Identification and Characterization of *Schizosaccharomyces pombe asp1*$^+$, a Gene That Interacts with Mutations in the Arp2/3 Complex and Actin

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ABSTRACT

The Arp2/3 complex is an essential component of the actin cytoskeleton in yeast and is required for the movement of actin patches. In an attempt to identify proteins that interact with this complex in the fission yeast *Schizosaccharomyces pombe*, we sought high-copy suppressors of the *S. pombe arp3*-c1 mutant, and have identified one, which we have termed *asp1*$^+$. The *asp1*$^+$ open reading frame (ORF) predicts a highly conserved protein of 921 amino acids with a molecular mass of 106 kD that does not contain motifs of known function. Neither *asp1*$^+$ nor its apparent *Saccharomyces cerevisiae* ortholog, *VIP1*, are essential genes. However, disruption of *asp1*$^+$ leads to altered morphology and growth properties at elevated temperatures and defects in polarized growth. The *asp1* disruption strain also is hypersensitive to Ca^+ ions and to low pH conditions. Although Asp1p is not stably associated with the Arp2/3 complex nor localized in any discrete structure within the cytoplasm, the *asp1* disruption mutant was synthetically lethal with mutations in components of the Arp2/3 complex, *arp3*-c1 and *sop2*-1, as well as with a mutation in actin, *act1*-48. Moreover, the *vip1* disruption strain showed a negative genetic interaction with a *las17*Δ strain. We conclude that Asp1p/Vip1p is important for the function of the cortical actin cytoskeleton.

IN recent years, actin-related proteins (Arps) have been the subject of intensive investigation. Sequence comparisons among Arps indicate that they comprise several families that have been highly conserved throughout evolution (Schroer *et al.* 1994; Frankel and Mooseker 1996; Poch and Winsor 1997). Arp1p, first identified in yeast, is a component of the dynactin complex and is important for microtubule organization (reviewed in Schroer 1994). Arp2p and Arp3p were also identified first in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively; these two Arps participate in the regulation of the actin cytoskeleton (reviewed in Machesky and Gould 1999). In *S. cerevisiae*, there are six other Arps (Poch and Winsor 1997), some of which (Arp4p, Arp7p, and Arp9p) appear to participate in chromatin structure or function (Weber *et al.* 1995; Jiang and Stillman 1996; Peterson *et al.* 1998).

The actin cytoskeleton of *S. pombe* contains three major types of F-actin structures that reorganize during the cell cycle (Marks and Hyams 1985; Marks *et al.* 1987). In interphase, actin is found in patches localized at the growing ends of cells. Actin cables that run the length of the cell are also present. At the onset of mitosis, a ring of F-actin forms in the medial region of the cells, which is believed to be analogous to the cleavage furrow of higher eukaryotes. During anaphase, actin patches become concentrated adjacent to the actin ring rather than at the cell ends. After completion of anaphase, the ring begins to constrict and the septum is deposited in the wake of the constricting ring. Upon exit from mitosis, actin patches once again localize to the growing ends of the cell (reviewed in Chang and Nurse 1996; Gould and Simanis 1997).

One component of the yeast actin patch is a complex containing both Arp2 and Arp3 proteins (reviewed in Machesky and Gould 1999). Originally identified in Acanthamoeba (Machesky *et al.* 1994), the Arp2/3 complex is now recognized to be highly conserved throughout evolution. In amoebas, yeast, and mammals, it is composed of seven polypeptides, including Arp2p and Arp3p, in apparent 1:1 stoichiometry with one another (Machesky *et al.* 1997; Mullins *et al.* 1997; Welch *et al.* 1997a; Winter *et al.* 1997). The other five subunits (termed Arcs) have been identified and named primarily according to their molecular weight, which varies between species (tabulated in Machesky and Gould 1999). In *S. cerevisiae*, they have been termed Arc40p, Arc35p, Arc19p, Arc18p, and Arc15p (Winter *et al.* 1997). These five subunits do not share significant sequence similarity with proteins of known function, although the p40/41 subunit, encoded by *S. pombe sop2*$^+$ and *S. cerevisiae* Arc40, contains four WD-40 motifs (Balasubramanian *et al.* 1996; Machesky *et al.* 1997; Welch *et al.* 1997a; Winter *et al.* 1997), domains that are thought to mediate protein-protein interactions (reviewed in Neer and Smith 1996). There is only one

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gene encoding each subunit in *S. pombe* and *S. cerevisiae*, but in mammals there are two Sop2p/Arc40p homologs (Balasubramanian *et al.* 1996; Machesky *et al.* 1997; Welch *et al.* 1997a), raising the possibility that this subunit might modulate the function of the complex (discussed in Machesky and Gould 1999).

