Formins Regulate Actin Filament Flexibility through Long Range Allosteric Interactions*

Received for publication, September 19, 2005, and in revised form, February 16, 2006. Published, JBC Papers in Press, February 20, 2006, DOI 10.1074/jbc.M510252200

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The members of the formin family nucleate actin polymerization and play essential roles in the regulation of the actin cytoskeleton during a wide range of cellular and developmental processes. In the present work, we describe the effects of mDia1-FH2 on the conformation of actin filaments by using a temperature-dependent fluorescence resonance energy transfer method. Our results revealed that actin filaments were more flexible in the presence than in the absence of formin. The effect strongly depends on the mDia1-FH2 concentration in a way that indicates that more than one mechanism is responsible for the formin effect. In accordance with the more flexible filament structure, the thermal stability of actin decreased and the rate of phosphate dissociation from actin filaments increased in the presence of formin. The interpretation of the results supports a model in which formin binding to barbed ends makes filaments more flexible through long range allosteric interactions, whereas binding of formin to the sides of the filaments stabilizes the protomer-protomer interactions. These results suggest that formins can regulate the conformation of actin filaments and may thus also modulate the affinity of actin-binding proteins to filaments nucleated/capped by formins.

Formins are evolutionarily conserved proteins (1, 2) that activate signaling pathways and nucleate actin filaments independently of the Arp2/3 complex (3–6). In mammalian cells, formins play a role in the formation of stress fibers, cell motility, signaling, gene transcription, and embryonic development (7–13). In yeast, formins organize cytoplasmic actin cables and the contractile ring (1, 3, 14–17). Formins are composed of multiple domains (2), which can include formin homology domains (18) (FH1, FH2, FH3), N-terminal GTPase-binding domain (GBD),3 and C-terminal diaphanous-autoregulatory domain (DAD). FH1 and FH2 domains are present in all formins (15). The proline-rich FH1 can interact with profilin, with factors involving the SH3 domain (19) and the Src family kinases (9, 14, 17, 19, 20). The FH2 domain is required for the interaction with actin, for the stabilization of microtubules, and for serum response factor activation (5, 9, 12, 21). Diaphanous-related formins involve GBD and DAD domains (22). In some diaphanous-related formins, binding of activated Rho relieves intramolecular interactions between the DAD and N-terminal sequences (19, 23).

Biophysical characterization of formin fragments from mammalian sources (from mouse, mDia1 (4, 24–26) and mDia3 (25)), from Saccharomyces cerevisiae (Bni1p and Bnr1p) (3, 24), and from Schizosaccharomyces pombe (Cdc12p) (27) established that they were potent actin nucleators in vitro and that the FH2 domain was essential for the nucleation. Recent structural studies have given insights into the molecular mechanisms responsible for the formin functions. The structures of the FH2 domains from mDia1 (25), from Bni1p (28), from the complex of actin with Bni1-FH2 (29), and from the complex of the GBD from mDia1 and Rho A (30) have been determined so far.

Despite the numerous studies characterizing the effect of formin fragments on the polymerization properties of actin, very little is known about the effect of formins on the conformation of actin. One of the few related observations was that the affinity of CapG protein for actin filaments decreased by a factor of ~100 in the presence of mDia1-FH2 fragments (26), and the weaker affinity was not due to the direct competition of the two proteins. This observation indicates that mDia1-FH2 (and also the FH1-FH2 fragment) may induce conformational changes at the barbed end of actin filaments. Supporting the existence of the formin effect on actin, the recently solved structure of the complex of Bni1p-FH2 and actin showed that a special conformational strain was generated when formin bound to the actin structure mimicking the barbed end (29).

In this work, we studied the effects of mDia1-FH2 fragments on the conformational properties of actin filaments by using temperature-dependent fluorescence resonance energy transfer (FRET) measurements. The results revealed that mDia1-FH2 fragments increased the flexibility of actin filaments. The magnitude of the effect of formin depended on the formin:actin concentration ratio. The characteristics of the formin concentration dependence indicated that more than one mechanism was involved in the formin effects. The results were interpreted assuming that formin binding to the barbed end made the actin filaments more flexible. This formin effect propagated through allosteric interaction to actin protomers far from the barbed end. At greater formin concentrations, the binding of formin fragments to the sides of the actin filaments could stiffen the interaction between neighboring actin protomers along the filaments. Based on these observations, we hypothesize that actin filaments serve as information channels in vivo. The formin-induced conformational changes in actin filaments can play a role in modifying and regulating the affinity of various actin-binding proteins for actin. This possible new mechanism could operate in the case of other actin nucleating factors as well and may provide a novel mechanism to regulate the formation of cytoskeletal protein complexes.
Formin Effects on Actin Filaments

