Current Biology, Vol. 13, 1820–1823, October 14, 2003, ©2003 Elsevier Science Ltd. All rights reserved. DOI 10.1016/j.cub.2003.90.057

Formin Leaky Cap Allows Elongation in the Presence of Tight Capping Proteins

Sally H. Zigmond,[1,*] Marie Evangelista,[3] Charles Boone,[3,4] Changsong Yang,[1] Arvin C. Dar,[5] Frank Sicheri,[6] Joe Forkey,[2] and Martin Pring[2]

[1]Department of Biology
[2]Department of Physiology
University of Pennsylvania
Philadelphia, Pennsylvania, 19104
[3]Department of Biology
Queen's University
Kingston, Ontario K7L 3N6
[4]Banting and Best Department of Medical Research and
Department of Molecular and Medical Genetics
University of Toronto
Toronto, Ontario M5G 1L6
[5]Program in Molecular Biology and Cancer
Samuel Lunenfeld Research Institute
Mount Sinai Hospital
Toronto, Ontario M5G 1X5
[6]Department of Molecular and Medical Genetics
University of Toronto
Toronto, Ontario M5S 1A8
Canada

Summary

Formins, characterized by formin homology domains FH1 and FH2, are required to assemble certain F-actin structures including actin cables, stress fibers, and the contractile ring. FH1FH2 in a recombinant fragment from a yeast formin (Bni1p) nucleates actin filaments in vitro [1, 2]. It also binds to the filament barbed end where it appears to act as a "leaky" capper, slowing both polymerization and depolymerization by ~50% [3]. We now find that FH1FH2 competes with tight capping proteins (including gelsolin and heterodimeric capping protein) for the barbed end. We also find that FH1FH2 forms a tetramer. The observation that this formin protects an end from capping but still allows elongation confirms that it is a leaky capper. This is significant because a nucleator that protects a new barbed end from tight cappers will increase the duration of elongation and thus the total amount of F-actin. The ability of FH1FH2 to dimerize probably allows the formin to walk processively with the barbed end as the filament elongates.

Results and Discussion

Bni1p FH1FH2 Protects Barbed Ends from Capping by Capping Protein or Gelsolin

Actin polymerization nucleated by Bni1p FH1FH2 was not inhibited by the presence of enough capping protein to rapidly cap free barbed ends (Figure 1A). Thus, either FH1FH2-induced filaments elongate only from their pointed ends, or elongation at the barbed end is protected from capping by capping protein. If the FH1FH2-induced filaments were elongating at their pointed ends, removal of FH1FH2 would increase the rate of elongation by ~10-fold (due to the faster on rate at the barbed end). We diluted the formin-nucleated filaments to decrease the FH1FH2 concentration below its binding constant for ends [1]. After dilution, the maximal rate of elongation increased 2-fold (see the Experimental Procedures in the Supplemental Data available with this article online). Addition of capping protein now severely inhibited the elongation (Figure 1B), indicating that FH1FH2 had dissociated and that elongation was occurring at the barbed end. These results confirm that Bni1p FH1FH2-induced filaments elongate at their barbed end at ~50% of free barbed-end rate (1, 2, 5) and further show that the presence of FH1FH2 protects the barbed end from capping by capping protein.

To further characterize the protection from capping, we examined the effects of FH1FH2 on elongation from preformed F-actin (spectrin-actin seeds, which provide a barbed end for actin assembly) in a G-actin concentration too low to allow FH1FH2 to nucleate new filaments. Incubating spectrin-actin seeds with FH1FH2 briefly (~30 s) before addition of actin decreased the rate of elongation as expected [1]. There was no further inhibition of elongation when either capping protein or gelsolin was included with the actin (Figures 1C and S1). Controls without FH1FH2 showed that both capping protein and gelsolin inhibited barbed-end elongation by ≥50%. Thus, even though FH1FH2 decreased the rate of elongation by ≤50%, it protected all of the ends from capping proteins. These results support the conclusion that FH1FH2 is a leaky cap and not merely a weak cap that inhibits partially because its concentration is not high enough to bind all of the ends.

The ability of FH1FH2 to protect against capping depends on the relative concentrations of FH1FH2 and capping protein and on the duration of incubation in both. In the previous experiment (Figure 1C), elongation was measured 2 min after adding capping protein to FH1FH2 bound barbed ends. When elongation was measured 30 min after adding capping protein, the rate of elongation was decreased; the extent of the decrease approached that expected at equilibrium (Figure 1D). A similar level of inhibition of elongation was achieved at a steady state independent of whether FH1FH2 or capping protein was added first (Figure S2).

