Aip1 Promotes Actin Filament Severing by Cofilin and Regulates Constriction of the Cytokineti c Contractile Ring*

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Background: Aip1 cooperates with cofilin to disassemble actin filaments.
Results: Aip1 increases the rate of filament severing by cofilin by binding the sides of actin filaments, and Δaip1 mutants have cytokinesis defects.
Conclusion: Aip1 promotes actin filament severing by the high concentrations of cofilin in cells.
Significance: We provide the first evidence that Aip1 promotes filament severing and illustrate its importance to cytokinesis.

Aip1 (actin interacting protein 1) is ubiquitous in eukaryotic organisms, where it cooperates with cofilin to disassemble actin filaments, but neither its mechanism of action nor its biological functions have been clear. We purified both fission yeast and human Aip1 and investigated their biochemical activities with or without cofilin. Both types of Aip1 bind actin filaments with micromolar affinities and weakly nucleate actin polymerization. Aip1 increases up to 12-fold the rate that high concentrations of yeast or human cofilin sever actin filaments, most likely by competing with cofilin for binding to the side of actin filaments, reducing the occupancy of the filaments by cofilin to a range favorable for severing. Aip1 does not cap the barbed ends of filaments severed by cofilin. Fission yeast lacking Aip1 are viable and assemble cytokinetic contractile rings normally, but rings in these Δaip1 cells accumulate 30% less myosin II. Further, these mutant cells initiate the ingestion of cleavage furrows earlier than normal, shortening the stage of cytokinetic ring maturation by 50%. The Δaip1 mutation has negative genetic interactions with deletion mutations of both capping protein subunits and cofilin mutations with severing defects, but no genetic interaction with deletion of coronin.

Actin filament polymerization and turnover drive a variety of essential cellular processes, including motility, endocytosis, cytokinesis, and the establishment of cell polarity. Regulation of filament disassembly maintains pools of monomeric actin available for polymerization. The small actin-binding protein cofilin, a member of the ADF (actin depolymerizing protein) family, promotes filament turnover by binding cooperatively to the sides of ADP-actin filaments and severing at interfaces between decorated and undecorated segments (1–4).

Cofilin is ubiquitous in eukaryotes and essential for viability of yeast, fruit flies, nematodes, and vertebrates where it contributes to endocytosis, cytokinesis, cell motility, neurite growth, and tissue formation (5). Mammals possess ADF and two cofilin isoforms: nonmuscle cofilin-1 and muscle cofilin-2. Fission yeast has one cofilin gene (6), making it favorable for studying the biological functions of cofilin. Fission yeast depends on actin filament severing by cofilin to remove filaments from sites of endocytosis (actin patches) and to produce actin filament fragments that promote assembly of new actin patches (7). Fission yeast cofilin also participates in both the assembly and disassembly of the cytokinetic contractile ring (6, 8).

Paradoxically, cofilin concentrations in cells, >10 μM (7), are high enough to stabilize rather than sever actin filaments (2). Cells might promote severing by phosphorylating a large fraction of the cofilin pool (9), which greatly reduces the affinity for actin filaments (10). Although some yeast cofilin is phosphorylated (11), the phosphorylation is not essential for the regulation of its cellular activities (12), so phosphorylation cannot be a universal mechanism. Alternatively, high cofilin concentrations in cells may sever newly polymerized filaments only transiently as the first few cofilins bind ADP-actin after phosphate dissociates, or other actin binding proteins might promote severing by enhancing the activity of cofilin or by competing with cofilin to reduce the density of cofilin on filaments (13).

Aip1 (Actin interacting protein 1), a candidate for regulation of the activity of cofilin, was discovered in a two-hybrid screen for budding yeast actin binding proteins (14, 15) and later identified in a broad spectrum of eukaryotes. The 65-kDa Aip1 protein consists of two seven-bladed β-propeller domains (16, 17), so it is also called WDR1 (WD domain repeat protein 1) in some species.

Aip1 participates in cell division, endocytosis and cellular locomotion. Cytokinesis is slow and sometimes fails in Dictyostelium lacking Aip1 (18) and mammalian cells depleted of Aip1 (19). Both budding yeast and Dictyostelium aip1 null mutants have endocytosis defects (18, 20, 21), whereas aip1 mutations reduce cellular motility in both Dictyostelium and human tissue cells (18, 22). Mutation or depletion of aip1 causes a wide range of developmental defects in multicellular organisms including muscles of nematodes and fruit flies (23, 24), epidermal cells of fruit flies (25), the immune system of mice (26), and root hairs of plants (27). In several cases aip1 mutant cells were noted to have abundant thick actin cables.

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Mutations of *aip1* interact genetically with cofilin mutations (14, 28) and genes for many other actin cytoskeletal proteins. A budding yeast *aip1 deletion mutation* had negative genetic interactions with mutations of *svr2/CAP* (29), capping protein and fimbrin (14, 30), and positive interaction with a mutation of tropomyosin (31). *Dictyostelium* *aip1* deletion mutations also had negative genetic interactions with a coronin deletion mutation (32), and the null mutation of one of the two *aip1* isoforms in nematode had positive genetic interactions with both tropomyosin and muscle myosin II mutations (33).

