INF2 Is a WASP Homology 2 Motif-containing Formin That Severs Actin Filaments and Accelerates Both Polymerization and Depolymerization*

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Formins are actin assembly factors for a wide variety of actin-based structures, including cytokinetic rings, certain types of stress fiber, filopodia, actin filaments around endosomes, and yeast actin cables (1) (reviewed in Refs. 2 and 3). To date, all direct interaction with actin occurs through the formin homology 2 (FH2) domain, which is dimeric and binds actin barbed ends. All FH2 domains studied possess the following activities: 1) acceleration of filament nucleation; 2) processive movement with the elongating filament barbed end, which can influence both elongation and depolymerization rate at this end; and 3) the ability to block barbed end capping by capping proteins such as gelsolin and heterodimeric capping protein. The FH1 domain, N-terminal to the FH2, binds profilin and accelerates elongation from FH2-bound barbed ends in a profilin-dependent manner (4, 5).

Although individual formins differ quantitatively in some activities, the overall effect of formins is to enhance actin assembly rate. In addition, some FH2 domains can bundle filaments (6–8). No actin binding ability has been identified outside the FH2 domain.

Most eukaryotes possess multiple formins, with mammals having at least 15 formin genes that segregate into seven classes based on FH2 domain phylogenetic analysis (9). One exclusively metazoan group, termed inverted formins (INFs), were thought to be unique in the placement of their FH1 and FH2 domains at the N terminus of the protein, as opposed to all other formins, which have additional domains N-terminal to the FH1. This difference has regulatory significance, because the N termini of many formins, including mammalian mDia1 and mDia2, contain a diaphanous inhibitory domain (DID), which is a stably folded region consisting of five armadillo repeats (10–14). In mDia1 and mDia2, DID binds tightly to the diaphanous autoregulatory domain (DAD), a stretch of 20 or so amino acids C-terminal to the FH2 (10–12, 15–17). The DID/DAD interaction strongly inhibits FH2 domain activity; thus formins that contain these domains are considered “autoinhibited.” For mDia1, autoinhibition is relieved when GTP-bound RhoA binds a GTPase binding domain that overlaps the N terminus of the DID (10, 12, 16). Many other formins possess DID and DAD sequences, suggesting that autoinhibitory regulation is widespread (18). Because no DID or DAD has been identified for an INF formin, the conclusion has been that other mechanisms regulate this group.

In this study, we found that the mouse formin INF2 has the unique ability to accelerate depolymerization, in addition to the nucleation and elongation activities common to formins. INF2 depolymerization activity requires its unique C terminus, containing an actin-binding WASP homology 2 motif (WH2), and can sequester actin monomers in 1:1 complexes. In addition, the C terminus is required for rapid filament severing by INF2. Both depolymerization and severing are inhibited by phosphate, suggesting that these activities require phosphate release.
from actin subunits after ATP hydrolysis. Mutation of the WH2 motif strongly inhibits depolymerization. Finally, we identify an N-terminal DID for INF2, suggesting its regulation by autoinhibition. The WH2 motif likely serves as a DAD based on its position and sequence similarity to other DADs. The closely related formin INF1 possesses neither DID nor DAD.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—INF2-(FH1-FH2-C) (amino acids 538–1274) INF2-(FH1-FH2) (538–993), and INF2-(C) (994–1274) (Fig. 1 and supplemental Fig. 2) constructs were generated by reverse transcriptase-PCR from RNA isolated by the TRIzol method (Invitrogen) from 300.19 murine Abelson leukemia virus-transformed pre-B cells (19). cDNA was synthesized using oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen). INF2-(C) was cloned into pGEX-KT vector (20) for bacterial expression as a glutathione S-transferase fusion protein. INF2-(FH1-FH2) was cloned into pET22b for expression as a C-terminally His6-tagged protein. INF2-(FH1-FH2-C) was cloned into pET22b for expression as an untagged protein. To mutate the three conserved leucine residues (amino acids 1008, 1009, and 1018) in the WH2 motif of INF2-(FH1-FH2-C) and INF2-(C), the QuikChange kit (Stratagene) was used. All three residues were converted to alanine. The INF2 sequence has been deposited in GenBank™ under accession number DQ834374.

**Protein Expression and Purification**—Rosetta 2 DE3 Escherichia coli (Novagen) were used for expression of all constructs, and expression was induced as described previously (6, 21). INF2-(FH1-FH2) was purified by Ni²⁺-nitrilotriacetic acid super flow chromatography (Qiagen) and dialyzed into Na150MEPD (150 mM NaCl, 0.1 mM MgCl₂, 0.1 mM EGTA, 2 mM NaPO₄, 0.5 mM DTT, pH 7.0), then stored at 4 °C. INF2-(C) was purified by glutathione-Sepharose 4B chromatography (GE Healthcare) followed by removal of the glutathione S-transferase. This procedure results in two bands that migrate at 55 and 37 kDa by SDS-PAGE. Mass spectroscopy and Edman degradation analysis showed that these bands represented the full INF2-(C) construct (calculated mass of 30.2 kDa) and a C-terminally truncated product, amino acids 994–1234, respectively. Full INF2-(C) was separated from the truncated product by SP-Sepharose chromatography (GE Healthcare). The anomalously high mass by SDS-PAGE might be because of the low pI (4.6) of INF2-(C). Purified INF2-(C) was dialyzed in Na50MEPD and stored at 4 or −70 °C.