Morphology of Elongating Filaments Confirms Protection against Capping

To monitor morphologically the effects of FH1FH2 on barbed-end elongation, a low concentration of F-actin seeds labeled with Alexa 488-phalloidin was allowed to elongate for 6 min in 0.5 μM G-actin in the presence of TRITC-phalloidin. The solution was diluted 10-fold and

*Correspondence: szigmond@sas.upenn.edu

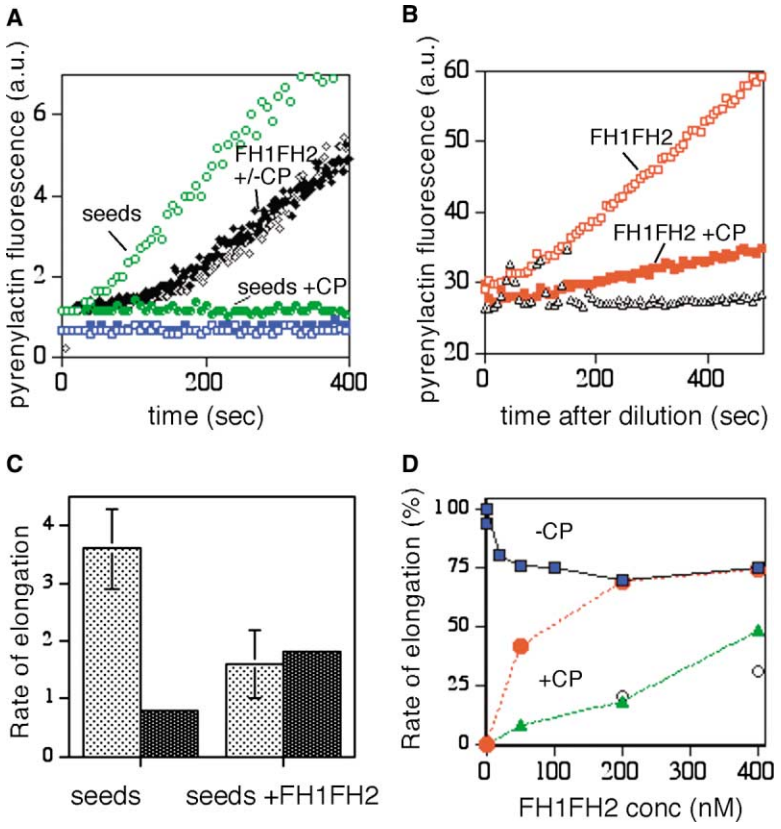


Figure 1. FH1FH2 Protects the Barbed End from Capping by Capping Proteins

(A) FH1FH2 (75 nM) was added to 2 μM Mg-actin (25% labeled) in the absence (open black diamonds) or presence of capping protein (10 nM) (closed black diamonds), and polymerization was followed from the pyrenylactin fluorescence. Spectrin-actin seeds (0.3 nM) were added to the pyrenylactin in the absence (open green circles) or presence of capping protein (closed green circles). Spontaneous nucleation and polymerization by actin alone (open blue squares) or with capping protein (closed blue squares) were also monitored.

(B) To determine the relative number of ends produced by FH1FH2 under the conditions described in Figure 2A, FH1FH2 (75 nM) incubated with Mg-actin for 150 s was diluted 25-fold into pyrenylactin (1 μM) without (open red squares) or with 10 nM capping protein (closed red squares). At the diluted concentration (3 nM), FH1FH2 did not nucleate new filaments in 1 μM actin over the time period involved (open black triangles).

(C) Spectrin-actin seeds (1 nM) were incubated for 3 min in the absence (left set) or presence of 200 nM FH1FH2 (right set) before addition of 0.6 μM Mg-pyrenylactin in the absence (light bars) or presence (dark bars) of 10 nM capping protein, and the rate of elongation, expressed as a percent of seeds alone, was determined between 0 and 120 s.

(D) Spectrin-actin seeds were incubated for 5 min with different concentrations of FH1FH2 and then assayed for rate of elongation in 1 μM pyrenylactin (closed blue squares) or then mixed with 10 nM capping protein for 30 s (closed red circles) or 30 min (closed green diamonds) before assaying elongation between 15 and 60 s (expressed as percent of seeds alone). The calculated steady-state values (using capping protein K_d = 0.3 nM, FH1FH2 K_d = 20 nM and 1 nM seeds) are shown by the open circles.

examined by epifluorescence microscopy. The elongation (identified by TRITC label alone) from the seeds (labeled brightly with Alexa and dimly with TRITC) was unidirectional (Figure 2A).