*Aip1* is proposed to enhance actin filament severing by cofilin and then cap the newly created barbed ends, but these biochemical activities are far from clear. When added to mixtures of actin filaments and cofilin, Aip1 from both budding yeast and nematodes increased the amount of actin in high speed supernatants (14, 34), originally interpreted as depolymerization. Direct observation by fluorescence microscopy showed that Aip1 reduced the average length of filaments in the presence of cofilin (35, 36). Other microscopic observations suggested that Aip1 promotes depolymerization (37). Some have proposed that a trimeric complex of cofilin, Aip1, and actin enhances the activity of cofilin (14), but no evidence exists for such a complex or direct physical interaction of Aip1 and cofilin except for yeast two-hybrid assays. Purified *Xenopus* Aip1 enhanced actin filament disassembly by cofilin, and the newly created barbed ends did not elongate in a bulk polymerization assay (35). This apparent capping activity of Aip1 depended on cofilin (35). Aip1 and capping protein complemented each other’s activities in an *in vitro* bead motility assay with budding yeast cellular extracts (38).

Here we compare the biochemical activities of fission yeast Aip1 (SpAip1) and human Aip1 (HsAip1). Both Aip1 proteins bind the sides of actin filaments and have modest effects on nucleation, filament elongation, and depolymerization. Aip1 enhances actin filament severing by cofilin but does not cap the severed fragments. Although fission yeast cells lacking Aip1 are viable, Δ*aip1* deletion mutants recruited less myosin II to their cytokinetic contractile rings, which initiated constriction prematurely.

**MATERIALS AND METHODS**

**Cloning**—SpAip1 cDNA was cloned from a fission yeast cDNA library. Human HsAip1 cDNA was cloned from a human full-length cDNA library (SC320182; Origene). Both cDNAs were subcloned into the maltose-binding protein (MBP)2 expression vector (NEB). A TEV protease cleavage site (ENLYFQG) was engineered between MBP and the recombinant proteins by PCR.

**Protein Purification**—Recombinant SpAip1 and HsAip1 proteins were expressed in BL21(DE3)-RIL cells (Agilent), induced with 0.5 mM isopropyl β-D-thiogalactopyranoside at 16 °C overnight. Cells were lysed by sonication in MBP buffer (400 mM NaCl, 20 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1 mM DTT) plus 0.5 mM PMSF and protease inhibitor mixture (Complete; Roche). Bacterial lysates were clarified and incubated with amylose resin (NEB) for 1 h at 4 °C on a rocking platform. The lysate and resin were poured into an empty column and washed with 2

The abbreviations used are: MBP, maltose-binding protein; SPB, spindle pole body; TIRF, total internal reflection fluorescence; LatA, latrunculin A.

### TABLE 1

| Name   | Genotype                                                                 | Source                     |
|--------|---------------------------------------------------------------------------|----------------------------|
| FY27   | *h*-leu1-32 ura4-D18 his3-D1 ade6-M216                                    | Lab stock                  |
| QC101  | *h*-aip1::ura4*   leu1-32 ura4-D18 his3-D1 ade6-M216                       | This study                 |
| QC107  | aip1::ura4*           sda1-mEGFP-KanMX6 rcl1-tdTomato-NatMX6               | This study                 |
| QC274  | rcl1-tdTomato-NatMX6 sda1-mEGFP-KanMX6                                   | Lab stock                  |
| QC109  | aip1::ura4*           rcl1-3GFP-KanMX6                                    | This study                 |
| QC289  | *h* wsp1Δ::KanMX6 leu1-32 ura4-D18 his3-D1 ade6-M216                      | Lab stock                  |
| QC313  | aip1::ura4*           wsp1::KanMX6                                       | This study                 |
| QC40   | *h*cdc12-112 ade6-M210 leu1-32 ura4-D18                                   | Lab stock                  |
| QC155  | aip1::ura4*           cdc12-112                                         | This study                 |
| KV7    | *h* acp2Δ::KanMX6 ade6-M216 his7-366 leu1-32 ura4-D18                    | Lab stock                  |
| KV112  | *h* acp2Δ::KanMX6 ade6-M216 his7-366 ura4-D18                            | Lab stock                  |
| QC152  | aip1::ura4*           acp2Δ::KanMX6                                      | This study                 |
| QC546  | *h* csr1::ura4*          leu1-32 ura4-D18 his3-D1 ade6-M?                  | This study                 |
| QC550  | csr1::ura4*             aip1::ura4*                                      | This study                 |
| QC102  | *h* adj1M2::KanMX6 ade6-M216                                            | Ref. 8                     |
| QC93   | *h* aip1::KanMX6-39nt1-mEGFP-acp1 leu1-32 ura4-D18 his3-D1 ade6-M216      | This study                 |
| QC517  | *h* aip1::KanMX6-P3nt1-aip1 leu1-32 ura4-D18 his3-D1 ade6-M216            | This study                 |
| QC518  | aip1::KanMX6-P3nt1-aip1 dfj1-M2-kanMX6                                   | This study                 |
| JW1558 | *h* kanMX6-P3nt1-mEGFP-lifeact ade6-M210 leu1-32 ura4-D18                 | Jianqui Wu                 |
| QC463  | kanMX6-P3nt1-mEGFP-lifeact aip1::ura4*                                   | This study                 |
| KGY978 | *h* arp3-1 ura4-D ade6-M210                                              | Lab stock                  |
| QC312  | aip1::ura4*           arp5-c1                                           | This study                 |
| MB9    | cdck8-27 his7-366 leu1-32 ura4-D18 ade6-M216                              | Lab stock                  |
| QC154  | aip1::ura4*           cdck8-27                                          | This study                 |
| QC168  | gfn1::ura4*          leu1-32 ura4-D18 his3-D1 ade6-M216                    | This study                 |
| QC311  | gfn1::ura4*           aip1::ura4*                                      | This study                 |
| QC341  | *h* end4::end4Δ663-1102-NatMX6                                          | Ref. 7                     |
| QC453  | aip1::ura4*           end4::end4Δ663-1102-NatMX6                           | This study                 |
| QC441  | ent·ent·Δ676-706-mEGFP-KanMX6                                           | Ref. 7                     |
| QC452  | aip1::ura4*           ent·ent·Δ676-706-mEGFP-KanMX6                        | This study                 |
| QC457  | *h* pan·panΔ1743-1794-NatMX6                                            | Ref. 7                     |
| QC454  | aip1::ura4*           pan·panΔ1743-1794-NatMX6                           | This study                 |
| TP190  | *h* leu1-32 ura4-D18 his3-D1 ade6-M216 myo1::KanMX6                      | Lab stock                  |
| QC314  | aip1::ura4*           myo1::KanMX6                                       | This study                 |
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10 column volumes of MBP buffer followed by five column volumes of TEV buffer (50 mM NaCl, 50 mM Tris-Cl, pH 7.4, 0.5 mM EDTA). The MBP-tagged recombinant protein was digested on the column with MBP-tagged TEV protease overnight at 4°C. The untagged recombinant protein was eluted from the column with three column volumes of MBP buffer, concentrated to 20 mM NaCl, 20 mM Tris-Cl, pH 8.0, 0.5 mM DTT, and gel-filtered on Sephacryl S-200 in the same buffer. Peak cofilin fractions were dialyzed against 10 mM Tris-Cl, 1 mM NaN₃, 1 mM EDTA, 250 mM NaCl, 2 mM DTT, pH 8.0, and gel-filtered on a 400-ml column of Sephacryl S-200. Peak cofilin fractions were dialyzed against 10 mM Tris-Cl, pH 7.5, 1 mM EGTA, 2 mM DTT; lysed by sonication; and clarified. The lysate supernatant was then applied to DEAE-Sepharose. The flow-through was collected; dialyzed against 10 mM NaCl, 10 mM Tris-Cl, and 0.2 mM EGTA; and gel-filtered on Sephacryl S-200 in the same buffer. Both fission yeast and human cofilin were dialyzed into