INF2-(FH1-FH2-C) was purified by SP-Sepharose chromatography, Q-Sepharose chromatography (GE Healthcare), and Superdex 200 26/60 size exclusion chromatography (GE Healthcare). Fractions were stored at 4 °C in Na150MEPD. The molecular mass of full-length protein is 81.4 kDa by mass spectroscopy, but the protein migrates at ~100 kDa by SDS-PAGE.

Rabbit muscle actin was purified from acetone powder and labeled with pyrenyliodoacetamide (22). Both labeled and unlabeled actin were gel-filtered on an S-200 column (23), which was essential for obtaining reproducible kinetic results. Human profilin I was expressed in bacteria and purified as described (21).

Buffers Used for Biochemical Studies—The following buffers were used frequently: G buffer (2 mM Tris, pH 8, 0.5 mM DTT, 0.2 mM ATP, 0.1 mM CaCl₂, and 0.01% Na₂EDTA); G-Mg buffer (same as G buffer but with 0.1 mM MgCl₂ instead of CaCl₂); 10X KMEI: 500 mM KCl, 10 mM MgCl₂, 10 mM EGTA, and 100 mM imidazole, pH 7.0; 10X NaMEI (same as 10X KMEI but with 500 mM NaCl instead of KCl); polymerization buffer (G-Mg buffer plus either 1X KMEI or 1X NaMEI). Polymerization buffer with 1X NaMEI was used for pelleting assays because dodecyl sulfate precipitates as the potassium salt. For some studies, polymerization buffer without ATP was used, when studying the nucleotide dependence of depolymerization. 10E/1M = 10 mM EGTA/1 mM MgCl₂, pH 7–8. Fluorescence buffer = 25 mM imidazole, pH 7–8, 25 mM KCl, 4 mM MgCl₂, 1 mM EGTA,100 mM DTT, 0.5% methylcellullose,18 μg/ml catalase, 3 μg/ml glucose, and 100 μg/ml glucose oxidase.

**Dual Color Filament Elongation Assay Using Fluorescence Microscopy**—Unlabeled actin (4 μM) was polymerized for 2 h in polymerization buffer at 23 °C and then stabilized with 4 μM Alexa 488-phalloidin (Molecular Probes). To start the reaction, 2 μl of polymerized actin was incubated with 4 μl of polymerization buffer for 1 min at 23 °C followed by simultaneous addition of: 1) 3 μl of rhodamine phalloidin (5 μM) in polymerization buffer, 2) 2 μl of actin monomers (20 μM) in G-Mg, and 3) 0.4X KMEI in G-Mg to bring the volume to 40 μl. Profilin was premixed with actin monomers in reactions that used profilin. Aliquots were removed at various times and diluted 80-fold in fluorescence buffer. Samples (1.5 μl) were adsorbed to 0.01% poly-L-lysine-coated 12-mm round glass coverslips and examined for Alexa488 and TRITC label through a Nikon-Eclipse TE-2000 microscope with a 100× 1.4 numerical aperture objective. Images were acquired using a Roper Cool-Snap camera. Lengths of red filaments (newly elongated) emanating from profilin-labeled actin were measured from final mixing and the start of data collection ranged between 10 and 15 s for each assay.

**Calculating Filament Concentration**—Filament concentration was determined from slopes of pyrene fluorescence curves at 50% polymerization as described previously (24). For several conditions (INF2-(FH1-FH2), INF2-(FH1-FH2-C), and INF2-(FH1-FH2-C) plus profilin, filament concentrations were adjusted to reflect the altered elongation rates measured in this study (Table 1).
Polymerization/Depolymerization by INF2

Labeling INF2-(C) and INF2-(FH1-FH2-C)—INF2-(C) was dialyzed for 2 h in 50 mM NaCl, 2 mM NaPO₄, pH 7, 0.5 mM MgCl₂, 0.5 mM EGTA. Fluorescein 5’-maleimide (Molecular Probes) was added to 100 μM, and the reaction was incubated on ice for 5 min. Labeling was stopped by adding DTT to a concentration of 10 mM. Labeled protein was separated from free dye by Superdex 200 10/30-size exclusion chromatography (GE Healthcare). INF2-(FH1-FH2-C) was labeled similarly using 200 μM fluorescein 5’-maleimide for 15 min.

Fluorescence Anisotropy Measurements—Fluorescein-labeled INF2-(C) (FL-INF2-(C)) was diluted to 22.2 nM in 1.11 M sulfate. The resulting beryllium fluoride (BeF)-bound filaments containing rhodamine-phalloidin (1 μM final concentration) was measured and used to calculate the depolymerization rate. Initial slopes were measured and used to calculate the depolymerization rate.

Critical Concentration Measurements—Actin stock (6 μM, 5% pyrene) was first converted to Mg²⁺ salt by incubating it at 23 °C for 2 min in 10 mM ATP. This stock actin solution was then diluted in the range of 5 to 1 μM in polymerization buffer containing 0.2 mM ADP instead of ATP and the appropriate concentration of INF2-(FH1-FH2-C). Phosphate (10 mM NaPO₄, pH 7.0) was added where indicated. Samples were incubated overnight in the dark at 23 °C, and pyrene fluorescence (excitation 365 nm, emission 410 nm) was recorded for 300 s. Concentrations of INF2-(C) tested were 1.5 and 3 μM (no effect on nucleotide exchange rate). Profilin was tested (1.5 μM) as a positive control and affected full nucleotide exchange within the 15-s dead time between mixing and fluorescence recording.