The distribution of Alexa-labeled filament lengths is shown in Figure 2B. By scoring only filaments labeled with Alexa, we avoided any filaments nucleated spontaneously or by FH1FH2 or capping protein. The median length of the TRITC labeled region was 4.9 μm (mean ± SD = 5.5 ± 3 μm). Seeds elongating in the presence of 200 nM FH1FH2 had a shorter, tighter length distribution, median 2.6 μm (2.9 ± 1.8 μm). This is consistent with the elongation of each filament being slowed by FH1FH2. Seeds elongating in the presence of 10 nM capping protein were rapidly inhibited, median 0.3 μm (0.5 ± 1.1 μm). FH1FH2 incubated with seeds for 1 min before addition of capping protein partially protected the ends from capping during the subsequent elongation period, increasing the median from 0.3 to 1.8 μm (1.9 ± 1.1 μm). Incomplete protection is expected over this time period because as the FH1FH2 comes off the end, it must compete with capping protein to rebind. These results support and extend the conclusions from the biochemical studies: FH1FH2 slows barbed-end elongation but protects the barbed end from capping by capping protein.

The maintenance of the leaky cap cannot be explained merely by a rapid on and off rate of FH1FH2 for the end. The maximal on rate for FH1FH2 is diffusion limited, i.e., 10/μM/s. The loss of FH1FH2 from the end of the filament during elongation reflects the sum of dissociation of FH1FH2 and movement of FH1FH2 to an interior position due to G-actin addition. The latter has an on rate of 5/μM/s (half the normal rate) so at 1 μM G-actin, a pseudo first-order rate of 5/s. This predicts a K_d of >(5/s)/(10/μM/s) or 0.5 μM (dependent on [G-actin]), which contrasts with the observed K_d of 20 nM. Similar arguments were made for insertin, a fragment of tensin, which is also a leaky cap [4].

We investigated whether the formin could move to keep up with a barbed end that was elongating rapidly. If it was unable to keep up, it would no longer inhibit elongation. Bni1p FH2 behaves similarly to FH1FH2 with the exception that it cannot utilize profilin-actin for nucleation [3] (Figures S3 and S4). This allowed us to use profilin-actin to increase the rate of elongation. The ratio of profilin to actin was adjusted to keep the free G-actin at 0.5 μM, and the total G-actin concentration was increased to 6 μM; under these conditions FH2 caused no nucleation. The rate of barbed-end elongation of control seeds was, as expected, proportional to the sum of the concentrations of free G-actin and profilin-actin (Figure 3). The extent of inhibition by FH2 was independent of the rate of elongation. Thus, the formin can keep up with a barbed end that is elongating at a rate >30 monomers/s ([G-actin + profilin-actin] k_{on}/2 = [6 μM] 5/μM/s).