For microscopy experiments, actin filaments in 50 mM PIPES, pH 6.8, 50 mM KCl, 0.2 mM CaCl₂, 0.2 mM ATP were labeled on lysines by incubating overnight at 4°C with a 1:13 molar ratio of actin to Alexa Fluor 488 carbocyclic acid succinimidyl ester (A-20000; Invitrogen). After depolymerization, clarification, and gel filtration on Sephacryl S-300, purified Alexa 488-actin monomers were typically ~30–50% labeled.

Recombinant fission yeast cofilin was purified (2) from bacteria induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 5 h, lysed by sonication, and clarified. Protein was precipitated with 70% ammonium sulfate, resuspended in buffer D (10 mM Tris-Cl, 1 mM Na₃VO₄, 1 mM EDTA, 250 mM NaCl, 2 mM DTT, pH 8.0), and gel-filtered on a 400-ml column of Sephacryl S-200. Peak cofilin fractions were dialyzed against DEAE buffer 2 (25 mM Tris-Cl, pH 8.4, 2 mM DTT), were loaded on a DEAE-Sepharose column, and eluted with a 500-ml gradient of 0–500 mM NaCl in DEAE buffer 2. Human cofilin 1 was expressed in pLysS cells induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 37°C. Cell pellets were resuspended in 10 mM Tris-Cl (pH 7.5), 1 mM EGTA, 2 mM DTT; lysed by sonication; and clarified. The lysate supernatant was then applied to DEAE-Sepharose. The flow-through was collected; dialyzed against 150 mM NaCl, 10 mM Tris-Cl (pH 7.5), 2 mM DTT; and gel-filtered on Sephacryl S-200 in the same buffer. Both fission yeast and human cofilin were dialyzed into

A, B, SDS-PAGE of standards and purified SpAip1 (A) and HsAip1 (B) stained with Coomassie Blue. C and D, cosedimentation of Aip1 with actin filaments. Samples of 1.5 μM SpAip1 (C) or 1.5 μM HsAip1 (D) were incubated with 1 μM actin filaments for 1 h and then centrifuged at 100,000 × g for 25 min. Supernatant and pellet samples were analyzed by SDS-PAGE and stained with Coomassie Blue. The SpAip1 samples were twice the volumes of the HsAip1 samples. E and F, fluorescence quenching to measure equilibrium binding of a range of concentrations of SpAip1 (E) or HsAip1 (F) to 1 μM 100% labeled pyrenyl-actin filaments in KMEI buffer at 25°C. The smooth curves are fits of the binding equation to the data yielding Kₐ values of 2.0 μM for SpAip1 and 3.0 μM for HsAip1. The inset in E shows fluorescence quenching of 1 μM 20% pyrenyl-actin filaments SpAip1 with the best fit (smooth curve) yielding a Kₐ of 1.2 μM. G, pelleting assay: 1 μM 94% labeled pyrenyl-actin filaments were incubated with a range of concentrations of SpAip1 and centrifuged at 100,000 for 25 min. Actin in the pellet samples were analyzed by SDS-PAGE and stained with Coomassie Blue (top panel) and quantified using NIH ImageJ (middle panel). E, the ratio of SpAip1 to actin in the pellet samples was quantified to measure the stoichiometry between SpAip1 and actin (bottom panel). H, pelleting assay: 2 μM actin filaments were incubated with either 15 μM HsCofilin or 10 μM HsAip1 or both and centrifuged at 100,000 × g for 1 h. Pellet samples were analyzed by SDS-PAGE and stained with Coomassie Blue. The intensities of the HsAip1 bands were quantified with NIH ImageJ.