The *sop2*$^+$ gene was first identified in a screen for extragenic suppressors of a conditional-lethal mutation in the *cdc3*$^+$ gene, *cdc3*-124 (Balasubramanian *et al.* 1996). The *sop2*-1 mutation was able to restore growth of the *cdc3*-124 strain at elevated temperatures. The *cdc3*$^+$ gene encodes profilin (Balasubramanian *et al.* 1994), a small actin-binding protein found in all eukaryotic cells (reviewed in Schluter *et al.* 1997). *In vitro*, profilin can interact with at least five ligands: monomeric actin, phosphoinositide 4,5-bisphosphate, poly-l-proline, the phosphorylated focal adhesion protein VASP, and the Arp2/3 complex (reviewed in Schluter *et al.* 1997). Profilin has been proposed to stimulate actin filament assembly by lowering the critical concentration of actin or by sequestering actin monomers when the barbed ends of actin filaments are capped (Pantaloni and Carlier 1993). It has also been proposed to stimulate actin filament assembly by accelerating the rate of exchange of nucleotide bound to actin (Goldschmidt-Clermont *et al.* 1992). In *S. pombe*, *cdc3*-profilin mutant strains are defective for cytokinesis because they cannot assemble an F-actin contractile ring (Balasubramanian *et al.* 1994). Profilin is important for the process of cytokinesis also in Dictyostelium, *S. cerevisiae*, and Drosophila (reviewed in Schluter *et al.* 1997).

In addition to the mutation in *sop2*$^+$, we found that a mutation within another subunit of the Arp2/3 complex, *arp3*-c1, was able to suppress the *cdc3*-124 mutant strain (McCollum *et al.* 1996). *sop2* and *arp3* mutants have similar phenotypes and exhibit a synthetic lethal genetic interaction. Both mutant strains shifted to their nonpermissive temperatures arrest heterogeneously throughout the cell cycle and display many defects associated with impaired cortical actin function. Cells with thickened septa accumulate and after prolonged incubation at restrictive temperature, both strains lyse (Balasubramanian *et al.* 1996; McCollum *et al.* 1996). *S. cerevisiae arp2* mutants were also reported to display multiple cortical actin defects, including defects in polarized growth, budding pattern, and endocytosis (Moreau *et al.* 1996, 1997); a disorganized actin cytoskeleton was also observed in a strain with mutant *S. cerevisiae arp3* (Winter *et al.* 1997). In the *S. pombe arp3*-c1 mutant, movement of actin patches to the medial region of the cell during mitosis was impaired, and we suggested, on the basis of this observation, that one function of the Arp2/3 complex is to promote actin patch movement (McCollum *et al.* 1996). This suggestion was confirmed in *S. cerevisiae* by analyzing the movement of a green fluorescent protein (GFP)-tagged component of the actin patch, Sac6p, in live cells (Winter *et al.* 1997).

In the two yeasts, Arp2/3 complex components have been localized by indirect immunofluorescence (Balasubramanian *et al.* 1996; Moreau *et al.* 1996; Winter *et al.* 1997) and immunoelectron microscopy (McCollum *et al.* 1996) principally to actin patches. Sop2p/Arc40p is also detected in cable structures in *S. pombe*; these cables most likely correspond to actin cables (Balasubramanian *et al.* 1996). There has also been a report that *S. cerevisiae* Arp2p localizes to nuclear pores, although the significance of this interaction remains to be determined (Yan *et al.* 1997). In higher eukaryotic cells, the Arp2/3 complex is concentrated at sites containing dynamic actin-based structures such as lamellopodia (Kelleher *et al.* 1995; Machesky *et al.* 1997; Welch *et al.* 1997a) and the surface of the motile intracellular pathogen *Listeria monocytogenes* (Welch *et al.* 1997b).