RESULTS

INF2 Contains an N-terminal DID—Previous searches of human and mouse databases suggested that the FH1 and FH2 domains of INF2 were at the N terminus (9). However, subsequent data base searches revealed sequences that extend the open reading frame (ORF) considerably at the N terminus in chimpanzee (GenBank™ accession number XM_522946), rat (XM_343116), dog (XM_547999), and cow (XM_580659). All
of these sequences are hypothetical protein entries derived from ORF predictions from the respective genomes. No similarly predicted ORFs exist for mouse or human at present.

To test whether mouse INF2 contains an extended N terminus, we conducted reverse transcriptase-PCR analysis using mouse 300.19 cell total RNA. We found an ORF similar to those predicted for chimpanzee, rat, dog, and cow. The identified mouse ORF encodes a 1274-amino acid protein (supplemental Fig. 1). Sequence analysis reveals that, in addition to the FH1 and FH2 domains, INF2 contains a clear DID at its N terminus (Fig. 1A) and a putative DAD about 40 amino acids C-terminal to the FH2, a position similar to that found in other formins (Fig. 1B). Thus, the domain structure of INF2 appears similar to formins that are regulated by autoinhibition.

INF2 FH2 Domain-containing Constructs Act Similarly to Other Formins—We tested the activities of two INF2 constructs (Fig. 1): one comprising the FH1 and FH2 domains (termed INF2-(FH1-FH2)) and one that also included the C-terminal region (INF2-(FH1-FH2-C)). Similar to other formins, these two FH2-containing constructs behave as dimers by analytical ultracentrifugation (supplemental Fig. 2).

All formin FH2 domains tested to date affect actin filament elongation rate, but vary greatly in the degree of these effects. We studied the effects of INF2-(FH1-FH2) and INF2-(FH1-FH2-C) on actin filament elongation using a dual-labeled fluorescence microscopy assay in which prepolymerized filaments seeds labeled with “green” phalloidin are incubated with actin monomers and “red” phalloidin (25). Both INF2 constructs inhibit the barbed end elongation rate by about 90% (Fig. 2A, Table 1) and display apparent Kd values of <10 nM for barbed ends (not shown). These high affinities are similar to those measured for mDia1, FRL1, and mDia2 (6, 21). In addition to slowing elongation, both INF2-(FH1-FH2) and INF2-(FH1-FH2-C) cause large increases in the number of short “red-only” filaments (supplemental Figs. 3 and 4), suggesting that they increase nucleation rate or cause filament severing.

As with other formins, the INF2 FH1 domain contains multiple profilin-binding sites. Because profilin-bound FH1 domain increases barbed end elongation rate for all formins tested (4, 26, 27), we examined the effects profilin on INF2-(FH1-FH2) and INF2-(FH1-FH2-C). The profilin and actin monomer concentrations used in these assays (5 μM profilin, 1 μM monomer) were such that almost all monomers were profilin-bound. This ratio of actin:profilin has also been found to
Polymerization/Depolymerization by INF2

| Table 1: Effects of INF2 on actin nucleation and elongation |
|-----------------|-----------------|-----------------|
| Conditiona      | Barbed end elongation rateb | Nucleation efficiencyc |
|                 | \( \mu m^{-1} s^{-1} \) | \( \text{Ratio} \) |
| Actin alone     | 8.2 ± 1.48 (252) | NM \( ^d \) |
| Actin + profilin| 8.8 ± 1.38 (210) | NM \( ^d \) |
| Actin + INF2-(FH1-FH2) | 0.90 ± 1.90 (212) | 0.51 ± 0.09 |
| Actin + INF2-(FH1-FH2) + profilin | 8.8 ± 1.34 (94) | 0.036 ± 0.006 |
| Actin + INF2-(FH1-FH2-C) | 0.73 ± 0.27 (40) | 0.93 ± 0.12 |
| Actin + INF2-(FH1-FH2-C) + profilin | 17.4 ± 5.3 (171) | 0.068 ± 0.006 |

a Actin = 1 \( \mu M \), INF2 constructs = 2–40 \( \mu M \), profilin = 5 \( \mu M \). For elongation experiments, 1.25 \( \mu M \) prepolymerized actin is also included.
b Barbed end elongation rate is calculated from measurements of “red” filaments off of “green” filament seeds in dual-color fluorescence microscopy assays (20 nM INF2 construct used). Errors are given as S.D. with the number of filaments measured shown in parentheses.
c Nucleation efficiency is calculated as the ratio of filaments produced to INF2 dimers present. The number of filaments produced was calculated from the polymerization slope at the point where 50% of the actin monomer has polymerized. Errors are given as S.D., based on filament concentrations for four INF2 concentrations in the linear range (root-mean-square deviation > 0.99 for linear fit).
d NM, not measured, because no INF2 was present. Nucleation efficiency is very low in the absence of INF2.

Promote maximal elongation rate for several forms (4). Profilin alleviates the elongation inhibition of INF2-(FH1-FH2), bringing the elongation rate back to that of the actin control (Fig. 2A, Table 1, supplemental Fig. 3). For INF2-(FH1-FH2-C) in the presence of profilin, filaments elongate twice as fast as actin alone (Fig. 2A, Table 1, supplemental Fig. 4). In addition, the number of filaments labeled with red phalloidin only is strongly decreased by profilin (supplemental Figs. 3 and 4), suggesting that profilin suppresses nucleation or severing activity for both constructs.