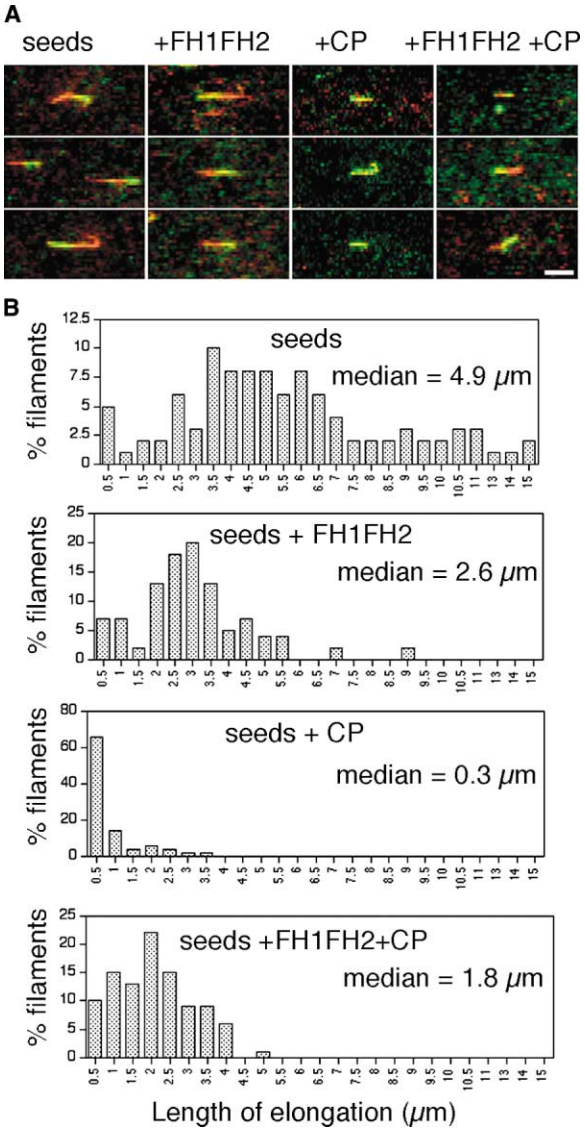


Figure 2. Filament Length Distribution Confirms FH1FH2 Inhibition of Elongation and Protection from Capping by Capping Proteins

(A) Shown are examples of filaments grown from Alexa-labeled spectrin-actin seeds (green) (see Experimental Procedures in the Supplemental Data) that were then allowed to elongate for 6 min with TRITC-phalloidin (red) and 0.5 μM G-actin alone (left three panels) or with 200 nM FH1FH2 (three left center panels), 10 nM capping protein (three right center panels), or both FH1FH2 and capping protein (three right panels). Scale bar = 5 μm.

(B) Length distribution of the TRITC-labeled portion of filaments elongated from seeds under conditions above. Y axis is the percent of total filaments with length in the bins described in μm on the x axis. The first bin is the fraction of filaments that grew 0.5 μm or less and the second bin, any that grew between 0.5 and 1 μm, etc. At least 50 filaments were measured for each variable.

The formin Cdc12 also appears to remain at the barbed end and prevent annealing, while the filaments are elongating rapidly in the presence of profilin-actin [5]. In *S. cerevisiae*, the actin cable, which is nucleated by a formin, is observed to elongate at >100 monomers/s [6].

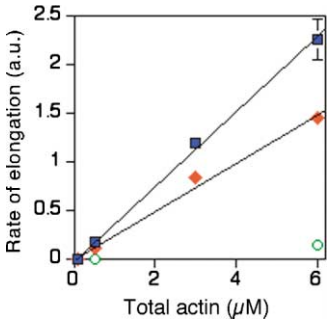


Figure 3. Inhibition of Elongation by FH2 Is Independent of Rate of Elongation

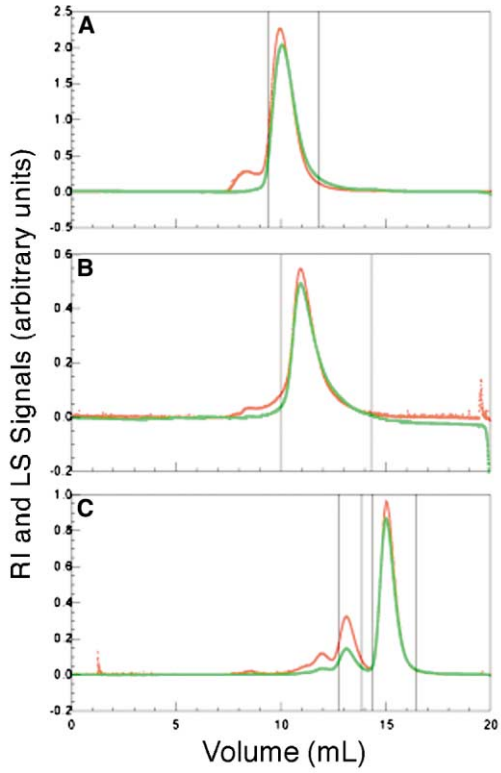
The initial rate of elongation (0–120 s) was determined for spectrin-actin seeds alone (closed blue squares) or in the presence of 200 nM FH2 (closed red diamonds) in 0.5, 3, or 6 μM G-actin (25% labeled) plus enough profilin to reduce the free G-actin in each case to 0.5 μM (determined by the concentration required to inhibit pointed-end elongation from gelsolin seeds). Controls, in the absence of seeds, monitored the extent of nucleation by FH2 in each condition (open green circles). The lines are best fits ($R^2 > 0.99$); the FH2 slope is 64% of control slope.