FIGURE 1. Fission yeast SpAip1 and human HsAip1 bind actin filaments. A and B, SDS-PAGE of standards and purified SpAip1 (A) and HsAip1 (B) stained with Coomassie Blue. C and D, cosedimentation of Aip1 with actin filaments. Samples of 1.5 μM SpAip1 (C) or 1.5 μM HsAip1 (D) were incubated with 1 μM actin filaments for 1 h and then centrifuged at 100,000 × g for 25 min. Supernatant and pellet samples were analyzed by SDS-PAGE and stained with Coomassie Blue. The SpAip1 samples were twice the volumes of the HsAip1 samples. E and F, fluorescence quenching to measure equilibrium binding of a range of concentrations of SpAip1 (E) or HsAip1 (F) to 1 μM 100% labeled pyrenyl-actin filaments in KMEI buffer at 25°C. The smooth curves are fits of the binding equation to the data yielding Kₐ values of 2.0 μM for SpAip1 and 3.0 μM for HsAip1. The inset in E shows fluorescence quenching of 1 μM 20% pyrenyl-actin filaments SpAip1 with the best fit (smooth curve) yielding a Kₐ of 1.2 μM. G, pelleting assay: 1 μM 94% labeled pyrenyl-actin filaments were incubated with a range of concentrations of SpAip1 and centrifuged at 100,000 for 25 min. Actin in the pellet samples were analyzed by SDS-PAGE and stained with Coomassie Blue (top panel) and quantified using NIH ImageJ (middle panel). E, the ratio of SpAip1 to actin in the pellet samples was quantified to measure the stoichiometry between SpAip1 and actin (bottom panel). H, pelleting assay: 2 μM actin filaments were incubated with either 15 μM HsCofilin or 10 μM HsAip1 or both and centrifuged at 100,000 × g for 1 h. Pellet samples were analyzed by SDS-PAGE and stained with Coomassie Blue. The intensities of the HsAip1 bands were quantified with NIH ImageJ.
KMEI buffer (50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 10 mM imidazole, pH 7.0), flash-frozen in aliquots, and stored at 
−8°C.

Equilibrium Binding Experiments—We measured the affinity of Aip1 for pyrenyl-actin filaments by fluorescence quenching. The reactants were mixed manually in KMEI buffer in a 96-well plate and incubated for 30 min at room temperature, and fluorescence was measured with a Spectra Max Gemini XPS (Molecular Devices, Downingtown, PA) plate reader with excitation measured with a Spectra Max Gemini XPS plate and incubated for 30 min at room temperature, and fluorescence was measured with a Spectra Max Gemini XPS (Molecular Devices, Downingtown, PA) plate reader with excitation at 365 nm and emission at 407 nm.

Bulk Assembly and Disassembly of Actin Filaments—We monitored the assembly of pyrenyl-actin filaments using a PTI Alphascan fluorimeter (Photo Technology International) with excitation at 365 nm and emission at 407 nm. At the start of each reaction, 2 μM pyrenyl-ATP-Ca²⁺-actin (10% pyrene-labeled) in buffer G was brought to 50 μM MgCl₂, 0.2 mM EGTA for 2 min at 25 °C to exchange Mg²⁺ for Ca²⁺ and then was polymerized in KMEI buffer. The concentration of barbed ends was calculated using the equation [ends] = assembly rate/(k⁺ [actin monomer]), where k⁺ is the elongation rate constant measured under the same conditions by TIRF microscopy (see below), and the actin monomer concentration is the total actin concentration minus the polymerized actin concentration.

Total Internal Reflection Fluorescence Microscopy—Glass flow chambers were prepared as described previously (41). Before introducing actin, each chamber was incubated for 1 min with two washes of 8 μl of 0.5% Tween 80 in high salt TBS (50 mM Tris-Cl, pH 7.5, 600 mM KCl), followed by two washes with 10 μl of high salt TBS, two 30-s incubations with 8 μl of 250 mM N-ethylmaleimide-inactivated chicken skeletal muscle myosin, two washes with 10 μl of high salt TBS, and two 30-s incubations with 8 μl of 10% BSA in high salt TBS. The standard microscopy buffer consisted of 10 mM imidazole, pH 7.0, 50 mM KCl, 1.0 mM EGTA, 1 mM MgCl₂, 1 mM EGTA, 0.3 mM ATP, 15 mM glucose, 50 mM DTT, 0.02 μM CaCl₂, 20 μg/ml catalase, 100 μg/ml glucose oxidase, and 0.5% methylcellulose (4,000 centipoise at 2% (w/v)).