MATERIALS AND METHODS

Protein Preparations and Modifications—A core formin mDia1-FH2 fragment (amino acids 826–1163) and a longer mDia1-FH2 fragment (amino acids 752–1163) involving the linker region between the FH1 and FH2 domains was prepared as described in Ref. 25. The former is a monomeric protein (25) and assigned in this study as mDia1-FH2monomer, whereas the latter is a dimer and indicated as mDia1-FH2dimer throughout the study. After the preparation, the formin fragments were kept in −80 °C in storing buffer (50 mM Tris/HCl, pH 7.6, 50 mM NaCl, 5 mM dithiothreitol, 5% glycerol). Mouse coflin-1 was purified as described in Ref. 31. Actin from rabbit skeletal muscle was prepared as described previously (32, 33). The results presented in this work were obtained using five independent formin and more than 10 independent actin preparations. Further purification of actin by a Sephacryl 300 column did not affect the results of our experiments (data not shown). The concentrations of actin and formin are given as monomeric concentrations throughout the study. The concentration of actin was determined by using the extinction coefficient of 0.63 mg ml−1 cm−1 at 290 nm (34). The mDia1-FH2monomer and mDia1-FH2dimer concentrations were determined by measuring the absorption at 280 nm in 6 M guanidine hydrochloride using the extinction coefficients of 7,680 M−1 cm−1 and 20,580 M−1 cm−1, which were estimated with ProtParam from the sequences (ExPASy Proteomics tools). Actin was labeled with pyrene (35), IAEDANS, or IAF (36, 37) on the Cys374 according to standard procedures. The concentrations of the probes were determined using the absorption coefficients of 22,000 M−1 cm−1 at 344 nm for pyrene, 6,100 M−1 cm−1 at 336 nm for IAEDANS, and 60,000 M−1 cm−1 at 495 nm for IAF. The labeling ratio, i.e. the molar ratio of the bound probe to the actin concentration, was 0.9−1.0 for pyrene, 0.8−0.9 for IAEDANS, and 0.6−0.7 for IAF.

Fluorescence Experiments—Monomeric calcium-actin was in 4 mM Tris/HCl, pH 7.3, 0.2 mM ATP, 0.5 mM dithiothreitol, and 0.1 mM CaCl2 after preparation. The bound calcium was replaced with magnesium by adding 200 µM EGTA and 50 µM MgCl2 and incubating the samples for 5−10 min. The magnesium-actin was polymerized with 0.5 mM MgCl2 and 10 mM KCl in either the presence or the absence of formin fragments. The actin concentration was 5 µM unless stated otherwise.

IAEDANS served as donor and IAF served as acceptor in the FRET experiments. Actin monomers separately labeled with either donor or acceptor were mixed in a 1:9 = donor-labeled:acceptor-labeled actin ratio. For FRET, reference samples were also prepared with 10% donor-labeled and 90% unlabeled actin. Then MgCl2 and KCl were added to the monomer actin to initiate polymerization, and the samples were incubated overnight at 4 °C before the fluorescence experiments. The volume of the storing buffer, regardless of whether it was added with or without formin, was kept constant (5%) in the samples.

To determine the efficiency (E) of the FRET, the fluorescence emission of the donor was measured in the absence (F_D) and presence (F_DA) of acceptor, and the following equation was applied.

\[ E = 1 - F_{DA}/F_D \] (Eq. 1)

Fluorescence experiments were carried out with a PerkinElmer Life Sciences LS50B spectrophotometer. To determine F_D and F_DA, the spectra of the samples were recorded using the excitation wavelength of 350 nm. The measured intensities were corrected for the inner filter effect using the absorption spectra of the samples (38), and the integrated intensities of the emission spectra between 440 and 460 nm in the absence and presence of acceptor were used as F_D and F_DA, respectively.

To obtain information regarding the dynamic and conformational properties of actin filaments, the value of E was determined at different temperatures between 6 and 30 °C, and a special FRET parameter, the normalized FRET efficiency (f'), was calculated using

\[ f' = E/F_{DA} \] (Eq. 2)

The temperature dependence of the f' is informative regarding the flexibility of the investigated protein (39, 40) and was used previously to characterize the effect of cations (41, 42), nucleotides (43), and pH (37) on actin filaments. For the interpretation of the 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 in this work.

The intensity of actin-bound pyrene increases by a factor of ~25 upon polymerization (44). Pyrene actin was used here to monitor the time dependence of the formation of actin filaments. In these experiments, 5% of the actin was labeled with pyrene. The excitation and emission wavelengths were 365 and 407 nm, respectively, whereas the optical slits were set to 5 nm in both the excitation and the emission paths. The elongation rate was obtained from the slopes of the pyrene intensity versus time curves at 50% completion of the polymerization. When actin (5% pyrene-labeled) was polymerized overnight at different concentrations, the actin concentration dependence of the pyrene intensity was used to determine the critical concentration of actin filament assembly.