We also examined effects of these constructs on actin polymerization from monomers, using pyrene-actin fluorescence. These assays are sensitive to both nucleation and elongation effects (18), but because we knew the elongation effects of these constructs, we were able to focus on nucleation effects. Both INF2-(FH1-FH2) and INF2-(FH1-FH2-C) accelerate polymerization of 1 \( \mu M \) actin, with INF2-(FH1-FH2-C) being the more potent (Fig. 2B). Calculations from slopes of these polymerization curves, corrected for the 90% inhibition of elongation (Fig. 2A), show that the number of filaments produced increases linearly for concentrations up to 40 \( \mu M \) INF2-(FH1-FH2) and up to 10 \( \mu M \) for INF2-(FH1-FH2-C) (not shown). In this linear range, the ratio of filaments produced per INF2 dimer is 0.51 for INF2-(FH1-FH2) and 0.93 for INF2-(FH1-FH2-C) (Table 1). Addition of profilin inhibits the nucleation rate by about 14-fold for both constructs (Table 1).

All of the form FH2 domains tested inhibit barbed end capping by proteins such as heterodimeric capping protein and gelsolin (21, 27–30). Both INF2 constructs also had this effect (not shown).

INF2-(FH1-FH2-C) Accelerates Filament Depolymerization—At higher concentrations that those tested in the proceeding experiments, INF2-(FH1-FH2-C) has a biphasic effect on polymerization of 1 \( \mu M \) actin monomers, with initial polymerization acceleration followed by depolymerization acceleration (Fig. 3A). In contrast, similar concentrations of INF2-(FH1-FH2) accelerate polymerization but not depolymerization. The depolymerization effect does not occur with FH1-FH2-C constructs of mDia1 or FRL1 (not shown). Addition of 10 \( \mu M \) P$_i$ does not affect the polymerization phase but abolishes the depolymerization phase (Fig. 3A). This concentration of P$_i$ shifts the equilibrium from ADP to ADP-P$_i$ subunits on actin filaments (31, 32), suggesting that the INF2-(FH1-FH2-C)-induced transition from polymerization to depolymerization is dependent upon P$_i$ release after ATP hydrolysis.

Given the effect of P$_i$ on the depolymerization ability of INF2-(FH1-FH2-C), we postulated that conducting similar experiments in ADP-containing buffer, instead of the ATP-containing buffer used in Fig. 3A, would intensify the depolymerization effect. In these experiments, the actin monomers were ATP-bound and only mixed with the ADP-containing buffer upon reaction initiation to minimize nucleotide exchange prior to polymerization. The effect of INF2-(FH1-FH2-C) is indeed intensified under these conditions (Fig. 3B), with as little as 200 \( \mu M \) inducing full depolymerization after extended incubation (Fig. 3C). This effect does not occur for INF2-(FH1-FH2) (Fig. 3, B and C), mDia1-(FH1-FH2-C) (Fig. 3C) or FRL1-(FH1-FH2-C) (not shown).

INF2-(FH1-FH2-C) has a similar depolymerization effect on prepolymerized filaments, with 200 \( \mu M \) causing complete depolymerization of 1 \( \mu M \) actin in ATP- (Fig. 3D) or ADP-containing buffer (not shown). This effect was detected by both pyrene-actin assay and a high speed pelleting assay (supplemental Fig. 5), showing that INF2-(FH1-FH2-C) does not simply quench pyrene fluorescence. Depolymerization activity is blocked by P$_i$ or BeF (Fig. 3E). BeF acts as a stably bound P$_i$, mimicking ADP actin filaments have properties similar to those of ADP-P$_i$ filaments (33). These results suggest that the depolymerization mediated by INF2-(FH1-FH2-C) requires P$_i$ release from actin filaments.

INF2(C) Sequesters an Actin Monomer—One possible mechanism for the depolymerization effect is through monomer sequestration by the INF2 C terminus. We tested this possibility by expressing a C-terminal construct (Fig. 1, INF2-(C)). This construct is monomeric, as measured by analytical ultracentrifugation (supplemental Fig. 2).

By multiple criteria, INF2-(C) sequesters actin monomers. INF2-(C) completely inhibits barbed end elongation (Fig. 4A). In addition, INF2-(C) inhibits polymerization from actin monomers (supplemental Fig. 6). Finally, the steady-state concentration of polymerized actin decreases linearly with increasing INF2-(C) concentration (Fig. 4B).

To measure binding affinity for actin monomers, we labeled INF2-(C) on cysteines with fluorescein-maleimide, creating FL-INF2-(C). Mass spectroscopy confirms that both of the INF2-(C) cysteines are fluorescein-labeled (not shown). By pyrene-actin polymerization assay, there is no significant difference between labeled and unlabeled INF2-(C) (supplemental Fig. 6A), suggesting that the label does not significantly change INF2-(C) affinity for actin. By fluorescence anisotropy, the apparent dissociation constant (K$_{app}$) of INF2-(C) for both ATP- and ADP-actin monomers is ~60 nm (Fig. 4C and not shown). Addition of the monomer-binding drug latrunculin A (Lat A) did not affect this affinity (not shown). Addition of profilin to the assays inhibited the actin-induced anisotropy...
increase (not shown), indicating that INF2-(C) and profilin cannot bind monomers simultaneously.

To assess binding stoichiometry, we conducted velocity and equilibrium analytical ultracentrifugation experiments using FL-INF2-(C) and a saturating concentration of Lat A-stabilized ATP-actin monomers. By monitoring absorbance at 490 nm during centrifugation, FL-INF2-(C) could be followed in the presence or absence of actin, the actin being undetectable at this wavelength. Velocity analytical ultracentrifugation indicates a single species of 1.9 S for FL-INF2-(C) alone (Fig. 4D), very similar to that obtained for unlabeled INF2-C (supplemental Fig. 2). In the presence of a 7-fold molar excess of actin, FL-INF2-(C) sediments as a single species of 3.5 S (Fig. 4D), indicating a single species of actin-bound INF2-(C) under these conditions. By equilibrium analytical ultracentrifugation, FL-INF2-(C) appears to form a 1:1 complex with ATP-actin (Fig. 4E). We obtained an apparent mass of 30.7 kDa for FL-INF2-(C) alone and of 74.6 kDa for FL-INF2-(C) with saturating actin (predicted masses are 31.7 kDa for FL-INF2-C and 73.5 kDa for a 1:1 complex between FL-INF2-(C) and actin monomer).