Biochemical studies have shown that purified Arp2/3 complex can bind to the sides of actin filaments (Mullins *et al.* 1997) and nucleate actin polymerization (Mullins *et al.* 1998; Welch *et al.* 1998). Further, addition of the *L. monocytogenes* ActA protein accelerates the ability of the Arp2/3 complex to nucleate actin filament formation (Welch *et al.* 1998). For this reason, efforts are being directed at identifying a eukaryotic protein with the properties of ActA (reviewed in Machesky and Way 1998). In a genetic approach to finding proteins that modulate Arp2/3 complex activity, we undertook a high-copy suppressor screen of the cold-sensitive *S. pombe arp3*-c1 mutation. We report here the isolation of the *asp1*$^+$ gene as such a suppressor. *asp1*$^+$ encodes a highly conserved protein of 106 kD that does not stably associate with the Arp2/3 complex and is localized diffusely throughout the cytoplasm. Neither *asp1*$^+$ nor its *S. cerevisiae* ortholog, *VIP1*, are essential genes, but the *asp1* disruption mutation displays temperature-sensitive morphological defects and hypersensitivities to a number of compounds in the media. Furthermore, the *asp1* disruption mutation is synthetically lethal with mutations in actin and Arp2/3 complex members. These data suggest that, like the Arp2/3 complex, Asp1p is important for cortical actin function.

MATERIALS AND METHODS

Yeast strains, methods, and media: Yeast strains used in this study are listed in Table 1. *S. pombe* strains were grown in yeast extract medium or minimal medium with appropriate supplements (Moreno *et al.* 1991). Crosses were performed on glutamate medium (minimal medium lacking ammonium chloride and containing 0.01 m glutamate, pH 5.6). Random spore analysis and tetrad analysis were performed as described (Moreno *et al.* 1991). Transformations were performed by electroporation (Prentice 1992). For nitrogen-deprivation experiments, cells were grown in minimal medium lacking nitrogen for 18 hr. To examine growth at low pH, adjustments

TABLE 1

Yeast strains used in this study

Strain	Genotype		Source
KGY28	h$^-$	972 wild type	Paul Nurse
KGY69	h$^+$	975 wild type	Paul Nurse
KGY246	h$^-$	*leu1*-32 *ura4*-D18 *ade6*-M210	Our stock
KGY247	h$^+$	*leu1*-32 *ura4*-D18 *ade6*-M210	Our stock
KGY249	h$^+$	*leu1*-32 *ura4*-D18 *ade6*-M216	Our stock
KGY435	h$^-$	*cdc3*-124 *ura4*-D18	Our stock
KGY680	h$^+$	*arp3*-cl *leu1*-32 *ura4*-D18 *his3*-237	Our stock
KGY1010	h$^-$	*act1*-48 *leu1*-32 *ura4*-D18 *lys1*-131	Our stock
KGY954	h$^-$	*asp1::ura4*$^+$ *leu1*-32 *ura4*-D18 *ade6*-M210	This study
KGY956	h$^+$	*asp1::ura4*$^+$ *leu1*-32 *ura4*-D18 *ade6*-M210	This study
KGY860	h$^-$	*sop2*-1 *leu1*-32 *ura4*-D18 *ade6*-M210	Our stock
KGY1086	h$^-$	*asp1::ura4*$^+$ *cdc3*-124 *leu1*-32 *ura4*-D18 *ade6*-M210	This study
KGY1962	h$^-$	*asp1::ura4*$^+$ *ura4*-D18	This study
KGY1350	*MAT*α*vip1*Δ*::HIS3 ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*1 ura3-52*		This study
KGY2142	*MAT***a** *las17*Δ*::LEU2 his3 leu2 trp1 ura3*		Alan Munn

to minimal medium were made as described (Saleki *et al.* 1997). For regulated expression of *asp1*$^+$ by the *nmt1* promoter (Maundrell 1993), cells were grown either in minimal media lacking thiamine to allow expression or with the addition of 5 μm thiamine to repress expression. Double mutant strains were constructed and identified by tetrad analysis or random spore analysis.

S. cerevisiae strains were grown in either synthetic minimal medium with the appropriate nutritional supplements or complex YP medium supplemented with 2% glucose as the carbon source. Genetic methods were as described (Guthrie and Fink 1991). Transformation of *S. cerevisiae* was performed by the lithium acetate method (Ito *et al.* 1983). The *las17*/*bee1* deletion strain (a gift from Dr. Alan Munn) was backcrossed three times to our strain background (s288c) before use in our experiments.

Plasmids and molecular biology techniques: All plasmid manipulations and bacterial transformations were by standard techniques (Sambrook *et al.* 1989). Essential features of plasmid construction are described. All sequencing of plasmid DNA was performed using Sequenase 2.0 (USB, Cleveland) or Thermo Sequenase (Amersham Life Sciences, Cleveland) according to manufacturer's instructions. Yeast genomic DNA was isolated as described (Moreno *et al.* 1991; Hoffman 1993).