Differential Scanning Calorimetry (DSC)—The thermal denaturation of actin filaments was monitored between 0 and 100 °C with a SETARAM Micro DSC-II calorimeter. The heating rate was 0.3 K/min, and the actin concentration was 60 µM. The experiments were carried out in the absence or presence of 3 µM mDia1-FH2dimer. Calorimetric enthalpy change (ΔH) of the endothermic transitions was calculated from the area under the heat absorption curve. Transition entropy change (ΔS) was calculated for the peak transition temperature (T_m) from the following equation.

\[ ΔS = ΔH/T_m \] (Eq. 3)

The Gibbs free enthalpy change was calculated for 22 °C from the following equation.

\[ ΔG = ΔH - TΔS \] (Eq. 4)

Determination of the Phosphate Release Rate—The rate of dissociation of phosphate from actin filaments was measured based on the method originally described by Webb (45) using the EnzChek phosphate assay kit (Molecular Probes). In the presence of P_i, the substrate 2-amino-6-mercapto-7-methylpurine riboside is converted enzymatically by purine nucleoside phosphorylase to ribose 1-phosphate and 2-amino-6-mercapto-7-methyl-purine. The enzymatic conversion of 2-amino-6-mercapto-7-methyl-purine riboside was followed by measuring the absorption at 360 nm. The experimental strategy was similar to that applied earlier by Carlier (46). First the bound calcium was replaced by magnesium in actin monomers, and then the actin at 100 µM was polymerized by the addition of 0.5 mM MgCl2 and 10 mM KCl in the absence or presence of formin. The polymerization was carried out for 2 min in the absence and for 1 min in the presence of formin. Then actin was added to the reaction buffer to a final concentration of 10 µM, and the absorbance at 360 nm was monitored. The reaction medium contained the standard buffer from the manufacturer (100 mM Tris-HCl, pH 7.5, 2 mM MgCl2, and 0.2 mM sodium azide) supplemented with 10 mM KCl.
Co-sedimentation Assays—To characterize the binding of formin to actin filaments, actin (5 μM; 200 μl) was polymerized overnight at 4 °C as in the FRET assays in the absence or presence of various mDia1-FH2dimer concentrations ranging from 0 to 5 μM. The samples were then centrifuged at 400,000 × g for 30 min at 20 °C with a Beckman Optima MAX benchtop ultracentrifuge and a TLA-100 rotor. The supernatants were separated from the pellets, and the pellets were resuspended in 200 μl of buffer. All supernatants and pellets were applied to a 12% SDS gel, and the gels were stained with Comassie Blue. The actin band intensities were determined with a Syngene bio-imaging system. The affinity (K) of formin to the sides of actin filaments was estimated by analyzing the [bound formin] versus [total formin] plots using the following equation (47).

\[
[A]_0 D^2 - ([A]_0 + [D]_0 + K) D + [D]_0 = 0 \quad \text{(Eq. 5)}
\]

where \([A]_0\) and \([D]_0\) are the total formin and actin concentrations, respectively, and \(D\) is the fraction of bound formin. The fraction of bound formin was calculated as the ratio of the intensity of formin bands to that of the actin bands in the pellets.

When the effect of formin on the interaction of actin filaments with cofilin was tested, preformed actin filaments (5 μM) polymerized either in the absence or in the presence of 0.5 μM mDia1-FH2dimer were incubated overnight at 4 °C with various concentrations of cofilin ranging from 0 to 5 μM. The samples were centrifuged at 400,000 × g for 30 min at 20 °C, and the supernatants were applied to 12% SDS gels to quantify the concentration of the depolymerized actin. The actin band intensities were determined using a Syngene bio-imaging system. The actin concentrations in the supernatants were determined as

\[
c_{\text{actin, sn}} = \frac{[B^\text{sn}]}{[B^\text{sn} + B^\text{pol}]} \times c_{\text{actin, total}} \quad \text{(Eq. 6)}
\]

where \(B^\text{sn}\) and \(B^\text{pol}\) are the actin band intensities in the supernatant and pellet, respectively, and \(c_{\text{actin, total}}\) is the total actin concentration (5 μM).

RESULTS

Our initial assays demonstrated that the fluorescence emission of IAEDANS-actin filaments (10% of the total 5 μM actin) decreased in the presence of acceptor due to the effect of FRET (Fig. 1A). In test experiments, the spectra were also recorded in the presence of 500 nM mDia1-FH2dimer (Fig. 1B). The results showed that the mechanism of FRET was effective to decrease the donor intensity in the presence of formin as well, which allowed the use of FRET method to examine the effects of formins on actin filaments.

"Temperature-dependent FRET Can Be Applied for Examining the Effects of Formins on Actin Filaments—" A characteristic feature of formins is that they accelerate the polymerization of actin solutions in vitro by enhancing the rate of the formation of actin nuclei and by promoting barbed end assembly. By following the time kinetics of the pyrene signal, we observed that 64 nM mDia1-FH2dimer accelerated the actin polymerization ~8-fold (Fig. 2A). Control experiments showed that mDia1-FH2dimer was effective in enhancing the polymerization rate by ~8-fold, to an extent similar to that observed with pyrene actin. When the IAEDANS emission was recorded in the presence of formin, the intensity decreased upon polymerization due to the appearance of FRET (Fig. 2B). In this case, the kinetics observed in the absence of formin was ~2-fold slower than that measured with pyrene actin, suggesting that the rate of association of acceptor-labeled actin monomers to the filaments was slower than that of the unlabeled ones. Formin (64 nM mDia1-FH2dimer) accelerated the polymerization of the double-labeled actin samples ~10-fold, which is close to the value measured with pyrene actin (~8-fold). These observations demonstrated that mDia1-FH2dimer retained its ability to accelerate the polymerization of actin after the actin was labeled with the fluorescent probes. Similar experiments at various formin concentrations (from 50 nM to 1.5 μM) corroborated this conclusion (data are not shown).