Polymerization was initiated by introducing actin into the chamber with or without Aip1 in microscopy buffer. For depolymerization and severing experiments, actin was polymerized in the chamber for ~5 min, and then the solution was replaced with a fresh sample of proteins (Aip1 and/or cofilin) in microscopy buffer, and imaging was continued. We generated time lapse movies of growing or shortening actin filaments using prism-style TIRF microscopy on an Olympus IX70 inverted microscope and a Hamamatsu C4742-95 CCD camera con-
trolled by MetaMorph software (Molecular Devices) (41). Specimens were illuminated for 10 s at intervals of 10 s for polymerization and depolymerization experiments and 5 s for severing experiments. Images were processed with NIH ImageJ software. For severing experiments, we monitored the elongation of each filament by time lapse imaging prior to introduction of cofilin and Aip1 into the chamber, allowing us to distinguish faster growing barbed ends from slowly growing pointed ends. For each sample, we measured the rates of barbed end elongation, shortening or severing of 10–15 filaments, typically over a span of at least 300 s.

Genetics and Molecular Genetics—The Δaip1 and Δcrn1 mutants were generated by replacing the ORFs of the fission yeast genes with the ura4+ cassette in pFA6a-URA4 vector using a PCR-based homologous recombination method (42). Overexpression of Aip1 or GFP-Aip1 was driven by the strong 3nmt1 promoter, which replaced the endogenous promoter in the genome, but only overexpression of Aip1 rescued the growth defect of cofilin mutants. Genetic crosses were carried out at 25 °C for 3 days before tetrad dissections. For dilution assays, we used the cell cultures grown at 25 °C with an A600 between 0.3 and 0.6. Table 1 lists the yeast strains used in this study.

Fluorescence Microscopy—Yeast cells were cultured by standard methods at 25 °C. To visualize actin filaments, cells were fixed with 4% formaldehyde in TEMK buffer (50 mM Tris-Cl, pH 7.4, 1 mM EGTA, 2 mM MgCl₂, 50 mM KCl) and permeabilized with 1% Triton X-100 in TEMK buffer before staining with 2 μM Bodipy-phallacidin (Invitrogen) in TEMK buffer. For imaging live cells, actively growing cultures in YE5s medium with A600 between 0.4 and 0.5 were harvested by centrifugation at 3000 rpm for 1 min and washed briefly three times with EMM5s before resuspending, applying to a 25% gelatin pad made with EMM5s and sealing under a coverslip. Cells were imaged with a 100× Plan Apochromat objective lens (NA 1.40) on an Olympus IX-71 microscope equipped with a
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CSU-X1 confocal spinning disk unit (Yokogawa) with either an Orca-ER camera (Hamamatsu) for fixed cells or electron multiplying charge-coupled device camera (Andor, iXon) for live cells. We measured the cytoplasmic concentration of Aip1 to be 1.0 ± 0.3 μM (n = 81) in aip1::Patip1-mGFP-aip1 cells (43) by confocal fluorescence microscopy (44).

RESULTS

We purified recombinant fission yeast Aip1 (SpAip1) and human Aip1 (HsAip1) (Fig. 1, A and B) and studied their interactions with actin filaments. These homologs share 33% sequence identity, and homology models based on the crystal structure of Saccharomyces cerevisiae Aip1 predict that both consist of two seven-bladed β-propeller domains.

Aip1 Binds to the Sides of Actin Filaments—Like Aip1 of other organisms (16, 35, 45, 46), both SpAip1 and HsAip1 pellet with chicken muscle actin filaments (Fig. 1, C and D). At saturation, the stoichiometry was 1 SpAip1 per polymerized actin subunit (Fig. 1G). We measured the affinities of these interactions with fluorescence spectroscopy by taking advantage of the fact that Aip1 binding quenches the fluorescence of pyrenyl-actin filaments (Fig. 1, E and F). Both fission yeast and human Aip1 reduced the fluorescence of 1 μM pyrenyl-actin filaments in a concentration-dependent fashion (Fig. 1, E and F) but did not depolymerize the filaments (Fig. 1G). Quenching was similar for 20 and 100% pyrenyl-labeled actin filaments (Fig. 1E, inset). Fitting binding curves to the dependence of the fluorescence quenching on Aip1 concentration gave K_d values of 2.0 μM for SpAip1 and 3.0 μM for HsAip1. Binding of HsAip1 to actin filaments was significantly lower in the presence of HsCofilin (Fig. 1H), showing competition between the two proteins for binding actin filaments.

Effects of Aip1 on Actin Polymerization and Depolymerization—Direct observations by total internal reflection fluorescence (TIRF) microscopy showed that actin filament barbed ends elongated at all concentrations of SpAip1 and HsAip1 that we tested (Fig. 2, A and A’). Submicromolar concentrations of SpAip1 increased the rate of barbed end elongation by ~50%, a small but reproducible effect, whereas higher concentrations slowed polymerization (Fig. 2A). In contrast, HsAip1 modestly slowed but did not completely halt barbed end elongation at all concentrations tested (Fig. 2A’). Thus, neither human nor fission yeast (recombinant) Aip1 caps barbed ends on their own at the concentrations tested.

