Fission Yeast Aip3p (spAip3p) Is Required for an Alternative Actin-directed Polarity Program

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Aip3p is an actin-interacting protein that regulates cell polarity in budding yeast. The *Schizosaccharomyces pombe*-sequencing project recently led to the identification of a homologue of Aip3p that we have named spAip3p. Our results confirm that spAip3p is a true functional homologue of Aip3p. When expressed in budding yeast, spAip3p localizes similarly to Aip3p during the cell cycle and complements the cell polarity defects of an *aip3Δ* strain. Two-hybrid analysis shows that spAip3p interacts with actin similarly to Aip3p. In fission yeast, spAip3p localizes to both cell ends during interphase and later organizes into two rings at the site of cytokinesis. spAip3p localization to cell ends is dependent on microtubule cytoskeleton, its localization to the cell middle is dependent on actin cytoskeleton, and both patterns of localization require an operative secretory pathway. Overexpression of spAip3p disrupts the actin cytoskeleton and cell polarity, leading to morphologically aberrant cells. Fission yeast, which normally rely on the microtubule cytoskeleton to establish their polarity axis, can use the actin cytoskeleton in the absence of microtubule function to establish a new polarity axis, leading to the formation of branched cells. spAip3p localizes to, and is required for, branch formation, confirming its role in actin-directed polarized cell growth in both *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*.

INTRODUCTION

Cell polarity is a fundamental process by which cells create specialized domains at their cortex in response to intracellular and extracellular cues. The regulation of cell polarity is achieved through the assembly of specialized cytoskeletal networks in spatially restricted regions at the cell cortex. Coordination between the actin and microtubule cytoskeletons leads to polarized secretion, resulting in polarized cell growth (Drubin and Nelson, 1996; Goode *et al.*, 2000). Eukaryotes differ in their reliance on the two cytoskeletal systems with respect to the establishment and maintenance of cell polarity. Both filament systems can act as tracks for the polarized delivery of biosynthetic material packaged in secretory vesicles, but some organisms rely more heavily on one system compared with the other for establishing the direction of polarized growth. Despite subtle differences, the mechanisms by which cells regulate their polarity are highly conserved from mammalian cells to relatively simple eukaryotes, such as the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*.

In budding yeast, the actin cytoskeleton is believed to play a major role in both the establishment and maintenance of cell polarity (for a review, see Bahler and Peter, 2000; Pruyne

and Bretscher, 2000). Microtubules are involved in migration and proper orientation of the nucleus, spindle function, and karyogamy during mating (Jacobs *et al.*, 1988; Botstein *et al.*, 1997) but appear to play no role in polarized growth (Hufaker *et al.*, 1988; Botstein *et al.*, 1997).

In contrast, fission yeast polarizes cell growth to the cell ends in a microtubule-dependent manner (Bahler and Peter, 2000). *S. pombe* cells first initiate growth at a single pole (called the old end), and then in early G2 they activate polarized growth at the new end as well, leading to bipolar growth. The switch from monopolar to bipolar growth is called NETO for "New End Take Off" (Mitchison and Nurse, 1985). As with budding yeast, actin is required for polarized cell growth in *S. pombe* but appears to be subservient to the microtubule cytoskeleton, at least for the bipolar growth pattern. For example, disruption of the microtubule cytoskeleton with the depolymerizing drug thiabendazole (TBZ; Walker, 1982) or by mutation of the tubulin genes (Toda *et al.*, 1983; Umesono *et al.*, 1983) transiently disrupts bipolar growth and activates polarization from the cell middle, leading to bent or branched cells. Furthermore, mutants have been isolated that disrupt microtubule organization, and these also lead to the branched cell phenotype (Verde *et al.*, 1995; Hirata *et al.*, 1998). Finally, deletion of the *tea1⁺* or *pom1⁺* genes, whose products localize to cell ends in a microtubule-dependent manner, also causes bent and branched cells (Mata and Nurse, 1997; Bahler and Pringle,

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Table 1. *S. cerevisiae* and *S. pombe* strains

Name	Genotype	Source
Y187	α <i>gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3</i> , 112 <i>GAL-lacZ</i>	Bai and Elledge
Y190	<i>a gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3</i> , 112, <i>URA::GAL-lacZ LYS2::GAL-HIS3cyh^r</i>	Bai and Elledge
DAY101X102	<i>a/a ura3-52/ura3-52 leu2Δ1/leu2Δ1 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 aip3-Δ2::HIS3/aip3-Δ2::HIS3</i>	Amberg, D
FY527	<i>h⁻ his3-D1 ade6-M216 ura4-D18 leu1-32</i>	Hallberg, R
Q868	<i>h⁻ leu1-32</i>	Young, P
Q1521	<i>h⁻ cdc25-22 leu1-32</i>	Young, P
Q1518	<i>h⁻ cdc10-129 leu1-32</i>	Young, P
Q1585	<i>h⁻ teal::ura4⁺ ura4-D18 leu1-32</i>	Young, P
I1100-2B	<i>h⁻ cdc10-129 leu1-32 ura4-D18</i>	Young, P
JA310	<i>h⁺ ade6-M216 ura4-D18 leu1-32</i>	Amstrong, J
HJY6	<i>h⁻ his3-D1 ade6-M216 leu1-32 ura4-D18 aip3spΔ::ura4⁺</i>	This study
HJY7	<i>leu1-32 ura4-D18 aip3spΔ::ura4⁺ cdc10-129</i>	This study

1998). The study of polarized growth in fission yeast has been frustrated by the inability to completely inhibit this process. Disruption of the microtubule cytoskeleton only transiently blocks growth from the cell ends. Even when the microtubule system is continually disrupted, it takes ~2 h for actin to return to the cell ends and for polarized growth to resume (Sawin and Nurse, 1998). Furthermore, loss of bipolar growth through microtubule disruption in many cells leads to activation of branch formation, an alternative polarity program mentioned above.