In this study, we applied temperature-dependent fluorescence experiments. Under any conditions, a fraction of the actin population, characterized by the critical concentration, is in monomeric form. Actin in its monomeric form cannot contribute to the measured transfer efficiency, and the temperature-induced changes in the critical concentration could insert errors to the interpretation of the FRET data. It was therefore important to test whether the temperature change between 6 and 30 °C shifted the equilibrium between the monomeric and filamentous forms of actin.

The critical concentration of actin was measured using the pyrene assay at 6, 22, and 30 °C (data are not shown). The critical concentration was 250–300 nM, in agreement with our previous observations (25), and proved to be temperature-independent. The experiments were repeated in the presence of 250 nM mDia1-FH2dimer (Fig. 2C). Neither the formin nor the temperature changed the critical concentration for actin assembly. The formin independence of the critical concentration is in agreement with previous observations (4, 26).

To test the effect of the applied donor and acceptor on the temperature dependence of the critical concentration, the FRET efficiency (E) was determined as a function of actin concentration. The method is based on the consideration that FRET cannot effectively occur between unpolymerized actin monomers, and thus the FRET efficiency can be used as the measure of actin filament concentration. Fig. 2D shows the data obtained at 22 °C. Fit to the actin concentration dependence of the FRET efficiency gave critical concentration of 421 ± 42 nM in the absence and 359 ± 67 nM in the presence of 250 nM mDia1-FH2dimer. These values are somewhat greater than the one obtained with the pyrene assay (250–300 nM). The higher critical concentration is most
likely a consequence of the association rate of donor- and acceptor-labeled actin, which was slower than that of pyrene-labeled (5%) monomers (Fig. 2B). Using the FRET method, the critical concentration of the actin found to be 397 ± 73 nm at 6 °C and 420 ± 48 nm at 30 °C in the absence of formin. In the presence of 250 nM mDia1-FH2dimmer, the critical concentration was 457 ± 60 nm at 6 °C and 420 ± 55 nm at 30 °C. These results indicate that the critical concentration of the actin labeled with the applied fluorophores remained temperature- and formin-independent.

In general, temperature-induced changes in the mean donor-acceptor distance could affect the temperature dependence of the relative $f'$. In the actin filament, one donor is interacting with more than one (maximum four) acceptor on neighboring protofilaments. Due to the helical symmetry of the actin filaments, any change in the position of the labeled residue (Cys374 here) parallel to the longitudinal filament axis will not cause changes of the mean donor-acceptor distance. It is only the radial coordinate (the distance between the residue and the longitudinal filament axis) that can modify this distance. We tested the value of radial coordinates using the method of Taylor et al. (48) by measuring the FRET efficiency in the absence and presence of mDia1-FH2dimmer ([formin]/[actin] = 1:10 ratio) at seven acceptor molar labeling ratios between 0 and 0.7 at 10, 22, and 30 °C. The results showed that the radial coordinate of the Cys374 residue was not affected by the temperature in either the absence or the presence of formin (data are not shown), indicating that the changes of this parameter were not responsible for the temperature dependence of the relative $f'$.

*mDia1-FH2dimmer* induces conformational changes in actin filaments—
To characterize the formin-induced conformational changes in actin filaments, we carried out temperature-dependent FRET experiments. The efficiency ($E$) of the FRET (Equation 1) and the normalized transfer efficiency ($f'$; Equation 2) were determined over the temperature range from 6 to 30 °C. In the absence of formin, the temperature dependence of the relative $f'$ measured at 5 μM actin showed monotonic increase to ~150% (Fig. 3A). In the presence of equimolar mDia1-FH2dimmer, the temperature dependence of the relative $f'$ was still monotonic but increased more steeply (to 175% at 30 °C) than in the absence of formin. This observation indicated that the mDia1-FH2dimmer fragment modified the conformation of actin filaments by making the filaments more flexible. When the experiments were repeated by keeping the 1:1 formin:actin ratio and increasing the concentration of both proteins to 10 μM,
the FRET results were essentially identical to those obtained at 5 μM actin and formin (Fig. 3A, inset). The actin concentration independence of the FRET results corroborated the conclusion from critical concentration experiments that the temperature cannot induce substantial shift in the monomer-filament equilibrium, and thus the temperature-dependent FRET data were not biased by this undesired temperature effect.