In the presence of HsAip1, more filaments appeared in TIRF microscopy experiments, so we analyzed the time course of spontaneous polymerization of 2 μM actin monomers with a range of concentrations of SpAip1 or HsAip1 (Fig. 2, B and B’) to measure the rates of nucleation (Fig. 2, C and C’). We normalized these curves to correct for quenching of the pyrene fluorescence. Calculating the number of filament ends from the time course had negative genetic interactions with deletion of the genes for each subunit of capping protein; Δaip1Δacp1 grew slowly, whereas Δaip1Δacp2 was not viable. C, overexpression (O/E) of Aip1 partially rescued the slow growth of adf1-M2 mutant. D, the Aip1 null mutation had no genetic interaction with the deletion mutant of coronin (crr1). E, tetrad dissection plates of Δcrr1 crossed with either adf1-M2 or adf1-M3 show that the Crn1 null mutation is synthetically lethal with cofilin mutants. Red circles highlight inviable double mutants.

### TABLE 2

| Gene    | Protein      | Mutations      | Genetic interaction         |
|---------|--------------|----------------|-----------------------------|
| aip1    | Cofilin      | aip1-M2, aip1-M3 | Synthetically lethal (8)     |
| arp3    | Arp2/3 complex subunit | arp3-M1        | None                         |
| acp1    | Capping protein subunit | Δacp1         | Slow growth at all temperatures |
| acp2    | Capping protein subunit | Δacp2         | Synthetically lethal         |
| cd8     | Tropomyosin  |                 | None                         |
| cdc12   | Formin       |                 | Partial rescue of growth defect at 30 °C |
| crn1    | Coronin      |                 | None                         |
| end4    | Endocytosis adapter | end4Δ663-1102 | None                         |
| ent1    | Endocytosis adapter | ent1Δ647-706 | None                         |
| gfn1    | Glial maturation factor | Δgfn1        | None                         |
| pan1    | Endocytosis adapter | Δpan1Δ743-1794 | None                         |
| myo1    | Myosin-1     | Δmyo1          | None                         |
| wsp1    | WASp         | Δwsp1          | Partial rescue of growth defect at 36 °C |

![FIGURE 4. Analysis of genetic interactions of Δaip1. A–D, ten fold serial dilutions of cells were grown for 2 days on YES5 plates at three temperatures, related to Table 2. A, the Aip1 null mutation Δaip1 partially rescued the growth defects of the cdc12-112 and Δwsp1 mutants. B, the Aip1 null mutation had negative genetic interactions with deletion of the genes for each subunit of capping protein; Δaip1Δacp1 grew slowly, whereas Δaip1Δacp2 was not viable. C, overexpression (O/E) of Aip1 partially rescued the slow growth of adf1-M2 mutant. D, the Aip1 null mutation had no genetic interaction with the deletion mutant of coronin (crn1). E, tetrad dissection plates of Δcrn1 crossed with either adf1-M2 or adf1-M3 show that the Crn1 null mutation is synthetically lethal with cofilin mutants. Red circles highlight inviable double mutants.](image-url)
A range of SpAip1 concentrations did not change the rate that subunits dissociated from barbed ends (Fig. 2D) or the total depolymerization rate of actin filaments in bulk assays (Fig. 2, E and F). This is additional evidence that (recombinant) Aip1 does not cap the barbed ends of actin filaments on its own, unlike capping protein, which caps barbed ends with low nanomolar affinity (47).

Aip1 Enhances Severing of Actin Filaments by Cofilin—Aip1 was reported to enhance cofilin-mediated filament severing (14, 48), but this process was not observed directly, so we examined the effects of Aip1 and cofilin on actin filament severing by TIRF microscopy (Fig. 3, A and B). To keep severing slow, we used 100 nM fission yeast cofilin, a concentration well above the 10 nM optimum. SpAip1 dramatically stimulated severing by 100 nM cofilin, with a maximum rate at 1.5 μM SpAip1 and lower rates at higher concentrations (Fig. 3C). At all SpAip1 concentrations tested ~80% of new barbed ends created by severing events depolymerized (Fig. 3D) at rates that decreased insignificantly with SpAip1 concentration (Fig. 3E). These depolymerization rates were higher than published values (2), likely because severing near barbed ends was difficult to distinguish from filament depolymerization.

HsAip1 stimulated severing by human cofilin 1 with an optimum concentration of 3.5 μM (Fig. 3C). We used 7 μM human cofilin 1 in these experiments, because of its lower affinity for actin filaments and its higher optimal concentration for severing. In the absence of HsAip1, basal levels of severing were similar to those of 100 nM fission yeast cofilin. HsAip1 did not increase the fraction of barbed ends of severed filaments that did not shorten, consistent with a lack of capping activity in the presence of cofilin (Fig. 3D). HsAip1 did not significantly slow the rate of depolymerization of barbed ends created by severing (Fig. 3E). These results and the evidence that HsAip1 competes with HsCofilin for binding actin filaments (Fig. 1H) suggest that Aip1 increases severing by reducing the binding density of cofilin on the filaments into a range that favors severing.