Aip3p is an actin-interacting protein that regulates cell and cytoskeletal polarity in budding yeast. Aip3p localizes to the bud site and the cortex of small buds and then relocates to form two rings in the mother-daughter neck. This localization pattern overlaps with regions of active actin polymerization on the plasma membrane. In *aip3 Δ* cells the actin cytoskeleton is disorganized and poorly polarized toward the bud, and as a result the cells are abnormally round and frequently extremely large. Disruption of actin organization leads to depolarized secretion and accumulation of secretory membrane intermediates. Loss of actin organization in *aip3 Δ* cells also causes defects in septation and cell separation, leading to chains of attached cells (Amberg *et al.*, 1997). *aip3 Δ* cells also have nuclear positioning/segregation defects, and Aip3p has been observed to bind to microtubules, suggesting that Aip3p is directly involved in nuclear positioning (David Pellman, personal communication).

The *S. pombe* genome sequencing project has revealed a gene that is structurally related to *AIP3*; we have named this gene *aip3⁺sp* (originally named *fat1⁺* [Jin and Amberg, 2000]). Here we show that spAip3p is a true functional homologue of budding yeast Aip3p. In *S. pombe* cells, spAip3p appears to be able to use both the actin and microtubule cytoskeletons for localization. Furthermore, we establish that, upon disruption of the microtubule cytoskeleton, *S. pombe* cells use the actin cytoskeleton to activate an alternative polarity program that requires spAip3p.

MATERIALS AND METHODS

Yeast Strains, Media, and Genetic Methods

Yeast strains are listed in Table 1. Standard genetic methods for *S. pombe* were used (Moreno *et al.*, 1991). *S. pombe* transformations

were performed by the lithium acetate-dimethyl sulfoxide (DMSO) method (Bahler *et al.*, 1998).

The *aip3sp* deletion allele was constructed by double-fusion polymerase chain reaction (PCR; Amberg *et al.*, 1995) using the following primers: DAO-PHAT1-1 (5'-TTAGCAATCCTCACAAATTCAA-3'), HJO-POM-11 (5'-GTCTGTACTGGGAAAACCTGGCGGGAAG-GGACTAATCTTCGAGA-3'), HJO-POM-12 (5'-TCCTGTGTGAAAT-TGTTATCCGCTAAGATGCCGAAGATGTTGAA-3'), and DAO-PHAT1-4 (5'-GATTCCGATGGTTCATATCTG-3'). The *ura4⁺* marker was amplified using primers KS-ura4 (forward) and KS-ura4 (reverse) and plasmid KS-ura4 as the template (Bahler *et al.*, 1998). The *aip3sp::ura4⁺* cassette was transformed into strain FY527XFY528; *ura4⁺* transformants were selected, sporulated, and dissected. Replacement of the *aip3⁺sp* locus was confirmed by PCR. Strain HJY7 was constructed by sporulation and tetrad dissection of strain HJY6X1100-2B.

Plasmid Constructions and DNA Manipulations

Plasmids encoding fusions of the *GAL4* DNA-binding domain (DBD) to SNF1 (pSE1112), the *GAL4* DBD to lamin (pAS1-lamin), and the *GAL4* activation domain (AD) to SNF4 (pSE1111) were provided by Steve Elledge (Baylor College of Medicine, Houston, TX). The construct encoding a fusion of the *GAL4* DBD to AIP3 (pDab213) was described elsewhere (Amberg *et al.*, 1997). The plasmids carrying fusions of the *GAL4* AD to ACT1 (pAIP70), a fusion of the *GAL4* DBD to ACT1 (pDab7), and its alanine-scan derivatives were previously described (Amberg *et al.*, 1995). Plasmid pDA290 was constructed by PCR amplification from *S. pombe* genomic DNA using primers DAO-PHAT1-5 (5'-CGCGGATC-CCAAATGTTTAATAACGGCGAT-3') and DAO-PHAT-6 (5'-CGCGTCGACTTAAGTTAGGCTTGCTCTCTC-3'). The PCR product was digested with *Bam*HI and *Sal*I and cloned into *Bam*HI- and *Xho*I-digested plasmid pACTII. Plasmid pHJ42 was constructed by PCR amplification from *S. pombe* genomic DNA using primers HJO-POM-6 (5'-GCGGCGGATCCATGTTTAATAACGGCGATAA-3') and HJO-POM-7 (5'-GCGGCTCTAGATTAAGTTAGGCTTGCTCTCTC-3'). The PCR product was digested with *Bam*HI and *Xba*I and cloned into the same sites of plasmid pTD125. The green fluorescence protein (GFP) expression vectors pSGP573 (Pasion and Forsburg, 1999) and pREP41GFP N (Craven *et al.*, 1998) were kindly provided by Dr. Forsburg (The Salk Institute for Biological Studies, La Jolla, CA) and Dr. Hagan (University of Manchester, Manchester, United Kingdom), respectively. Plasmid pHJ31 was constructed by PCR amplification from *S. pombe* genomic DNA using primers HJO-POM-1 (5'-GCGGCGTCGACGATGTTTAATAACGGCGATAA-3') and HJO-POM-3 (5'-GCGGCGGATCCGCTTAAGTTAGGCTTGCTCTCTC-3'). The PCR product was digested with *Bam*HI and *Sal*I and cloned into plasmid pSGP573 digested with *Bgl*II and *Sal*I. Plasmid pHJ43 was constructed by PCR amplification from *S. pombe* genomic DNA using

primers HJo-POM-1 (5'-GCGGCGTCGACGATGTTTAATAACGGC-GATAA-3') and HJo-POM-3 (5'-GCGGCGGATCCGCTTAAGTTAG-GCTTGCTCTTC-3'). The PCR product was digested with *Bam*HI and *Sall*I and cloned into plasmid pREP41GFP N digested with *Bam*HI and *Sall*I.

Cytology

Microscopic analysis was performed on a Zeiss Axioskop (Carl Zeiss, Oberkochen, Germany) with Plan-APOCHROMAT 40× and 100× objectives. Cells were visualized either by differential interference contrast or epifluorescence with a standard fluorescein isothiocyanate filter set (Chroma Technology, Brattleboro VT). Images were captured with a SPOT2 camera (Diagnostic Instruments, Sterling Heights, MI) and downloaded directly into Adobe Photoshop (Adobe Systems, San Jose, CA). Actin rhodamine-phalloidin staining of *S. pombe* cells was adapted from a standard protocol (Marks and Hyams, 1985). Calcofluor and 4,6-diamidino-2-phenylindole (DAPI) staining were used as described by Verde *et al.* (1995). For immunofluorescence, cells were fixed with 4% formaldehyde and 0.2% glutaraldehyde for 60 min and processed as described by Mata and Nurse (1997). For anti-tubulin staining, TAT1 monoclonal antibody was used (Woods *et al.*, 1989; gift from Dr. Gull, University of Manchester, Manchester, United Kingdom) at a 1:10 dilution, followed by detection with Cy3-conjugated AffiniPure goat anti-mouse IgG, F(ab')₂ (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:100. For immunofluorescent detection of GFP-spAip3p, lyophilized rabbit anti-GFP antibody (gift of Pam Silver, Dana-Farber Cancer Institute, Boston, MA) was used at a 1:2000 dilution, followed by a 1:2000 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (ICN Pharmaceuticals, Aurora, OH).