In control measurements, the FRET experiments were carried out under various salt conditions. At 1 mM MgCl₂ and 50 mM KCl, the effect of mDia1-FH2dimmer was smaller on the temperature dependence of the relative FER by about a factor of 2 than measured under low salt conditions (0.5 mM MgCl₂ and 10 mM KCl). When the ionic strength was further increased by using 2 mM MgCl₂ and 100 mM KCl, the formin effect further decreased to ~30% of the effect observed under low salt conditions. Considering these observations, we performed the formin concentration-dependent FRET experiments under low salt conditions.

The temperature-dependent FRET experiments were also carried out by keeping the actin concentration constant (5 μM) and varying the mDia1-FH2dimmer concentration between 5 nM and 5 μM. Fig. 3B shows the results obtained at 500 nM mDia1-FH2dimmer. At this formin concentration, the relative F value showed a steep increase to ~270% (Fig. 3B). Repeated experiments at 500 nM mDia1-FH2dimmer (n = 3) gave relative F values between 250 and 290%. The change of the F value was greater at 500 nM mDia1-FH2dimmer than at 5 μM, indicating that the effect of mDia1-FH2dimmer depended on the formin concentration.

To interpret the formin concentration dependence of the FRET results, we choose the value of the relative F at 30 °C as the measure of the actin filament flexibility. This parameter increases monotonically with the increasing flexibility but is not linearly proportional to that (39, 40). The dependence of this flexibility parameter on the formin:actin ratio is presented in Fig. 4. The flexibility of actin filaments was increased by the formin fragment at all formin:actin ratios. The effect of mDia1-FH2dimmer increased with the increasing formin concentration up to ~750 nM, which corresponded to a 0.15:1 = formin:actin ratio. Above 750 nM, the effect of formin became smaller with increasing formin concentration.
For the interpretation of the FRET results, we considered the ability of formin fragments to bind to the sides of actin filaments (see "Discussion"). It was recently reported that mDia1-FH2 fragments bind to the sides of actin filaments with an affinity of ~3 μM (4). To test whether our mDia1-FH2dimer construct behaves similarly under the conditions applied here, co-sedimentation assays were carried out (Fig. 4B). At 5 μM mDia1-FH2dimer, the sum of formin band intensities in the pellet and supernatant was identical to the sum of actin (5 μM) band intensities after correcting for the difference in the molecular weights (data are not shown), indicating that Coomassie Blue stained equal masses of the two proteins identically. Although mDia1-FH2dimer did not pellet in the absence of actin (Fig. 4B, lane i), in the presence of actin, formin was found in the pellets, indicating that mDia1-FH2dimer bound to the sides of actin filaments (Fig. 4B). The concentration of formin in the pellets increased with increasing total formin concentrations (Fig. 4B). Although this tendency did not perfectly define a hyperbola, the data could be fitted with Equation 5 assuming that formin binds to the sides in a 1:1 stoichiometry (Fig. 4B, solid line). The hyperbola fit gave a binding affinity of 2.0 ± 0.2 μM. This affinity is tighter than that (3 μM) reported by Li and Higgs (4). Considering that their study was carried out at greater salt concentrations (1 mM MgCl2 and 50 mM KCl) than applied here (0.5 mM MgCl2 and 10 mM KCl), the difference between the two estimates suggests that the affinity of mDia1-FH2 for the sides of actin filaments is salt-dependent.

The effect of mDia1-FH2monomer on actin filaments was also described by the FRET method. Fig. 4C shows the dependence of actin filament flexibility on the mDia1-FH2monomer concentration. The actin filaments were more flexible in the presence of mDia1-FH2monomer than in the absence of it, although the transient formin concentration dependence found with mDia1-FH2dimer (Fig. 4A) was not observed in experiments with the mDia1-FH2monomer.

**Formin Decreases the Thermal Stability of Actin**—In previous studies, the dynamic behavior of actin resolved by spectroscopic assays often correlated with its thermal stability (e.g. Ref. 49). To test whether it was the case here, we measured the heat denaturation of actin filaments (60 μM) using differential scanning calorimetry. The experiments were carried out in the absence or presence of 3 μM mDia1-FH2monomer, i.e. at formin:actin ratio of 1:20. The results showed that the peak temperature of the endothermic melting (Tm) decreased from 61.9 °C in the absence of formin to 60.4 °C in the presence of formin (Fig. 5A). The relatively small formin-induced decrease of Tm was reproducible in repeated experiments (n = 3) and indicated that the thermal stability of actin filaments decreased in the presence of formin. The calorimetric enthalpy change (∆H), the entropy change (∆S), and the Gibbs free enthalpy (∆G) values were smaller when the experiments were carried out in the presence of formin (Table 1), supporting the conclusion that the filaments were thermodynamically less stable in the presence of mDia1-FH2monomer than in the absence of it.