For expression of *asp1*$^+$ under control of the *nmt1* promoter, *Nde*I and *Bam*HI sites were introduced at the initiating methionine codon and after the stop codon, respectively, by site-directed mutagenesis using the Bio-Rad Muta-Gene kit after the genomic clone had been subcloned into pSK+. The following oligonucleotides were used for mutagenesis: for *Nde*I, 5′-CGTAGTTGAATAATAACATATGATTCAAAA-3′ and for *Bam*HI, 5′-CATTAATTAACCGCTTGGATCCTTTTTACG-3′. This allowed subcloning of the *asp1*$^+$ ORF into the pREP series of fission yeast expression plasmids (Basi *et al.* 1993; Maudrell 1993). Additionally, the *Nde*I-*Bam*HI ORF fragment of *asp*$^+$ was fused in frame to GFP in the vector pREP41GFP.

Disruption of *asp1*$^+$ and *VIP1*$^+$: *asp1*$^+$ was disrupted by the one-step gene disruption method using the genomic clone of *asp1* in which the *Hin*dIII fragments had been replaced with the *ura4*$^+$ gene. This fragment was used to transform the diploid strain of genotype *ade6*-M210/*ade6*-M216 *leu1*-32/*leu1*-32 *ura4*-D18/*ura4*-D18 *h*$^+$/*h*$^-$ to uracil prototrophy. Transformants were replica plated five times at 1-day intervals to medium containing uracil to allow loss of any autonomously replicating DNA molecules carrying the *ura4*$^+$ gene. Transformants were subsequently replica plated to medium lacking uracil, and colonies that had remained uracil prototrophs were treated as putative stable integrants. Putative integrants were induced to sporulate by shifting to malt extract plates. Tetrads dissected from one stable integrant displayed a 2:2 segregation of Ura+:Ura− colonies. Southern analysis of genomic DNA from the Ura+ colonies confirmed that this strain contained the *asp1* disruption.

The *vip1*-Δ*1::HIS3* null allele was generated by the polymerase chain reaction (PCR) as described (Baudin *et al.* 1993). Primers 5′-GGACGCTGAGCAGACTAACATGGGAACTAACTCTGGCAGATTGTACTGAG-3′ and 5′-CGGAGGTAGATGATGAAGATCCGGACGAGGCAGGACGCTCCTT ACGCATCTG-3′ were used to amplify the *HIS3* gene from pRS313. The underlined portions of each primer correspond to *HIS3*-flanking sequence. The resulting PCR product contains the complete *HIS3* gene flanked by 35 bp of *VIP1* 5′-sequence and 37 bp of *VIP1* 3′ sequence. When substituted at the *VIP1* locus, this DNA fragment deletes the *VIP1* gene except the initial 152 codons. The diploid strain YPH274 was transformed with this DNA fragment, and two independent transformants (KGY1323, KGY1324) that contained a *vip1*-Δ*1::HIS3* allele substituted correctly at one of the two *VIP1* loci were identified by PCR. These strains were sporulated, and tetrads were dissected and analyzed.

Fluorescence microscopy: All fluorescence microscopy was performed using a Zeiss (Thornwood, NY) axioscope and the appropriate set of filters. DNA, F-actin, and cell-wall material were visualized using 4,6-diamidino-2-phenylindole (DAPI), rhodamine-conjugated phalloidin, and calcofluor, respectively. Stainings were performed as described (Balasubramanian *et al.* 1997). For visualization of endocytosis, the fluorescent dye FM 4-64 was used as described (Vida and Emr 1995). Strains were grown in YE medium at 32° and 36° to midlog phase, concentrated 100-fold by centrifugation, and preincubated in fresh YE medium at 32° or 36°. FM 4-64 (Molecular Probes, Eugene, OR) was added to 16 μm from a stock solution of 16 mm in dimethyl sulfoxide, and cells were incubated for 15 min on ice, or at 32° or 36°. Cells were then washed into fresh YE medium and incubated on ice or at 32° or 36° for 10, 20, or 30 min. Cells were mounted on slides and visualized immediately.

Antibody production and immunoblotting: The 1331-bp

*Nco*I-*Hin*dIII fragment of *asp1*$^+$ (see Figure 2A) was cloned into the vector pRSETB (Invitrogen, San Diego) for production of a his(6)-tagged fusion protein in bacteria. The insoluble fusion protein was purified from inclusion bodies by SDS-PAGE, and antibodies were produced in rabbits by Cocalico Inc. Antibodies were affinity purified as previously described (Olmsted 1981).