Two-Hybrid Analyses

The two-hybrid analyses were performed as described by Rodal *et al.* (1999). Strain Y190 carrying constructs encoding fusions to the GAL4 DBD were mated to strain Y187 carrying constructs encoding fusions to the GAL4 AD. Transformants were lined, spotted, or spread as lawns on selective medium. Mating was carried out by replica plating the Y190 and Y187 derivatives together onto yeast selective media. The selected diploids, carrying both DBD and AD fusion constructs, were replica plated to media containing 25, 50, and 100 mM 3-AT (Sigma Chemical, St. Louis, MO) and incubated at 25°C.

Branching Experiments

The branching experiments with *cdc10-129* and *aip3spΔ cdc10-129* were performed as described by Sawin and Nurse (1998). *Cdc10-129* and *aip3spΔ cdc10-129* mutant cells were incubated in a 36°C water bath for 4 h from a starting density of OD₍₅₉₅₎ = 0.07–0.1; 1:100 volumes of 10 mg/ml TBZ (dissolved in DMSO just before use) was then added, and the cultures were incubated for 30 min at 36°C. The cultures were then shifted down to 25°C for up to 4 h.

Expression of GFP-spAip3p

GFP-spAip3p was expressed at different levels from the thiamine-repressible *nmt1* promoters (Maundrell, 1990). For the moderate expression levels of GFP-spAip3p under the strongest or the mid-strength of *nmt1* promoter, cells were grown in media supplemented with 5 μg/ml or 0.016 μg/ml thiamine, respectively. GFP-spAip3p was visualized in cells at early-log phase. For GFP-spAip3p observed on the microtubules, cell cultures either underwent prolonged incubations or started with a density of OD₍₅₉₅₎ = 0.3–0.4 before the temperature shift. For full overexpression of GFP-spAip3p under the full or midstrength of *nmt1* promoter, thiamine was removed from the culture medium.

RESULTS

spAip3p Is a Functional Homologue of Aip3p

We previously identified a sequence from the *S. pombe*-sequencing project that showed strong similarity to the N terminus of Aip3p (Jin and Amberg, 2000). Recently, the full sequence of this *AIP3* homologue became available (*S. pombe* genome-sequencing project). We have named the *S. pombe* gene *aip3⁺sp* (*S. pombe* homologue of *AIP3*) because there is strong similarity between Aip3p and spAip3p throughout much of their lengths (highest E value in a Blast alignment = 5×10^{-33}). *AIP3* encodes a 788-amino acid protein with a molecular mass of 98 kDa (Amberg *et al.*, 1997), whereas *aip3⁺sp* encodes a predicted protein of 1385 amino acids with an expected size of 152 kDa. The sequence alignment of spAip3p and Aip3p is shown diagrammatically in Figure 1A.

Aip3p can be divided into two domains, each of which is conserved in spAip3p, but spAip3p has an additional central domain (shown as gray in Figure 1A) that is unique to spAip3p. The greatest similarity is in their N termini (Figure 1A, black regions, 36% identity and 46% similarity). We refer to this region as the addressing domain because it is the minimal sequence of Aip3p necessary to mediate normal localization. Note that the N terminus of spAip3p can substitute for the N terminus of Aip3p for this localization function in *S. cerevisiae* (Jin and Amberg, 2000). The C termini (Figure 1A, white plus diagonal region) are also conserved (26% identity and 44% similarity), and contained within this region is a sequence (indicated with diagonal stippled lines in Figure 1A) that shows weak homology to a large number of coiled-coil-containing cytoskeletal proteins (Amberg *et al.*, 1997). Both proteins contain predicted coiled-coil domains (Lupas *et al.*, 1991; Berger *et al.*, 1995) in the conserved C-terminal domains, and spAip3p has an additional predicted coiled-coil domain within its central unique domain (labeled cc). We have previously shown that Aip3p is associated with secretory vesicles as a peripheral membrane protein (Jin and Amberg, 2000). Given this, it is interesting that spAip3p contains a possible transmembrane domain (indicated as tm in Figure 1A) in the unique domain as predicted by the programs TM Pred (Hofmann and Stoffel, 1993) and Top Pred2 (von Heijne, 1992). Neither of these programs identify a potential transmembrane domain in the Aip3p sequence.

We next focused on whether spAip3p is a true, functional homologue of Aip3p. An important feature of Aip3p is its ability to facilitate the assembly of a polarized actin cytoskeleton, perhaps through its ability to interact with actin. We similarly showed that spAip3p was able to interact with *S. cerevisiae* actin in a two-hybrid assay (Figure 1B). Because there is an extremely high level of conservation between budding and fission yeast actin, we believed that it was unnecessary to test the ability of spAip3p to interact with *S. pombe* actin. We previously showed (Amberg *et al.*, 1997) that the Aip3p coiled-coil-containing domain can mediate oligomerization of Aip3p, but as can be seen here, spAip3p cannot interact with Aip3p. We have yet to test for self-association of spAip3p but expect that the function of the coiled-coil domains in both proteins is to mediate oligomerization.