We also conducted velocity analytical ultracentrifugation experiments on fluorescein-labeled INF2-(FH1-FH2-C) to assess its monomer binding ability. Our labeling protocol resulted in an average of 1.5 fluoresceins/INF2-(FH1-FH2-C) and did not affect the ability of INF2-(FH1-FH2-C) to polymerize or depolymerize 1/0.9262 M actin (not shown). FL-INF2-(FH1-FH2-C) sediments similarly to the unlabeled protein (4.4 S), and inclusion of a 7-fold molar excess of ATP- or ADP-actin monomers increases its sedimentation coefficient to 8.0 S (Fig. 4F), consistent with a mass increase of two actin monomers as analyzed by the program, Sedfit. Because INF2-(FH1-FH2-C) is dimeric, this result supports 1:1 binding by the C-terminal region of INF2-(FH1-FH2-C).

Actin monomer-binding proteins have variable effects on nucleotide exchange from monomers, with some accelerating (profilin (34)), some decelerating (cofilin (35)), and some having no effect (WASP WH2 motif (36)). We tested the effect of INF2-(C)
on nucleotide exchange rate and found it to have no effect (not shown, described under ‘Experimental Procedures’).

Monomer Sequestration Does Not Explain Depolymerization Effect—If monomer sequestration is the sole mechanism for INF2-(FH1-FH2-C)-mediated depolymerization, then the depolymerization rate should be similar to the depolymerization rate of actin alone, because sequestering proteins do not affect the rate of depolymerization (37). We measured the rate of INF2-(FH1-FH2-C)-mediated depolymerization using pyrene-actin assays. The depolymerization rate increases linearly with increasing INF2-(FH1-FH2-C) concentration (Fig. 5), with an observed rate of 12.7 nM/s at 400 nM INF2-(FH1-FH2-C). The initial concentration of actin filaments in these depolymerization reactions was 0.194 nM (calculated as described under ‘Experimental Procedures’). Assuming simple sequestration of monomers, the expected depolymerization rate of these filaments would be 1.45 nM/s, using previously measured barbed end (7.2 s⁻¹) and pointed end (0.27 s⁻¹) depolymerization rate constants for ADP-actin (38). However, little depolymerization should occur from the barbed end, because INF2-(FH1-FH2) slows this rate substantially (supplemental Fig. 7), similar to other FH2 domains (16, 39). Thus, INF2-(FH1-FH2-C) causes filament depolymerization at a rate that is 8.8-fold (if both ends are free) and 240-fold (if only pointed end is free) faster than that expected for a protein that is only sequestering monomers.

We further tested the role of sequestration on INF2-(FH1-FH2-C)-mediated depolymerization by examining critical concentration. Because INF2-(FH1-FH2-C) binds monomers in a 1:1 ratio, the critical concentration of actin should not rise higher than the concentration of INF2-(FH1-FH2-C) present in the assay. However, the critical concentration rises from about
0.1 μM in the absence of FH1-FH2-C to 1.6 and 2.4 μM in the presence of 0.4 and 0.8 μM INF2-(FH1-FH2-C), respectively (Fig. 6A). These increases are largely blocked by the addition of Pi (Fig. 6B). Thus, sequestration explains neither the rapidity of depolymerization by INF2-(FH1-FH2-C) nor its effect on critical concentration.

INF2-(FH1-FH2-C) Is a Potent Filament-severing Protein—One possible explanation for the rapidity of INF2-(FH1-FH2-C)-induced depolymerization is that this construct severs filaments, creating more pointed ends for depolymerization. We examined severing by fluorescence microscopy of rhodamine-phalloidin-labeled filaments (21, 25). INF2-(FH1-FH2-C) rapidly severs filaments, with maximal detectable severing occurring in less than 1 min (Fig. 7). Severing is detectable at a ratio of one INF2/20 actin molecules. Because of the resolution limit of light microscopy (about 0.2 μm), these measurements are weighted to large scale changes in filament length, with severing of shorter filaments or severing from filament ends being less well detected.

The C-terminal region is required for severing, because INF2-(FH1-FH2) does not sever (Fig. 7C). Furthermore, equimolar ratios of INF2-(FH1-FH2) and INF2-(C) do not sever (not shown), indicating that this activity requires the two regions to operate in cis. As with the depolymerization activity, severing is blocked by the inclusion of Pi (Fig. 7D). Severing activity implies that INF2-(FH1-FH2-C) can bind filament sides. High speed pelleting assays confirm tight side binding by INF2-(FH1-FH2-C), whereas neither INF2-(FH1-FH2) nor INF2-(C) displays such activity (not shown).

A recent publication showed evidence that the FH2 domain of mDia1 modestly accelerates Pi release (40). Because both severing and depolymerization appear to require ADP-actin,
INF2-(FH1-FH2-C) might also accelerate Pi release. However, we found that INF2-(FH1-FH2-C) does not affect Pi release rate significantly, either when filaments assemble in the presence of INF2-(FH1-FH2-C) or when filaments are preassembled (not shown).

