982. Fluorescence anisotropy of labeled F-actin: influence of divalent cations on the interaction between F-actin and myosin heads. *Biochemistry.* 21:3661–3665.

42. Miki, M., P. Wahl, and J. C. Auchet. 1982. Fluorescence anisotropy of labelled F-actin. Influence of Ca^{2+} on the flexibility of F-actin. *Biophys. Chem.* 16:165–172.

43. Li, F., and H. N. Higgs. 2003. The mouse formin mDia1 is a potent actin nucleation factor regulated by autoinhibition. *Curr. Biol.* 13:1335–1340.

44. Romero, S., C. Le Clainche, D. Didry, C. Egile, D. Pantaloni, et al. 2004. Formin is a processive motor that requires profilin to accelerate actin assembly and associated ATP hydrolysis. *Cell.* 119:419–429.

45. Goode, B. L., and M. J. Eck. 2007. Mechanism and function of formins in the control of actin assembly. *Annu. Rev. Biochem.* 76:593–627.

46. Wegner, A. 1979. Equilibrium of the actin-tropomyosin interaction. *J. Mol. Biol.* 131:839–853.

47. Fowler, V. M., and V. Bennett. 1984. Erythrocyte membrane tropomyosin. Purification and properties. *J. Biol. Chem.* 259:5978–5989.