We extended our analysis by examining whether spAip3p interacts with actin in the same manner as Aip3p. We previously measured the ability of 35 alanine scan alleles of actin to interact with Aip3p in a two-hybrid assay (Amberg *et al.*, 1997). In this manner we can derive a "binding footprint" of any given actin-binding protein on the surface of yeast actin (Amberg *et al.*, 1995). We hypothesize that, if Aip3p and spAip3p display similar binding footprints on actin, then the function of this interaction is likely to be conserved. Displayed in Figure 1C is a comparison of the binding footprints for spAip3p and Aip3p on the crystal structure of actin. Five mutations affect binding of both

Figure 1. spAip3p is a functional homologue of Aip3p. (A) The sequences of Aip3p and spAip3p are compared diagrammatically. The addressing domain is shown in solid black, the conserved C-terminal domain is shown in solid white with the diagonal lines to indicate the region that is homologous to other cytoskeletal proteins, the domain unique to spAip3p is indicated in gray, the coiled-coil domains are marked with "cc," and the potential transmembrane domain of spAip3p is marked with "tm." (B) Two-hybrid analysis was performed with a fusion of *aip3⁺sp* to the *GAL4* AD. The DBD fusions were transformed into strain Y190 and were mated to strain Y187 containing fusions to the *GAL4* AD. Diploids were selected, and activation of the *HIS3* two-hybrid reporter was assessed on medium containing 25 mM 3-amino 1,2,4 triazole. (C) The Aip3p- and spAip3p-actin interactions were tested against 35 alanine scan alleles of actin using the two-hybrid system (Amberg *et al.*, 1995). A Van der Waals view of the front and back surfaces of actin is presented. The side chains altered by mutations that disrupt only the Aip3p-actin interaction are indicated in yellow, those that disrupt only the spAip3p-actin interaction are green, and those that disrupt both Aip3p- and spAip3p-actin interactions are red. (D) GFP-spAip3p was expressed in a diploid *S. cerevisiae aip3Δ/aip3Δ* strain from Cen plasmid pHJ42 and visualized by fluorescence microscopy(left) and DIC (right).

spAip3p and Aip3p to actin (*act1-119*, *act1-124*, *act1-133*, *act1-103*, and *act1-120*), two mutations affect only Aip3p (*act1-131* and *act1-123*), and two mutations affect only spAip3p (*act1-112* and *act1-134*). Despite the minor differences, the close proximity of each of these mutations suggests that Aip3p and spAip3p have similar mechanisms for binding to actin.

Finally, we determined whether expression of spAip3p in *S. cerevisiae* can complement the phenotype of an *aip3Δ* strain. We previously showed that a chimeric protein carrying the spAip3p N terminus fused to the Aip3p C terminus could localize correctly in *S. cerevisiae* (Jin and Amberg,

Figure 1 (legend on facing page).

2000). Here we asked whether full-length spAip3p can localize like Aip3p in budding yeast. A GFP-spAip3p fusion protein was constructed and expressed in an *aip3Δ/aip3Δ* strain, and as shown in Figure 1D, its localization was extremely similar to that of Aip3p. spAip3p localized to regions of polarized actin assembly and polarized growth: the presumptive bud site, the cortex of small buds, and as two rings in the necks of large budded cells. Furthermore, spAip3p appears to be functional at sites of polarized actin assembly as indicated by a correction of the cell morphology defects of this *aip3Δ* strain (see the differential interference contrast microscopy [DIC] panel in Figure 1D). In addition, spAip3p corrected many of the other defects of the *aip3Δ* strain, including defects in septation, actin organization, and nuclear segregation (Jin and Amberg, unpublished observations). Collectively, the results presented in Figure 1 confirm that spAip3p is a true, functional homologue of Aip3p.

spAip3p Is a Regulator of S. pombe Cell and Cytoskeletal Polarity

To investigate the localization of spAip3p in *S. pombe*, a GFP-spAip3p fusion protein was constructed under the con-

trol of full- or medium-strength thiamine-repressible *nmt1* promoters (Maundrell, 1990). Visualization of GFP-spAip3p localization, at moderate expression levels (with high concentrations of the thiamine repressor, see MATERIALS AND METHODS), in wild-type cells is shown in Figure 2. spAip3p was found either at both cell ends or as two rings in the cell middle. Using DAPI staining, we found that 95% of those cells with GFP-spAip3p localization to the cell middle had divided nuclei (Figure 2, B and C). This indicated that spAip3p localization to the cell middle occurs at late stages of the cell cycle (anaphase/telophase), some time after actin polarization to the cell middle (Su and Yanagida, 1997). In cells that appeared to have just completed septation, localization of spAip3p was rapidly re-established at what is called the old end (see lower right of Figure 2A and left of Figure 2B). Note that in these cells localization appeared to be stronger at the new end, possibly because a pool of spAip3p remains after cytokinesis and septation in the last cell cycle. Right after cell division, many regulators of cell polarity, including actin, localize to only one end (called the old end), resulting in monopolar growth. After S phase, actin and other regulators of cell polarity move to

Figure 2. In *S. pombe* cells, spAip3p localizes to cell ends and the cell middle. GFP-spAip3p was expressed at moderate levels from plasmid pHJ31 in the wild-type *S. pombe* strain FY527. The fluorescence signal of the GFP-spAip3p fusion protein was overlaid on the DIC image of the same cells (A). The nuclei of live cells expressing GFP-spAip3p were stained with DAPI and GFP-spAip3p (B) and nuclei (C) were visualized by fluorescence microscopy.

both cell ends, a process called NETO, resulting in bipolar growth. In contrast spAip3p appeared to be at both ends of the cell even before NETO (Figures 2 and 3). To confirm this, we examined GFP-spAip3p localization (under moderate

expression levels) in a *cdc10-129* mutant (Nurse *et al.*, 1976) arrested at, and then released from, the pre-NETO stage of the cell cycle (Figure 3, A–D). When cells were arrested at the pre-NETO state (Figure 3A), GFP-spAip3p was found at

Figure 3. spAip3p localizes to both cell ends before NETO. (A–D) GFP-spAip3p was expressed at moderate levels from plasmid pHJ43 in the *cdc10-129* mutant strain Q1518. This strain was shifted to 36°C for 4 h to synchronize the cells at pre-NETO (A). The cells were released from the pre-NETO arrest by shifting the culture to 25°C, and GFP-spAip3p localization was monitored by fluorescence microscopy (B–D). GFP-spAip3p localization was visualized at post-NETO (40 min after the shift to 25°C; B), at cytokinesis (180 min after shift down; C), and just after completion of cytokinesis (220 min after shift down; D). Cells arrested at pre-NETO were also stained with calcofluor, and GFP-spAip3p (E) and the cell wall (F) were visualized by fluorescence microscopy.

both cell ends with apparently stronger localization at one end. To identify the growing (old) end, we stained the cells with calcofluor and found that in all cases the greater concentration of spAip3p is found at the new (nongrowing) end. One representative example is shown in Figure 3, E and F. This result agrees with our hypothesis that a pre-existing pool of spAip3p remains at the new end after septation/cell separation. After the cells were released from the cell cycle arrest, they promptly underwent NETO, and GFP-spAip3p localized evenly to both cell ends (Figure 3B). At mitosis, when growth is redirected to the cell middle, GFP-spAip3p was lost from the cell ends, and it localized to the cell middle, forming two bands (Figure 3C). At septation, GFP-spAip3p localization was rapidly re-established at the old ends, remaining in the former cell middle (now the new end) even after cytokinesis had been completed (Figure 3D). Therefore, at those stages of the cell cycle during which spAip3p was found at the cell ends, it was always at both of the cell ends.