INF2 DAD Acts as a WH2 Motif—The INF2 putative DAD bears some resemblance to a WH2 motif (41–43), including the presence of three highly conserved leucine residues (Fig. 1B). We tested the relevance of this sequence for actin binding to INF2-(C) by mutating these three leucines to alanines, which is effective in abolishing WH2 function for other proteins (44). Mutant INF2-(C) displays no measurable affinity for actin monomers in the fluorescence anisotropy assay (Fig. 4C) and does not inhibit actin polymerization (supplemental Fig. 6B). These results suggest that the DAD does indeed act as a WH2 motif. Furthermore, this motif is the major region in the C terminus that binds actin monomers, given the potent effect of the mutation. We doubt that this mutation causes a global misfolding of INF2-(C), because the WH2 motifs of other proteins are unstructured when not bound to actin (45) and INF2-(C) is predicted to have little overall tertiary structure.

We next tested the effects of the triple mutation on INF2-(FH1-FH2-C) activity. Mutant INF2-(FH1-FH2-C) accelerates actin polymerization but has no effect on depolymerization (Fig. 8, A and B). In severing assays, mutant INF2-(FH1-FH2-C) still severs, but the resulting filaments are significantly longer than for the wild-type protein (Fig. 8, C–G). Interestingly, a similar effect occurs in the severing assay when wild-type INF2-(FH1-FH2-C) is mixed with phalloidin-stabilized filaments (Fig. 8, C–G). Phalloidin prevents depolymerization from either end of actin filaments (46). The significance of this finding is addressed under “Discussion.”

Effect of Profilin on INF2-(FH1-FH2-C) Depolymerization Activity—Because profilin competes with INF2-(C) for actin monomer binding, we postulated that the presence of profilin might inhibit the depolymerization activity of INF2-(FH1-FH2-C). However, profilin actually accelerates depolymerization from prepolymized filaments in the presence of INF2-(FH1-FH2-C) (Fig. 9A). Furthermore, the depolymerization reaches an intermediate plateau value in the presence of profilin as opposed to the steady decline toward complete depolymerization in its absence. Thus, profilin accelerates depolymerization but does not allow complete depolymerization.

These depolymerization assays were conducted in the presence of 0.2 mM free ATP, which is one of our standard conditions for actin dynamics assays. We postulated that the intermediate plateau of depolymerization was the result of two profilin activities: 1) its ability to accelerate nucleotide exchange on actin monomers (34, 35) and 2) its ability to accelerate barbed end elongation in the presence of INF2-

3 Roberto Dominguez, personal communication.
Thus, the intermediate plateau occurs because of profilin-enhanced nucleotide exchange of depolymerized ADP-actin monomers with ATP followed by profilin- and FH1-enhanced barbed end addition of these ATP-monomers. These two activities counteract the depolymerization effect of INF2-(FH1-FH2-C), causing a new equilibrium to be established between monomer addition at the barbed end and depolymerization at the pointed end.

To test this hypothesis, we conducted depolymerization experiments in the absence of ATP. Under these conditions, profilin accelerates depolymerization without the intermediate plateau (Fig. 9B). Adding back low concentrations of ATP causes the intermediate plateau to reappear, with the duration of the plateau being proportional to the concentration of ATP added (Fig. 9B). Thus, profilin enhances the rates of both depolymerization and barbed end elongation by INF2-(FH1-FH2-C). From these experiments, we conclude that INF2-enhanced depolymerization occurs at the pointed end.

**DISCUSSION**

Our results suggest that INF2 is unique among formins in its ability to accelerate both the polymerization and depolymerization of actin filaments. INF2-mediated depolymerization requires P, release from the filament, because added P, blocks the effect. Two factors contribute to the INF2 depolymerization activity. First, the INF2 severing ability accelerates the rate of depolymerization by increasing the number of depolymerizable ends. Second, the C-terminal region of INF2 sequesters an actin monomer in a 1:1 complex, with the DAD/WH2 motif contributing significantly to this interaction. Mutation of the WH2 motif blocks the depolymerization effect of INF2-(FH1-FH2-C) but does not completely inhibit severing.

**Comparison of INF2 Properties with Other Formins**

The FH1 and FH2 domains of INF2 have effects on actin that are in the same range as those of other formins (reviewed in Refs. 18 and 47). INF2-(FH1-FH2) accelerates filament assembly with high potency. This assembly ability is comparable with those of mDia1 and mDia2, which are potent nucleators, more than 100-fold higher than FRL1 (6, 21). Filament assembly could be due to nucleation of new filaments or severing existing filaments. INF2-(FH1-FH2) does not sever, so the effect is presumably due to nucleation. INF2-(FH1-FH2) slows barbed end elongation by 90%, which places it in the following series relative to other formins: Cdc12 (100% inhibition), INF2 (90%), mDia2 (75%), FRL1 (50%), Bni1p (25%), and mDia1 (0%) (4, 6, 21, 26, 29, 39). Profilin has reciprocal effects on nucleation and barbed end elongation by INF2-(FH1-FH2), increasing elongation 10-fold and decreasing nucleation 14-fold. INF2-(FH1-FH2) antagonizes barbed end capping by heterodimeric capping protein.