**Formin Accelerates the Dissociation of Phosphate from Actin**—The dynamic properties of actin filaments were reported previously to correlate with the rate of phosphate dissociation from them (e.g. Ref. 50).
Here we tested the effect of formin on the rate of phosphate dissociation from actin filaments by using the method of Webb (45). A prerequisite for these experiments is that the formation of actin filaments is much faster than the phosphate release rate. To achieve this aim, the actin was first polymerized at relatively high concentration (100 μM). Under the applied conditions, more than 90% of the actin was in polymer form within the first 2 min as indicated by pyrene fluorescence (data are not shown). In the presence of formin, more than 90% of the actin polymerized in ~1 min. The filaments formed during this polymerization phase contained more Pi than in a dynamic equilibrium, which resulted in a burst phase of phosphate release during the first 10–20 min of the experiments (Fig. 5B, inset). A subsequent phase was also observed that followed linear tendency and was attributed to the phosphate release during the slow turnover of actin filaments. The absorption transients were analyzed by fitting the sum of a single exponential and a linear function to the experimental data (Fig. 5B, inset). In the presence of formin, the linear component of the fit appeared to be slower by approximately a factor of 2. This finding is in accordance with previous observations that mDia1-FH2 is a barbed end capper and slows down the association and dissociation of actin monomers to or from filaments. The amplitude of the exponential component was in the range of 0.05–0.07, which corresponded to 6–8 μM P, according to the calibration curve obtained with standard phosphate solutions. The rate determined from the exponential component corresponded to the first order phosphate release rate from actin filaments. In the absence of formin, the phosphate release rate was 1.85 × 10⁻³ s⁻¹. This value is in close agreement with previous estimates for this parameter (e.g. 1.98 × 10⁻³ s⁻¹ (51)). The phosphate release rate from actin filaments was increased by formin (Fig. 5B) and followed a formin concentration dependence similar to that observed for the filament flexibility in the FRET assays (Fig. 4A). These observations indicated that there was a direct correlation between the flexibility of actin filaments and the rate of phosphate dissociation from actin filaments.

### DISCUSSION

#### The Interpretation of the FRET Results

To characterize the effect of mDia1-FH2 on the dynamic properties of actin filaments, temperature-dependent FRET experiments were carried out. The basis of the application of this method is that the value of f⁺ is sensitive to the average distance between the donor and acceptor and also to the amplitude of the relative motion of these two probes (39, 40). Increasing temperature increases the rate and amplitude of the relative fluctuations of the donor and acceptor. Due to the inverse 6th power dependence of the transfer efficiency on the donor-acceptor distance, these effects of the increasing temperature result in an increase of f⁺ (39, 40). When the probes are located in a more flexible protein environment, the increase of the amplitude of the donor-acceptor fluctuations is greater, and thus the increase in the value of f⁺ is greater as well. Accordingly, the steeper temperature dependence of f⁺ reflects the more flexible form of the protein. Note that due to the nature of this method, the relative f⁺ is sensitive to all kinds of motions at any time scale that can influence the donor-acceptor distance. Thus the term flexibility does not exclusively correspond to either the torsional or the bending or any other specific filament flexibilities here. The separation of the effects of these different modes of motions on the temperature dependence of f⁺ is very difficult. This FRET method was previously applied in numerous cases to describe conformational changes in actin (37, 42, 43, 49, 52). In the present study, due to the applied experimental strategy, there was no actin monomer containing both donor and acceptor, and thus FRET was always occurred between probes on different actin protomers in the filaments. This strategy assured that the inter-protomer dynamics of the filaments were described.

We found in control experiments that the mDia1-FH2dimer fragment studied here interacted properly with actin over the investigated temperature range. Donor or acceptor labeling of actin did not diminish the formin-actin interactions. Neither formin nor the change in temperature caused substantial shift in the monomer-filament equilibrium. We concluded that the observed formin-induced changes in the temperature dependence of the f⁺ were attributed to the conformational changes within the actin filaments. The exact nature of these conformational changes is, however, not yet known, and we cannot exclude at this stage that apart from intramolecular conformational transitions, the binding of formin alters the supra-molecular filament structures.

#### The Effect of mDia1-FH2 on Actin Filaments

The temperature dependence of the f⁺ was steeper in the presence of mDia1-FH2dimer than in the absence of it at all investigated formin concentrations (Fig. 4), indicating that mDia1-FH2dimer increased the flexibility of actin filaments. We found that the effect of mDia1-FH2 on the flexibility of actin filaments was more pronounced at 0.5 mM MgCl₂ and 10 mM KCl than at 1 mM MgCl₂ and 50 mM KCl. This observation suggests that the formin effect may be smaller at physiological ionic strength. The increase in the flexibility detected by the FRET assay was accompanied by the decrease of the thermal stability of the filaments (Fig. 5A). The formin-induced flexibility change strongly depended on the applied formin concentration (Fig. 4A). The largest formin effect was observed at ~750 nM mDia1-FH2dimer, which corresponds to a 0.15:1 formin:actin protomer ratio. The formin concentration dependence of the actin flexibility presented in Fig. 4 suggests that the effect of mDia1-FH2dimer on actin involves at least two mechanisms.