To further investigate the function of spAip3p, we examined the effects of spAip3p overexpression on cell morphology and actin organization. When thiamine is removed from the medium, the expression level of GFP-spAip3p under the full-strength *nmt1* promoter was 30-fold higher than under our moderate expression level conditions (based on Western analysis, Jin and Amberg, unpublished observations). Overexpression of GFP-spAip3p was not lethal, but cell morphology was dramatically affected. Many (45%) of the cells dis-

play a pear- or lemon-shaped morphology (Figure 4B) compared with overexpression of GFP alone (Figure 4A). This phenotype suggested that growth had become depolarized in one end of the cell. When we looked at localization of GFP-spAip3p in the overexpressing cells, we found that all of the protein was located in a small number of large aggregates (Figure 4B). There did not appear to be a correlation between the location of the aggregates and the morphology defects, although the aggregates were frequently located near the cell ends. The organizations of the microtubule and actin cytoskeletons were examined in cells overexpressing GFP-spAip3p. The cytoplasmic microtubules appear to be normal, although given the cell morphology defects it was difficult to determine at the cell ends (Figure 4C). In contrast, the actin cytoskeleton was grossly aberrant; actin cables were not evident and actin patches were completely depolarized (Figure 4D). We hypothesize that in the overexpressing cells spAip3p is sequestering (via association) additional important regulators of *S. pombe* actin polarity.

spAip3p Localization to Cell Ends Is Dependent on the Microtubule Cytoskeleton

There is a fundamental difference between the regulation of cell polarity in *S. cerevisiae* and that in *S. pombe*. In budding yeast, cell polarity is dependent only on the actin cytoskeleton; disruption of microtubules has little effect on polarized

Figure 4. Overexpression of spAip3p disrupts the actin cytoskeleton and cell polarity in *S. pombe*. GFP was overexpressed (no thiamine in the medium) from plasmid pSGP573 (A), or GFP-spAip3p was overexpressed from plasmid pHJ31 (B–D) in wild-type *S. pombe* strain FY527. The cells were visualized by DIC (A) or DIC plus fluorescence microscopy (B) or stained with the anti-microtubule antibody TAT1 (C) or rhodamine-phalloidin (D) and visualized by fluorescence microscopy.

growth (Huffaker *et al.*, 1988; Botstein *et al.*, 1997). In contrast, fission yeast appears to rely heavily on microtubules for polarized growth. Disruption of microtubules leads to the transient loss of cell growth from the cell ends (for a review, see Sawin, 1999; Bahler and Peter, 2000) and a tran-

sient loss of actin and other regulators of cell polarity, such as Ral3p (a homologue of budding yeast Bem1p) and Tea1p, from the cell ends (Sawin and Nurse, 1998). Curiously, disruption of microtubule-mediated polarized growth (by disruption of microtubules themselves or microtubule-binding

Figure 5. spAip3p localization to cell ends is microtubule dependent, and localization to the branch tips is microtubule independent. GFP-spAip3p was expressed at moderate levels from plasmid pHJ43 in the *cdc10-129* mutant *S. pombe* strain Q1518, and the cells were visualized by fluorescence microscopy. (A) The cells were visualized 4 h after a shift to 36°C in the absence of TBZ (–TBZ). (B) Cells were then treated with 100 μ g/ml TBZ for 30 min while maintaining the culture at 36°C. (C) Finally, the cells were shifted to 25°C for 2 h while maintaining 100 μ g/ml TBZ in the medium.

proteins) induces *S. pombe* cells to polarize from their middles to form branched and/or bent cells (Mata and Nurse, 1997; Sawin and Nurse, 1998). Note that actin and other cell polarity proteins localize (as expected) to the tips of these branches (Sawin and Nurse, 1998). We previously showed that Aip3p localization in budding yeast is dependent on actin-based polarized secretion. Given fission yeast's heavier reliance on microtubules for polarized growth, we asked whether spAip3p is dependent on microtubules for polarized localization in *S. pombe*.

Previously, conditions were established for efficient branch formation upon microtubule disruption in *S. pombe*. First, cells are arrested at pre-NETO (by shifting a *cdc10-129* mutant to 36°C), followed by treatment of the cells with the microtubule-depolymerizing drug TBZ, and subsequent release from the cell cycle arrest by shifting to 25°C. After such a treatment, cells undergo a transient loss of polarization at the cell ends, and ~30% of the cells form branches (Sawin and Nurse, 1998). Apparently the capacity to form branches is highest when cells are at the pre-NETO stage of the cell cycle. As we described above, when GFP-spAip3p was expressed in a *cdc10-129* mutant strain shifted for 4 h to 36°C, GFP-spAip3p was seen at the cell ends (Figure 5A). Upon treatment with TBZ (at 36°C) for 30 min, polarization of GFP-spAip3p to the cell ends was lost (Figure 5B). When these cells were released from the cell cycle arrest by shifting the cultures to 25°C in the presence of TBZ, a high frequency of branched cells was observed after 1.5–2 h and GFP-

spAip3p was seen in the branch tips (Figure 5C). This suggests that spAip3p localization to cell ends is microtubule dependent, whereas spAip3p localization to branch tips is microtubule independent.