FH2 Is a Tetramer

To be processive, a cap, like a motor, presumably has to maintain a foothold on the filament while it moves. An oligomer of at least two FH2 domains may be essential for the FH2 to walk along the filament, with one molecule keeping the complex attached to the filament while the other repositions itself. Static light scattering (SLS) analysis provides an absolute measurement of the size of the complex independent of molecular shape [7]. An experimental molecular weight of 260,500 Da (±3000) was observed for FH1FH2, which indicates that FH1FH2 adopts a tetrameric configuration since the polypeptide molecular weight is 67,600 Da (Figure 4A). FH2 alone also formed a tetramer based on SLS analysis (Figure 4). FH1FH2 fragments of the mammalian formin mDia1 also oligomerize [8], and Bni1p FH2 crystallizes as a dimer (M. Eck, personal communication).

How Does FH1FH2 Work As a Leaky Cap?

Together, these data support the view that the Bni1p cap is processive and walks along with the end as it elongates (Movie S1). Actin subunits at the elongating barbed end contain ATP or ADP-Pi. However, the selective affinity of Bni1p for the elongating barbed end does not depend on the presence of ATP or ADP-Pi since Bni1p also partially inhibits depolymerization when the actin subunits at the filament end contain ADP (5). Rather, it appears that the Bni1p FH2 prefers a binding surface only available at the barbed end of the filament. During elongation, as the barbed end binds the next G-actin, the site binding FH2 switches from "end" to "internal," which decreases its affinity for FH2. But as the FH2 comes off this site, it remains attached to the filament's other side by the second FH2, allowing the free FH2 to reattach to the new end. The ability to move rapidly is reasonable for a processive cap given that the on rate is now effectively an internal rearrangement of an existing molecular complex. As a result, it is indepen-



D

Sample ID	Experimental MW	Theoretical monomer MW	Oligomeric state
FH1FH2	260,500+/-3400	67,600	Tetrameric
FH2	211,750+/-6300	56,500	Tetrameric
BSA	133,800+/-1800	66,000	Dimeric
BSA	64,520+/- 900	66,000	Monomeric

Figure 4. FH1FH2 Behaves as a Tetramer in Solution

(A) Elution profile of FH1FH2 on a Superdex 200 (24 mL) gel filtration column. Red and green profiles correspond to static light scattering (LS) and refractive index (RI) detection, respectively. The peak between the vertical bars was analyzed to obtain the experimental molecular weight in daltons.

(B) Elution profile of FH2 on a Superdex 200 (24 mL) gel filtration column.

(C) Elution profile of BSA standard on a Superdex 200 (24 mL) gel filtration column.

(D) Experimental molecular weight calculations from the light scattering analysis. Values (in daltons) correspond to the peaks analyzed between the vertical bars of chromatograms in (A), (B), and (C). Theoretical monomer molecular weight was calculated from the protein sequence.

dent of diffusion, and would be expected to have first-order kinetics, without any theoretical limit of rate.

Physiological Significance of Protection against Capping

The ability of a nucleator to increase the amount of F-actin depends on the number of filaments created and the rate and duration of their elongation. Thus, in the presence of tight capping proteins in vivo, transient protection may make a crucial contribution. A filament nucleated by FH1FH2 will have its barbed end occupied by FH1FH2. Until the FH1FH2 comes off (a half-time of ~30 s), the end is protected from capping protein and can elongate, albeit at a reduced rate. The duration of protection is longer than the estimated half-time of free filaments in neutrophil cytoplasm (~1 s) [9]; comparable values in yeast are not known. Furthermore, when the formin comes off the barbed end, whether capping protein or another formin binds depends on their local concentrations. In yeast, Bni1p is concentrated at the bud tip where the barbed ends of actin cables are also found. Thus, one Bni1p molecule after another may occupy the barbed end for an extended period of time allowing these filaments to continue to elongate even while anchored to the bud tip.

Supplemental Data

Supplemental Data including experimental procedures, additional data, and an animation illustrating the properties of a processive cap are available online at http://www.current-biology.com/cgi/content/full/13/20/1820/DC1/.

Acknowledgments

S.H.Z. and C.Y. were supported by NIH grant AI19883 and M.E., C.B., A.C.D., and F.S. are supported by NCIC grants. We thank Y. Goldman for use of his microscope facility, Evan M. Goldman for processive cap animation, and Michael Joyce for excellent technical assistance.

Received: July 2, 2003
Revised: September 8, 2003
Accepted: September 8, 2003
Published: October 14, 2003

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