Genetic Interactions of Δaip1—Cells lacking Aip1 grew normally (Fig. 5A), but genetic crosses revealed negative interactions between Δaip1 and mutations of a subset of genes for proteins involved with endocytosis (Fig. 4; Table 2). The strongest negative interactions were with two cofilin mutations that reduce the severing activity of cofilin (7). Reciprocally, overexpression of Aip1 partially rescued the slow growth of these cofilin mutant strains (Fig. 4C). The Δaip1 mutation also had
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strong negative genetic interactions with deletion of either subunit of capping protein, Acp1 and Acp2 (Fig. 4B). Although both interacted with Δaip1, the capping protein deletion mutations did not interact with the cofilin mutant adj1-M2, and overexpression of Acp2 did not rescue the growth defect of adj1-M2. Aip1 had no genetic interaction with genes for several other proteins that participate in endocytosis (Arp2/3 complex, Ent1p, End4p, and Pan1p), including the coronin deletion mutant Δcrn1, even though Δcrn1 is synthetically lethal with the cofilin mutants (Fig. 4E). The Δaip1 deletion had positive genetic interactions with temperature-sensitive formin mutations (Fig. 4C). Mutations in Aip1 enhanced the severing of actin filaments by cofilin in vitro (7, 8). Because Aip1 enhances the severing of actin filaments by cofilin in budding yeast (49), we tested whether Aip1 influences the dynamics of cables in fission yeast. Actin cables labeled with LifeAct-GFP overexpression of actin cables in budding yeast relies on the activity of contractile rings accumulated Rlc1p at similar rates in (49) when several proteins are recruited to rings. Maturing foreshortening the maturation period before constriction

Contributions of Aip1 to Cytokinesis—The severing of actin filaments by cofilin plays a critical role in both endocytosis and cytokinesis in fission yeast (7, 8). Because Aip1 enhances the severing of actin filaments by cofilin in vitro, we expected that it participates in these two cellular processes as well. Another study presents an analysis of Aip1 in endocytosis (43). Our study is focused on the contribution of Aip1 to cytokinesis. Mutations in aip1 genes cause cytokinesis defects in many organisms (18, 22, 25), although a mechanistic understanding is lacking.

We used time lapse fluorescence microscopy to compare the assembly, maturation, constriction, and disassembly of actomyosin contractile rings in fission yeast cells with and without Aip1 (Fig. 6A and Table 3). We used cells expressing both a contractile ring marker, the myosin II regulatory light chain Rlc1p-tdTomato, and a spindle pole body (SPB) marker, Sad1p-mGFP, to track these events in time. Separation of the SPBs was defined as time 0.

The time course of contractile ring assembly did not differ significantly (log rank test) in wild type and Δaip1 cells (Figs. 6B and C and Table 3). However, newly formed contractile rings in Δaip1 cells had fewer Rlc1p molecules than the wild type cells (Fig. 7D).

Surprisingly, contractile rings began to constrict earlier in Δaip1 cells than wild type cells (Fig. 7, A and B, and Table 3), foreshortening the maturation period before constriction (49) when several proteins are recruited to rings. Maturing contractile rings accumulated Rlc1p at similar rates in Δaip1 and wild type cells, but the rings in Δaip1 cells ended up with ~30% fewer Rlc1p molecules, because they started with fewer Rlc1p molecules and because constriction started early (Fig. 7D).

As in wild type cells (49), the total fluorescence of Rlc1p-tdTomato in rings of Δaip1 cells was constant through most of ring constriction (Fig. 7D, gray shaded area). Contractile rings in Δaip1 cells constricted at 0.42 ± 0.01 μm/min (n = 10), slightly faster than rings in wild type cells (0.31 ± 0.01 μm/min, n = 10, p < 0.001) (Fig. 7C). As a result, ring constriction in Δaip1 cells takes ~5 min less than in wild type cells (Table 3). Overall, cytokinesis was ~16 min (24%) faster in Δaip1 cells than wild type cells, because the maturation period was shorter and the rings constricted faster.

TABLE 3

Comparison of the time for cytokinesis stages in aip1+ and Δaip1 cells

| Genotype | Ring assembly | Ring maturation | Ring constriction |
|----------|---------------|-----------------|------------------|
| aip1+    | 11.1 ± 0.5 (n = 89) | 22.6 ± 6.1 (n = 53) | 34.2 ± 4.8 (n = 66) |
| Δaip1    | 11.9 ± 0.4 (n = 83) | 11.4 ± 3.1 (n = 51) | 29.3 ± 2.4 (n = 59)* |

*p < 0.0001 (two-tailed Student’s t test).
When the circumference of rings decreased to \( \approx 3 \mu m \) (Fig. 8A, shaded area), myosin II marked by Rlc1p-tdTomato rapidly dispersed into the cytoplasm as constriction finished. This loss of Rlc1p-tdTomato fluorescence followed single exponential time courses with similar rate constants in wild type and \( \Delta aip1 \) cells (Fig. 8B). Because rings finished constricting \( \approx 5 \) min faster in \( \Delta aip1 \) than wild type cells, dissociation of Rlc1p-tdTomato was incomplete in most \( \Delta aip1 \) cells (Fig. 8C). The contractile ring remnants marked with Rlc1p-tdTomato moved away from the division plane through the cytoplasm toward the poles before disappearing after \( 8 \) min (Fig. 8C). In contrast, such movements of ring remnants were only seen in \( 40\% \) of wild type cells and lasted only \( \approx 4 \) min on average. Fast imaging of the ring remnants labeled by Rlc1p-3GFP confirmed their rapid movement in the cytoplasm of \( \Delta aip1 \) cells (Fig. 8D).