At low concentrations (10 nM or below), the INF2-(FH1-FH2-C) construct has properties similar to INF2-(FH1-FH2), with two variations (see Table 1). First, profilin has a more dra-
Polymerization/Depolymerization by INF2

**Figure 9. Effect of profilin on depolymerization by INF2-(FH1-FH2-C).** A, depolymerization in the presence of ATP. Prepolymerized filaments (5% pyrene label) were diluted from 1.05 to 1.0 μM in the presence or absence of 200 nM INF2-(FH1-FH2-C) and/or 5 μM profilin as indicated (polymerization buffer with ATP). Pyrene fluorescence was monitored. Arbitrary units. B, depolymerization dependence on ATP. Assays were conducted similar to that described in A, except that ATP was removed from the polymerization buffer and actin filaments and then the indicated μM concentrations of ATP were added back. The curves labeled 0, 0.5, 1, and 2 all contained 200 nM INF2-(FH1-FH2-C), 5 μM profilin, and the indicated μM concentrations of ATP. Similar results were obtained in two independent experiments.

...subunits (step 3). The P_i releases from these subunits allowing INF2 to sever the filament (steps 4 and 5). INF2 remains at the barbed end of its severed filament, and the C terminus accelerates depolymerization of actin subunits from the pointed end, which is dependent upon the WH2 motif (step 6). Although this model outlines a broad framework of INF2 activities, it raises a number of questions concerning the details of each step.

**Question 1: Can Filament Severing Alone Account for the Rapid Depolymerization Effect?**—Severing increases the number of pointed ends available for depolymerization, but additional consequences of severing might be important. Our results show that one effect of INF2 is to raise the critical concentration from that of ATP- or ADP-P_i-actin (about 0.1 μM) to a value closer to that of ADP-actin (1.9 μM). Kinetic modeling studies suggest that both barbed and pointed ends can maintain caps of ATP- and/or ADP-P_i-actin subunits at steady state, whereas the bulk of the filament is in the ADP-actin state (48, 49). Therefore, severing could serve to expose ADP-actin at pointed ends, accelerating the shift to the ADP-actin critical concentration. However, our results show that the WH2 motif mutant can still seve but is defective in depolymerization, suggesting the existence of a severing-independent depolymerization mechanism.

**Question 2: Where Does Severing Occur?**—Like other formins, INF2 binds filament barbed ends. Severing could occur near the barbed end, resulting in a short filament that is then a substrate for pointed end depolymerization (mechanism 1). Alternately, severing could occur along the length of the filament, mediated by INF2 bound to the filament side (mechanism 2). We found that INF2-(FH1-FH2-C) co-pellets with phalloidin-stabilized filaments, suggesting that it can bind sides. However, INF2-mediated severing is extremely rapid and is not inhibited by phalloidin. Thus, it is possible that co-pelleting results from repeated barbed end severing by successive INF2 molecules binding the newly exposed barbed end.

**Question 3: Does INF2-mediated Depolymerization Occur from the Barbed End, the Pointed End, or Both?**—Our results on INF2-(FH1-FH2) show that the INF2 FH2 domain, like those of other formins, slows barbed end depolymerization substantially. After INF2-(FH1-FH2-C) severs a filament, we hypothesize that either it remains at the new barbed end (for mechanism 1), or another INF2 binds the new barbed end (mechanism 2). Either way, INF2 would slow barbed end depolymerization.

The effect of profilin also suggests pointed end depolymerization. Profilin accelerates the depolymerization rate, but net depolymerization reaches a plateau until ATP is depleted. Profilin acceleration of depolymerization could be because of its ability to compete with the INF2 C terminus for monomer binding, thus recharging the C terminus and accelerating the depolymerization cycle. Profilin could also accelerate barbed end depolymerization by reversal of its mechanism for acceleration of barbed end elongation (5). In dilution-induced depolymerization assays similar to those in supplemental Fig. 7, we found that profilin modestly increases depolymerization rate (not shown). The plateau derives from two opposing forces: 1) profilin- and FH1-me-
 actin subunit dissociation from the pointed end (severs the filament, and remains at the newly created barbed end. The C terminus of INF2 mediates accelerated

hydrolysis or phosphate release. Phosphate release enables severing (ADP-Pi-actin subunits (brown arrowheads). The inorganic phosphate product of ATP hydrolysis releases from the subunit (step 4), which is now ADP-actin (yellow arrowheads). We have no evidence that INF2 influences ATP hydrolysis or phosphate release. Phosphate release enables severing (step 5) by one of two possible mechanisms: 1) INF2 at the barbed end severs a short filament a few subunits from the barbed end followed by capping of the newly created barbed end by a second INF2; or 2) additional INF2 binds to the filament side, severs the filament, and remains at the newly created barbed end. The C terminus of INF2 mediates accelerated actin subunit dissociation from the pointed end (step 6).

Polimerization/Depolymerization by INF2

FIGURE 10. Model of INF2-mediated polymerization/depolymerization. INF2-(FH1-FH2-C) (blue symbol of the FH2 domain dimer with C-terminal extension containing the WH2 motif) accelerates nucleation (step 1) of ATP-actin (red arrowheads) and moves processively with the barbed end as monomers are added during elongation (step 2). Actin subunits hydrolyze bound ATP after their addition to the filament (step 3), becoming ADP-Pi-actin subunits (brown arrowheads). The inorganic phosphate product of ATP hydrolysis releases from the subunit (step 4), which is now ADP-actin (yellow arrowheads). We have no evidence that INF2 influences ATP hydrolysis or phosphate release. Phosphate release enables severing (step 5) by one of two possible mechanisms: 1) INF2 at the barbed end severs a short filament a few subunits from the barbed end followed by capping of the newly created barbed end by a second INF2; or 2) additional INF2 binds to the filament side, severs the filament, and remains at the newly created barbed end. The C terminus of INF2 mediates accelerated actin subunit dissociation from the pointed end (step 6).