Screens for morphological mutants of fission yeast have led to the identification of several genes required for microtubule-based polarized growth (Snell and Nurse, 1994; Verde *et al.*, 1995). One such gene was called *tea1*⁺ because loss of Tea1p function leads to T-shaped (branched) cells that are very similar in appearance to branched cells formed by TBZ treatment. Tea1p is associated with microtubules *in vivo* and is microtubule dependent for its localization to cell ends. Furthermore, loss of *tea1*⁺ leads to disorganization of the microtubule cytoskeleton when cells are grown at 36°C: the microtubules fail to accurately terminate at the cell ends (Mata and Nurse, 1997). Therefore, the primary defect in *tea1* mutants is thought to be the disruption of microtubule-based cell polarization. Furthermore, branch formation appears to be a common response to disruption of the microtubule cytoskeleton. Therefore, we sought to confirm the role of the microtubule cytoskeleton in localization of spAip3p to cell ends by examining GFP-spAip3p localization in a *tea1* Δ strain (Figure 6). GFP-spAip3p was expressed at moderate levels (see above) in a *tea1* Δ strain grown at 25°C, conditions under which microtubule function is not grossly affected. As expected, GFP-spAip3p localization was largely normal (Figure 6A). After cells were shifted to 36°C for 4 h, ~20% of cells were T shaped and GFP-spAip3p was

Figure 6. Disruption of the microtubules in a *tea1Δ* mutant affects localization of spAip3p to cell ends but not to the cell middle or the branch tips. GFP-spAip3p was expressed at moderate levels from plasmid pHJ43 in the *tea1Δ* mutant *S. pombe* strain Q1585. GFP-spAip3p fluorescence was examined in cells grown at 25°C (A) and in cells grown at 36°C for 4 h (B).

absent from the cell ends and had moved to the newly formed branch tips (Figure 6B). In contrast, GFP-spAip3p localization to the cell middle was unaffected by disruption of the microtubule cytoskeleton. We conclude that spAip3p localization to cell ends is microtubule dependent and that the mechanisms for spAip3p delivery to the cell middle and branch tips involves an alternative (non-microtubule based) mechanism. These results are consistent with our observations that TBZ treatment of wild-type cells affected localization of GFP-spAip3p to cell ends but not to the cell middle (Jin and Amberg, unpublished observations).

spAip3p Localization to the Cell Middle Is Actin Dependent

Given that Aip3p is reliant on actin-based secretion for its localization in *S. cerevisiae*, we hypothesized that some aspects of spAip3p localization may also require actin-based processes. We were particularly interested in spAip3p localization to the cell middle that we previously showed is microtubule independent. Therefore, we introduced the GFP-spAip3p-expressing construct into a *cdc25-22* mutant strain, and the cells were arrested at G2/M by shifting them to 36°C for 4 h (Russell and Nurse, 1986). The cells were then treated with DMSO or the actin-depolymerizing agent Latrunculin A (Lat-A; Ayscough *et al.*, 1997) dissolved in DMSO for 10 min and then released from the cell cycle block by shifting the culture to 25°C. One hour after release from the G2/M arrest, 80–90% of the control cells displayed both actin and GFP-spAip3p rings in the cell middle (Figure 7, A and B). In contrast, none of the cells treated with Lat-A showed F-actin or GFP-spAip3p in the cell middle (Figure 7, C and D), even 2 h after release of the cell cycle block.

Treatment of wild-type cells with Lat-A had no effect on localization of spAip3p to cell ends only to the middle (Figure 7, E and F). Therefore, similarly to what has been shown for *S. pombe* Cdc42p (Merla and Johnson, 2000), spAip3p localization to the cell middle is dependent on actin filaments. In summary, our localization studies suggest that spAip3p has roles in polarized growth at the cell tips, branch tips, and the cell middle. We further conclude that spAip3p uses a microtubule-based system for localization to cell ends and an actin-based delivery system for localization to the cell middle and the branch tips.

spAip3p Localization Is Reliant on the Secretory Pathway

We previously showed that Aip3p in *S. cerevisiae* requires the secretory pathway for its delivery and that it is physically associated with late secretory vesicles (Jin and Amberg, 2000). Considering the functional similarity of Aip3p and spAip3p, and the ability of spAip3p to localize correctly in *S. cerevisiae* cells, we asked whether spAip3p uses the secretory pathway to localize in *S. pombe* cells. To do so, we introduced the GFP-spAip3p-expressing construct into a temperature-sensitive *ypf2* mutant and examined spAip3p delivery at the nonpermissive temperature. Ypf2p is a small GTPase and is a homologue of budding yeast Sec4p (Craighead *et al.*, 1993), which we previously showed to be necessary for Aip3p delivery in budding yeast cells (Jin and Amberg, 2000). GFP-spAip3p localization was normal at 25°C (Figure 8A), but the protein was absent from both cell tips and the cell middle when cells were shifted to 37°C (Figure 8B), confirming conservation of secretory pathway mediated delivery of Aip3p.

Figure 7. Localization of spAip3p to the cell middle is F-actin dependent. (A–D) GFP-spAip3p was expressed at moderate levels from plasmid pHJ43 in the *cdc25-22* mutant *S. pombe* strain Q1521. The cells were shifted to 36°C for 4 h to arrest the culture at G2/M. The cells were then incubated for 10 min with 1:200 (vol/vol) DMSO (A and B) or with Lat-A dissolved in DMSO at a final concentration of 100 μ M (C and D). Finally, the cells were shifted to 25°C for 1 h and stained with rhodamine-phalloidin (A and C) or GFP-spAip3p was visualized by fluorescence microscopy (B and D). GFP-spAip3p localization was also examined in the wild-type (wt) strain FY527 treated with 100 μ M Lat-A for 1 h (E and F).