**DISCUSSION**

Previous work provided evidence that Aip1 helps cofilin disassemble actin filaments and might cap the newly formed barbed ends. Our evidence suggests that Aip1 enhances severing by limiting binding of cofilin to ADP-actin filaments. Aip1 did not cap or slow depolymerization of the new barbed ends under the conditions of our assays. Our experiments on fission yeast show that Aip1 participates in the turnover of actin cables and constriction of actomyosin contractile rings.

**Aip1 Enhances the Severing Activity of Physiological Concentrations of Cofilin**—Our evidence supports the hypothesis that Aip1 enhances the severing of actin filaments by competing with cofilin for binding sites on the actin filament, as originally shown for phalloidin and myosin (13). Competition by Aip1 lowers the binding density of high concentrations of cofilin into a range where severing is favored. The \( 20 \mu M \) concentration of cofilin in the cytoplasm of fission yeast (7) is more than 3 orders of magnitude higher than the optimal concentration for steady state severing (2). High concentrations of cofilin can sever newly polymerized filaments transiently before they saturate the filament (50), so competition from Aip1 should prolong this period of severing.

However, other mechanisms must participate in cells, because the 1 \( \mu M \) cytoplasmic concentration of Aip1 is lower than the 20 \( \mu M \) concentration of cofilin, and the ratio of GFP-Aip1 molecules to cofilin in endocytic actin patches is \( \approx 1:20 \) (7, 43), much lower than what is required to enhance the severing activity of cofilin in our in vitro experiments. Another potential competitor, fimbrin, is present in greater numbers than Aip1 (44). The \( \beta \)-propeller protein coronin also facilitates severing.
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**FIGURE 8. Defective dissociation of myosin II from contractile rings in Δaip1 cells.** A, time courses of the circumference (open squares) and the fluorescence intensity (red circles) of contractile rings in a wild type cell expressing Rlc1p-tdTomato. Time is relative to the separation of SPBs at time 0. The shaded area highlights the rapid dissociation of Rlc1p from the contractile rings as their circumference decreased to < 4 μm. B, time courses of the average fluorescence intensities of Rlc1p-tdTomato that is dissociating from the rings of (red circles) aip1Δ and (blue squares) Δaip1 cells; both data sets fit with single exponential curves (solid lines). C and D, time series of fluorescence micrographs (reverse contrast, maximum intensity projections) of a wild type (aip1Δ) and a Δaip1 cell expressing Rlc1p-tdTomato (C) and a Δaip1 cell expressing Rlc1p-3GFP (D). Arrowheads mark ring remnants dissociating from the ring and moving toward the cell poles in Δaip1 cells. Cells are outlined in dashed lines. The time intervals are 60 s in (C) and 30 s in (D). Bar, 5 μm.

filaments by cofilin (51), and deletion of coronin was synthetically lethal with our cofilin severing mutants (Fig. 4E), but not with deletion of Aip1.

In addition to direct competition, binding of the two β-propeller domains of Aip1 may change the flexibility of actin filaments, making them more favorable for severing by cofilin. In addition, a complex of Aip1 and cofilin may sever actin filaments more efficiently than cofilin alone (30, 52).

Aip1 purified from Xenopus eggs capped the barbed ends of actin filaments severed by cofilin (35), but we detected no capping activity in our preparations of recombinant human or fission yeast Aip1. Similarly, purified recombinant worm Aip1 did not cap the ends of actin filaments (16). On the other hand, quantitative analysis of actin patch dynamics in cells with aip1Δ and capping protein deletions supports the hypothesis that both proteins cap actin filaments (43). The simplest explanation is that Aip1 cooperates with other factors in cells to cap actin filaments and that Aip1 prepared from Xenopus eggs contained those factors that helped to cap the filament ends.

The Role of Aip1 in the Turnover of Actin Cables and Actomyosin Contractile Rings—Our evidence that Aip1 contributes to the turnover of actin cables and contractile rings in fission yeast adds to previous work showing that Aip1 mutations lead to a wide range of defects in many model organisms. Actin cables in fission yeast are bundles of actin filaments nucleated by formin. Cables turn over slower in Δaip1 cells than in wild type cells. The Δaip1 cells are also hypersensitive to LatA, similar to budding yeast with other deletion mutations, including Δcap2 (capping protein), Δtpm1 (tropomyosin), Δasa6 (fimbrin), and Δsla2 (Hip1R) (31, 53), which can be explained by slower turnover of actin filaments in these mutants.

We discovered that Aip1 is also important for the initiation of contractile ring constriction. The contractile rings of Δaip1 mutants constrict prematurely after a shortened maturation stage of ring assembly. Nothing about the known activities of Aip1 or the assembly of contractile rings predicted this striking phenotype, so the Δaip1 mutation has revealed a new feature regulating cytokinesis. Further studies of this function of Aip1 may provide clues in understanding its role in regulating the contractility of both body wall muscle and the somatic gonad of C. elegans (33, 54). Additionally, Δaip1 mutants display a striking phenotype with ring remnants moving away from the division site at the end of cytokinesis as a result of slightly accelerated ring constriction.

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