#### A Model for the Interaction of Actin and mDia1-FH2dimer

It was established previously (4, 24, 25) that mDia1 fragments could bind to actin in two distinct ways, either at the barbed end or on the side of the filaments. We interpret our results considering these two binding mechanisms. The affinity for the binding of dimeric mDia1 fragments to...
the barbed end falls into the 20–50 nm range (4, 26), whereas side binding is characterized with a much weaker affinity of ~2–3 μM (4) (Fig. 4B). A scheme in Fig. 6 outlines the binding geometries involved in the interpretation.

At low mDia1-FH2 dimer concentrations (<750 nM), the binding of mDia1-FH2 dimer to the barbed end is dominating over the side binding due to two reasons: 1) the affinity is much tighter for the barbed end than for the side binding and 2) the formin:actin stoichiometry allows the saturation of only the minority of the filament side-binding sites. Increasing the formin concentration within the 0–750 nM range resulted in a saturation of the filament ends. The formin dependence up to 750 nM (Fig. 4) can be explained by assuming that formin binding to the barbed end increases the actin filament flexibility. The mDia1-FH2 used here is dimeric, which means that for example, at a 1:10 to side-binding sites ratio, there are at least 20 actin protomers for each formin dimer. However, this estimate puts only a lower limit to the number of actin monomers in a polymer capped by one single formin dimer.

Due to the actin nucleation activity of formins, the number of actin filaments formed initially in the presence of formins exceeds substantially the number of filaments generated in the absence of formin. A previous study from the Pollard group showed that end-to-end annealing was effective to produce long filaments from fragmented short actin filaments (53). The rate of annealing depends on the number of filaments. In our experiments, the number of shorter filaments was relatively high immediately after the initiation of polymerization by formins, suggesting that the annealing progressed rapidly. Andrianantoandro et al. (53) also showed that end-to-end annealing occurred, although at a lower rate, even in the presence of capping protein, which binds the barbed end of filaments tightly. They observed that in the presence of capping protein 24 h after fragmentation of actin filaments, the filament length distribution was re-established and was similar to that observed 1 h after fragmentation in the absence of capping protein. This observation was explained by the gated reaction theory based on the slow diffusion of actin filaments and the dynamic equilibrium between the capping protein and the barbed end of the filaments (53). Because the mDia1-FH2 fragments were reported to be in a rapid equilibrium with the filament ends (26), and because in this study, we incubated the formin-actin samples overnight before the experiments, it is likely that at the time of the FRET measurements, the length distribution of actin filaments was similar in the absence and presence of formin.

According to these considerations, there were hundreds of actin protomers for each formin dimer bound to a barbed end of a filament. The fact that the change of the flexibility could be observed in our assays indicates that one dimer must have changed the conformation of many actin protomers. It is assumed that the barbed end produced long range allosteric conformational changes along the actin filaments. The current set of data does not allow the determination of the ratio of the concentration of actin protomers influenced by formin to the total actin concentration, i.e., the range of the allosteric interactions cannot be determined.

It was reported previously in numerous cases that effects of ligands and actin-binding proteins propagated along actin filaments through long range allosteric interactions (e.g., Refs. 54–59). Related to the present study, a long range allosteric effect of gelsolin, another barbed end-binding protein, was observed. The Egelman group (57) showed that when actin filaments were nucleated in the presence of gelsolin, the altered conformational state of the filament could be detected throughout the whole filament. In accordance with this observation, the Thomas laboratory (58) reported gelsolin-induced long range cooperativity in actin filaments where a conformational change induced by the binding of a single gelsolin molecule to the barbed end is propagated throughout the actin filament. These observations and our present findings indicate that it is probably the inherent property of actin filaments to be able to transport the conformational changes initiated at the barbed end through many protomer-protomer interactions to distant locations along the actin polymers.

It was also suggested (e.g., Ref. 59) that the regulatory effect of actin-binding proteins on the polymerization state of actin is tightly correlated with the various, often co-existing, conformations of actin protomers and with the modifications in their interactions. Recently, Orlova et al. (59) suggested that actin-depolymerizing proteins such as ADF/cofilin exert their effect on actin filaments by inducing conformational transitions, which result in a less stable state of the filaments. These less stable states are proposed to exist in earlier phases of polymerization as well. In the light of our data, it seems possible that cofilin is more effective (Fig. 5C) at depolymerizing the more flexible (Fig. 3) and less stable (e.g., thermodynamically, Fig. 5A) actin filaments generated in the presence of formin because the activation energy required to shift the conformational changes to that established by cofilin was lower in the presence of formin than in the absence of it.

The altered interaction of actin filaments with cofilin in the presence of formin indicates that the formin-induced conformational changes can have functional consequences. In accordance with this conclusion, we also showed in this study that another functional feature of actin, one of the steps of the ATPase cycle, the phosphate release, was modified by the binding of formins. The close correlation between the formin dependence of the actin flexibility (Fig. 4A) and the phosphate release rate (Fig. 5B) suggests that the dissociation of P_i from the looser actin filaments formed in the presence of formin is thermodynamically more favorable than that in the absence of formin.