It is interesting that spAip3p is dependent on both the actin and microtubule cytoskeletons for aspects of its localization and yet is completely reliant on the secretory pathway for localization. This leads us to hypothesize that spAip3p uses both actin- and microtubule-based secretion for delivery to the cell cortex. Interestingly, we found that when GFP-spAip3p was overexpressed (not to levels that produce cell morphology defects as shown in Figure 4B, see MATERIALS AND METHODS), we observed what appeared to be localization on microtubules. We sought to confirm this colocalization by using indirect immunofluorescence to simultaneously detect both microtubules (Figure 9A) and GFP-spAip3p (Figure 9B). As can be clearly appreciated in the overlay shown in Figure 9C, under these conditions spAip3p can be observed on microtubules.

spAip3p Is Required for Branch Formation, an Actin-directed Alternative Polarity Program

We theorize that, upon disruption of the microtubule cytoskeleton, *S. pombe* cells can access an alternative polarity program leading to branch formation. To determine whether F-actin is required for this alternative program, we disrupted actin filaments in cells predisposed to branch formation. First, we examined branch formation in *tea1Δ* mutants shifted to 36°C with or without Lat-A. Four hours after the cells were cultured at 36°C, 19% of the cells treated with DMSO formed branched cells (Figure 10, A and G), whereas only 1% of the cells treated with Lat-A formed branches (Figure 10, C and G). The Lat-A treatment did not appear to kill the cells. When we washed out the Lat-A and incubated the cells at 36°C in the absence of drug, 20% of the cells were

Figure 8. spAip3p localization is dependent on the secretory pathway. GFP-spAip3p was expressed at moderate levels from plasmid pHJ43 in the *ypt2* mutant *S. pombe* strain JA310. GFP-spAip3p localization was examined by fluorescence microscopy after growth at 25°C (A) or after a 2-h shift to 37°C (B).

able to form branches (Figure 10, D and G). Second, we examined branch formation in a TBZ-treated *cdc10-129* mutant. *cdc10-129* cells were shifted to 36°C to arrest them at pre-NETO and then divided them into two aliquots, one of which was treated with only TBZ for 30 min, and the other was treated with Lat-A 15 min after the addition of TBZ. The cultures were then shifted to 25°C to induce branch formation. The culture treated with TBZ alone displayed 33% branched cells (Figure 10E), whereas the group treated with TBZ and Lat-A displayed only 1% of branched cells (Figure 10F). These results confirm that, in the absence of microtubule function, *S. pombe* cells can activate an alternative polarity program that appears to be directed by the actin cytoskeleton.

Because Aip3p is involved in actin-based polarized growth in *S. cerevisiae*, we asked whether its homologue spAip3p is involved in branch formation. For these experiments, we constructed an *aip3spΔ* strain that was viable and displayed no gross defects in growth or cell polarity under normal laboratory conditions. Furthermore, by both actin and calcofluor staining the *aip3spΔ* strain was not defective for NETO (Jin and Amberg, unpublished observations). We then crossed the *aip3spΔ* strain with a *cdc10-129* strain to isolate double mutants. The *aip3spΔcdc10-129* strain showed highly reduced percentages of branched cells after synchronization at pre-NETO and TBZ treatment as described above (33% compared with 2%, Figure 11). Expression of GFP-spAip3p in the *aip3spΔcdc10-129* cells complemented the branching defect (28% branch formation), confirming the

role of spAip3p in branch formation and the functionality of the GFP-spAip3p fusion protein. Therefore, *S. pombe* appears to rely primarily on the microtubule cytoskeleton to establish and maintain a bipolar axis of polarized growth. If microtubule function becomes compromised, the cells can then activate an alternative pathway that relies solely on actin to direct the formation of a new polarity axis, 90° to the old axis. spAip3p is required to establish this new axis, presumably by facilitating actin assembly at the site of branch formation/extension.

DISCUSSION

In most eukaryotes, there is a complex and poorly understood interplay between the microtubule and actin cytoskeletons for the establishment, maintenance, and execution of polarized cell growth. The yeast *S. cerevisiae* is unique in this respect because there appears to be no involvement of microtubules in polarized growth; they are delegated to the responsibility of nuclear segregation (Botstein *et al.*, 1997). In budding yeast, a set of metastable markers (the *BUD* gene products) identify where polarized growth will be established by causing localized activation of the small GTPase Cdc42p and its effectors. These effectors then induce the polarized nucleation and assembly of actin filaments, which are further organized into actin cables whose role is to direct secretion into the growing bud/daughter cell (for review, see Bahler and Peter, 2000). For these reasons, *S. cerevisiae*

Figure 9. spAip3p can be observed on microtubules. GFP-spAip3p was expressed from plasmid pHJ43 in the *cdc10-129* mutant *S. pombe* strain Q1518 grown at 25°C in the presence of 0.016 μ M thiamine. The cells were shifted to 36°C for 4 h and fixed, and indirect immunofluorescence was used to visualize microtubules (A) and GFP-spAip3p (B). An overlay of A and B is displayed in C.

has proven to be a wonderful system in which to study actin-based regulation of cell polarity. In mammalian cells, the situation is much more complex and more difficult to study. In general, it appears that microtubules are responsible for determining the direction of polarized growth/extension, whereas actin is responsible for executing polarized growth based on microtubule-based cues. For example, in migrating cells microtubules align along the axis of cell movement with their plus ends oriented out toward and into the leading edge (Heidemann *et al.*, 1981). Disruption of microtubules in these cells leads to a loss in polarized growth and the formation of membrane ruffles in all directions (Vasiliev *et al.*, 1970; Goldman, 1971; Vasiliev, 1991). In these respects, *S. pombe* cells appear to be more like mammalian cells. Disruption of microtubules in fission yeast causes a loss in actin polarization to the cell ends, leading in turn to the transient loss of polarized growth (Sawin and Nurse, 1998; Bahler and Peter, 2000).

Investigations into fission yeast cell polarity have identified two classes of proteins involved in microtubule-regulated polarized growth. The proteins of first class localize to the tips of the cell ends and are required for orientation and attachment of microtubules to the ends. The proteins of second class also localize to the cell ends but are dependent on the microtubules for their localization. An attractive model is that factors involved in polarized actin assembly are delivered to the cell tips via the action of microtubule-based motor proteins. In support of this model, we have found that the fission yeast homologue of Aip3p is delivered to the cell ends in a microtubule-dependent manner. In *S. cerevisiae* Aip3p is required for the efficient establishment and maintenance of a polarized actin cytoskeleton. We have shown that Aip3p is delivered to sites of polarized actin assembly by the secretory pathway due to a peripheral