The fact that other severing molecules, such as gelsolin, do not display the depolymerization ability of INF2 suggests that the WH2 motif might influence monomer disassembly rate. Kinetic modeling studies suggest that the ADP-actin pointed end disassembly rate constant is a limiting factor to depolymerization (48), so any mechanism that increases this rate constant will increase depolymerization rate. All other WH2 motifs studied interact with the barbed end side of the actin monomer (41, 43), so the INF2 WH2 motif would have to actively insert into the filament if it were to accelerate pointed end depolymerization. As such, depolymerization might be a direct consequence of multiple rounds of severing that occurs repeatedly at the filament pointed end.

Question 5: Is P Release the Switch from Polymerization to Depolymerization?—This conclusion derives from the fact that 10 mM NaPO₄ effectively blocked the depolymerization effect. We feel that other possible interpretations are less likely. It is doubtful that Pₐ binding to ADP-actin monomers affects this system. The affinity of Pₐ for ADP-actin monomers is much lower (100 mM dissociation constant (32)) than for ADP-actin in filaments (1.5 mM dissociation constant (31)), so that few ADP-actin monomers bind Pₐ in our assays. Furthermore, few ADP-actin monomers are present early in the process when depolymerization is most rapid. The increased ionic strength contributed by the NaPO₄ is probably not a factor, because an equivalent increase of NaCl does not affect depolymerization (not shown). We took care that both the polymerization buffer and the NaPO₄ were at pH 7.0 to avoid pH effects. We cannot rule out a direct inhibitory effect of Pₐ on INF2-(FH1-FH2-C).

Question 6: Does INF2 Accelerate P Release Rate?—Our results suggest that, unlike ADF/cofilin (33, 50), INF2 does not dramatically accelerate bulk Pₐ release rate. However, our assays were not able to detect effects at specific regions of filaments, such as barbed or pointed ends, so the possibility of localized Pₐ release acceleration remains.

N Terminus of INF2

We originally defined the INF group as metazoan formins having two similar features: 1) phylogenetic similarity in FH2 domains; and 2) the placement of FH1 and FH2 domains near the N terminus (as opposed to all other formins in which these domains are oriented toward the C terminus (9)). Nine metazoan sequences fit these criteria: two from mouse, three from puffer fish, two from Ciona (a tunicate), one from Drosophila, and one from Caenorhabditis elegans.

The results from the current study demonstrate that mouse INF2 extends considerably to the N terminus of the FH1 and thus is not “inverted.” So, is the inverted formin group a mirage, because of incomplete data bases or insufficient examination of these data bases? Our current answer is “no.” INF1, a mammalian formin closely related to INF2 in its FH2 domain, does not appear to possess a similar N-terminal extension. In both humans (GenBank™ accession AK092402) and mouse (AK173244), the 5′-untranslated region sequence contains stop codons in all three reading frames. These INF1 data base entries are for sequenced cDNAs (from human placenta and mouse brain, respectively) and are not predicted ORFs from genomic analysis. In these INF1 sequences, the FH1 domain starts at about amino acid 30, and almost 700 residues lie C-terminal to the FH2. The situation is similar for predicted INFs from Drosophila and C. elegans, whereas the puffer fish and Ciona sequences are not complete.

INF2 Regulation

The fact that INF2 has sequences of DID and DAD homology strongly suggests that it is regulated by autoinhibition, similar to mDia1 and mDia2 (10, 15–17). In addition, the
DAD appears to serve a second function: that of an actin monomer-binding WH2 motif. In additional experiments, we have found that a synthetic peptide of the INF2 DAD/WH2 binds monomers tightly. Interestingly, DAD peptides from mDia1 and mDia2 display no measurable affinity for monomers, and inclusion of the DAD sequence in mDia1 FH1-FH2 domain constructs does not alter their effects on actin polymerization (10). The reason for this difference could be that both mDia1 and mDia2 DADs contain a leucine-to-phenylalanine substitution at a key position for actin binding in WH2s (Fig. 1B). No DAD sequences from the FRL, DAAM, and FHOD formsins contain this key leucine. For this reason, we predict that INF2 is unique in this DAD/WH2 property, although testing of other formsins is needed.

Interestingly, INF2 appears unusual even among INFs in its possession of a DAD/WH2. The only other INF formim to contain a WH2 is one of the identified Ciona INFs (Ci6 (9)). Because the other INFs lack an N-terminal DID, the absence of a DAD is reasonable. On the other hand, the absence of a WH2 motif suggests either that these INFs do not possess the polymerization ability of INF2 or that they use different motifs for this activity.

Atomic structures exist for both the DAD-DID complex (13, 14) and the WH2-actin monomer complex (41, 43). These structures strongly suggest that INF2 DAD-WH2 could not bind the DID and actin monomer simultaneously. Therefore, we predict that the autoinhibitory DID/DAD interaction would block both actin polymerization (through the FH2) and depolymerization (requiring the WH2). The molecules that could disrupt INF2 DAD/DID interaction are unknown. Given their similarities with mDia1 and mDia2, Rho GTPases are prime candidates.

Extrapolation of the dual polymerization/depolymerization activities of INF2 to cellular conditions is premature at this point. However, INF2 activities would be predicted to generate short and highly transient filaments. Identification of activating factors will significantly advance elucidation of cellular function.

Our findings for INF2 reinforce a common theme with formsins. Despite gross similarities in FH2 domain function, formsin isoforms differ in their overall effects on actin dynamics. For this reason, careful characterization of each formsin is essential.

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