The changes in the actin flexibility above 750 nM cannot be explained by further barbed end binding of the formin fragment as most of the barbed ends are formin-bound at 750 nM. On the other hand, the flexibility in the 750–5000 nm range follows a tendency opposite to that observed below 750 nM mDia1-FH2 dimer. The simplest way to interpret the FRET results above 750 nm formin is to assume that binding of the dimeric mDia1-FH2 dimer to the sides of the actin filaments stabilizes the protomer-protomer interactions. The structural details of the interaction between the sides of the actin filaments and formin fragments are unknown. Our results suggest that in these interactions, one formin...
dimer is interacting with at least two actin protomers, which could form the bases of the stabilization of the filaments.

The results of the FRET experiments can therefore be explained by assuming that a formin “cap” at the barbed end increases the flexibility of the filament, whereas formin “cramps” linking actin protomers along the sides of actin filaments can stabilize the molecular interactions between neighboring protomers and make the filaments stiffer. The superposition of these two effects was observed in the FRET measurements.

We attempted to test this hypothesis by using the mDia1-FH2monomer fragment (Fig. 4C), which was characterized previously (25). The hypothesis predicted that if the binding of mDia1-FH2monomer can induce the loosening of the actin filaments, the mDia1-FH2monomer concentration dependence of the effect should correlate with the affinity of this mDia1-FH2monomer fragment to the filament barbed ends. The affinity of mDia1-FH2monomer to the barbed end is ~2 μM, and it binds the sides of actin filaments with an ~3 μM affinity (25). The filament flexibility increased over the investigated concentration range up to 12.5 μM mDia1-FH2monomer (Fig. 4C). Although the fact that the affinities of mDia1-FH2monomer for the filament end- and side-binding sites are similar complicates the interpretation of the FRET data, the observation that high mDia1-FH2monomer concentrations were required to increase the flexibility of the actin filaments is in agreement with our model. The lack of the decrease of the formin effect at high formin concentrations indicates that the mDia1-FH2monomer fragment bound to the sides of actin filaments was not able to stabilize the interaction between neighboring protomers, suggesting that this property is only characteristic for the formin dimers.

A Possible New Mechanism to Regulate the Formation of Cytoskeletal Complexes—The function of the actin cytoskeleton requires the coordinated work of many regulatory and structural proteins. In many cases, the proteins involved in these complexes can be attributed to specific actin nucleation factors. It is important to note that in cells, a group of proteins localizes to formin-nucleated actin structures, whereas other proteins are typically associated with actin filaments nucleated by the Arp2/3 complex (for a summary, see Ref. 60). However, the mechanism by which, e.g. ADF/cofilins, preferentially localize to Arp2/3-nucleated actin structures and tropomyosins localize to formin-nucleated structures is not understood. One possible mechanism of regulation would be based on the effect of actin nucleation factors on the conformation of actin filaments. One can envisage that these nucleation factors can change the affinity of actin-binding proteins to actin filaments and thus determine which proteins are involved in the corresponding complexes. Due to the nature of their function, actin nucleation factors are the first to bind to the newly generated filaments, and the problem of timing the sequence of the binding of the actin-binding proteins would be overcome by the regulatory role of these nucleation factors. A prerequisite for this mechanism is that the nucleation factors can change the conformation of actin filaments. These effects should be long range allosteric effects as the nucleation factors bind to the ends of filaments. Our FRET results provided evidence that formin fragments can have substantial and allosteric effects on the conformation of actin filaments by binding to the barbed end. This conclusion is independent of the validity of proposed explanation (Fig. 6). Based on this observation, we propose that a special mechanism is involved in the regulation of the formation of cytoskeletal protein complexes in which the conformational changes induced by the nucleation factors in actin filaments play an essential role in determining the affinity of actin-binding proteins for actin filaments. Although further experiments are required to test this hypothesis, this novel mechanism would be one of the first examples of how actin filaments can serve as regulatory information channels in living cells.

Conclusions—We showed here that the binding of mDia1-FH2dimers to actin increased the flexibility of actin filaments by long range allosteric interactions. In accordance with this observation, the filaments became less resistant to heat denaturation in the presence of formin. These conformational changes were accompanied by the modifications of functional properties of actin as its interaction with cofilin and the rate at which phosphate dissociates from actin filaments were also influenced by the binding of formin.

The present results, on the other hand, raised and left many questions unanswered. Further experiments will be required to determine the distance to which formin at the barbed end can allosterically modify the conformation of actin protomers. Also to be tested is how abundant this formin effect is among different members of the formin family. One would wonder whether other actin nucleation factors, such as the Arp2/3 complex, can also modify the conformation of actin filaments. Also, finally, the confirmation of our hypothesis describing the new mechanism, which can serve to regulate the formation of cytoskeletal complexes, requires further investigations.

Acknowledgment—We thank Alfred Wittinghofer for providing us with the mouse mDia1 clones.

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