association with late secretory vesicles (Jin and Amberg, 2000). We believe Aip3p assists in the formation of short actin filaments that subsequently get organized into two prevalent actin cytoskeletal structures of budding yeast, the actin cortical patches and the actin cables (Amberg *et al.*, 1997; Jin and Amberg, unpublished observations). It is the polarized orientation of actin patches and cables that are responsible for the polarized delivery of secretory vesicles and hence polarized cell growth. Very recently, it was shown that the Cdc42p effector Gic2p is responsible for seeding Aip3p on the plasma membrane at the very beginning of the cell cycle (M. Peter, personal communication). We believe that this seed of Aip3p initiates actin polymerization at the bud site, probably in concert with its binding partner Bni1p, a formin and homologue of the *S. pombe* protein Cdc12p (Evangelista *et al.*, 1997; Chang, 2000). Once the cell has initiated actin polymerization, actin cables and patches are formed from these filaments leading to polarized delivery, via the secretory pathway, of more Aip3p and reinforcement of the actin polymerization signal. Similarly, spAip3p appears to interact with important regulators of actin assembly and organization. Overexpression of spAip3p in fission yeast cells causes gross defects in polarized growth, leading to pear-shaped cells and complete depolarization of the actin cytoskeleton. This phenotype is similar to that observed for *cdc42* (Miller and Johnson, 1994), *arp2*, *arp3* (Balasubramanian *et al.*, 1996; McCollum *et al.*, 1996; Machesky and Gould, 1999), *cdc3* (profilin; Balasubramanian *et al.*, 1994), and *cdc8* (tropomyosin) mutants (Balasubramanian *et al.*, 1992) in *S. pombe*. We hypothesize that, when spAip3p is overexpressed, it sequesters important regulators of actin organization into nonfunctional and mis-localized aggregates (Figure 4).

Figure 10. Branching is an actin-dependent alternative polarity program. The *tea1Δ* strain Q1585 was shifted to 36°C for 4 h in the presence of 100 μ M Lat-A (C) or an equivalent volume of DMSO (A). The DMSO or Lat-A was washed out of the culture and replaced with prewarmed medium for 1 h (B and D). Cell morphology was visualized by DIC, the percentages of branched cells to total were quantified from 200–300 cells, and the percentages of branched cells were plotted (G). Q1518 (*cdc10-129*) mutant cells were shifted to 36°C for 4 h, TBZ was added to a final concentration of 100 μ g/ml, and the cells were incubated for an additional 30 min at 36°C and shifted down to 25°C for 2 h (E). In a parallel culture, 15 min after addition of TBZ, Lat-A was added to a final concentration of 100 μ M, and the culture was incubated for 15 min at 36°C and shifted down to 25°C for 2 h (F). The cells were visualized by DIC.

Our results with spAip3p in *S. pombe* suggest that it may be delivered to cell tips by microtubule- and actin-based polarized secretion. Although the sequence of spAip3p predicts one potential transmembrane domain, because spAip3p can fully replace Aip3p in *S. cerevisiae*, it seems unlikely that the *S. pombe* version is an integral membrane protein. However, like Aip3p, which appears to be peripherally associated with membranes (Jin and Amberg, 2000), spAip3p may also associate with the periphery of secretory and plasma membranes. In agreement with this model, we have found that a conditional mutant in the small GTPase Ypt2p (Craighead *et al.*, 1993) is defective for spAip3p delivery to the plasma membrane at both the cell tips and the cell middle (Figure 8). In addition, we have frequently observed, under conditions of overexpression, localization of spAip3p to microtubules (Figure 9). In this way, *S. pombe* cells appear to be more like mammalian cells. For example, it has been observed that individual secretory vesicles can hop between and be moved on either actin filaments or microtubules (DePina and Langford, 1999). We believe that spAip3p localization may be a good reporter for both microtubule-based and actin-based polarized secretion in *S. pombe* cells.

A long-standing mystery concerning the regulation of *S. pombe* polarity has been the inability to completely disrupt polarized growth by disruption of the microtubule cytoskeleton. The universal *S. pombe* response to disruption of its microtubule-based polarity systems is to either form a new axis of polarized growth from the cell middle (branching to form T-shaped or bent cells) or eventually re-establish polarized growth at the cell ends in a microtubule-independent process. In the experiments presented in this report, we support the model that branch formation is an alternative polarity program that is purely established by actin-based systems in response to disruption of the microtubule cytoskeleton. Perhaps *S. pombe* evolved this backup plan to deal with environmental conditions that are unfriendly to the microtubule cytoskeleton. For example, microtubules are cold sensitive to temperatures a free living *S. pombe* cell may frequently encounter, and many competing microbes secrete microtubule inhibitors (e.g., benomyl). If an *S. pombe* cell were to encounter such conditions while in or soon after S phase, it would require a new daughter cell body in which to segregate the replicated nucleus. This may explain why arresting cells at pre-NETO before microtubule disruption leads to greater percentages of branched cells. A key com-

Figure 11. spAip3p is required for branching. *S. pombe* mutant strains Q1518 (*cdc10-129*) (A and C) and HJY7 (*cdc10-129 aip3spΔ*) (B and D) were shifted to 36°C with prewarmed medium for 4 h to arrest the cells at pre-NETO. (A and B) TBZ was added to a final concentration of 100 μ g/ml and the cells were incubated for an additional 30 min at 36°C. (C and D) Cells were then released from the pre-NETO block by shifting the cultures to 25°C for 4 h. The percentages of branched cells were calculated before release of the pre-NETO block (time 0) and at 1, 2, 3, and 4 h after shifting the culture to 25°C; for each time point, N = 300–400 cells.

ponent of this actin backup system is spAip3p; in its absence, branch formation occurs at a much reduced efficiency.

Our current model is that, upon disruption of the MT cytoskeleton, the actin backup plan becomes activated. This

involves recruitment of spAip3p to the cell middle, a normal and microtubule-independent location for spAip3p localization. In *S. cerevisiae*, before bud emergence and actin polarization, Aip3p is recruited to the bud site by Gic2p in asso-

ciation with GTP-Cdc42p. *S. pombe* Cdc42p has recently been shown to localize to the cell middle (Merla and Johnson, 2000) and may be playing a similar role, through a Gic2p homologue, in recruiting spAip3p to the cell middle upon MT disruption. Once a seed of spAip3p is established at the cell middle, it may cooperate with Cdc12p (a homologue of the formin Bni1p), which is known to localize to the cell middle (Chang, 2000), to initiate the polarized assembly of actin filaments. Once this occurs, cables become elongated along this new axis, polarized secretion is directed to this new site, and polarized growth is re-established to build the branch. Finally, at mitosis the daughter nucleus is segregated into the branch and the actin cytoskeleton is redirected to execute cytokinesis.

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