Protein lysates were prepared in NP-40 or SDS-lysis buffers as previously detailed (Gould *et al.* 1991). Proteins were resolved by 6–20% gradient SDS-PAGE and transferred to polyvinylidene fluoride membranes. The blots were incubated with anti-arp3 (McCollum *et al.* 1996) or anti-Asp1p sera at 1:5000 dilution for 2 hr and then incubated for 1 hr with peroxidase-conjugated anti-rabbit IgG (Sigma Chemical Co., St. Louis) at 1:10,000 dilution. Reactive proteins were visualized by enhanced chemiluminescence.

Glycerol gradient analysis: For glycerol gradient analysis, cells were grown to midlog phase in YE at 32°. Approximately 2×10^8 cells were collected by centrifugation, and native lysates in NP-40 buffer were prepared as described (Gould *et al.* 1991). Lysates were layered on 5–20% glycerol gradients prepared in NP-40 buffer. Gradients were ultracentrifuged at 40,000 rpm for 19 hr in a Beckman (Fullerton, CA) SW50.1 rotor. Molecular weight markers were fractionated on gradients prepared and spun in parallel. Fractions were collected from the bottom of the gradients, run on 6–20% gradient SDS-polyacrylamide gels, and immunoblotted as described above.

RESULTS

***asp1* is a high-copy suppressor of *arp3*-c1:** To identify *arp3*$^+$-interacting genes, a genomic library was transformed into the cold-sensitive *arp3*-c1 mutant. In addition to plasmids containing *arp3*$^+$ (McCollum *et al.* 1996), a plasmid containing a second genomic locus was recovered that reproducibly rescued growth of the *arp3*-c1 mutant at 19° (Figure 1A). The ends of this ~5-kb genomic clone were sequenced, and a search of the Sanger Center *S. pombe* genome sequencing project database revealed that this region of *S. pombe* chromosome 3 on cosmid c962 had been sequenced. A single ORF, consisting of 2760 bp, was present within the 4886-bp genomic clone, and it encoded a predicted protein product of 921 amino acids with a molecular mass of 106 kD (Figure 1). We have termed this gene *asp1*$^+$ (*a*rp, *s*op, *p*rofilin interactor) for reasons described below. A database search with the predicted protein sequence revealed highly related proteins in a variety of species (Figure 1B). In *S. cerevisiae*, ORF YLR410w encoded a protein that was 55% identical and 68% similar to Asp1p. We have termed this ORF *VIP1*. ORF YLR410w is predicted to be longer by 152 amino acids at its N terminus than what we have indicated in Figure 1, and we did not include these amino acids when calculating identity. Other sequences displaying a high level of identity to Asp1p were encoded by a human cDNA with accession no. AB002375 and a *Caenorhabditus elegans* ORF, locus CELF46F11, accession no. U88173. The predicted human and *C. elegans* proteins do not have predicted extensions at their N termini relative to Asp1p but are predicted to have much longer C termini. Domains of known function were not detected within Asp1p, although amino acids 555–600 are predicted to form a coiled-coiled domain.

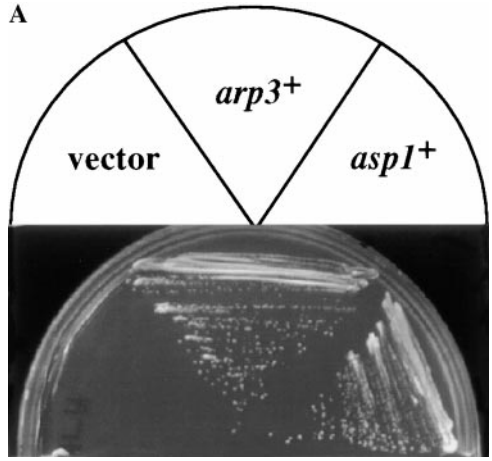
***asp1*$^+$ and *VIP1* are not essential genes:** Deletion constructs of the *asp1*$^+$ genomic clone were used to localize the rescuing activity to an ~1-kb fragment (Figure 2A), indicating that only the N terminus of Asp1p was required for its ability to suppress the *arp3*-c1 mutant. To further analyze *asp1*$^+$ function in *S. pombe*, the method of one-step gene disruption was used to generate a disruption of the *asp1* gene in *S. pombe* cells as described in materials and methods. An ~2.5-kb *Hin*dIII fragment of the *asp1*$^+$ gene, encompassing all of the *arp3*-c1 minimal rescuing fragment, was replaced with the *ura4*$^+$ selectable marker in diploid *S. pombe* cells (see Figure 2A). Haploid Ura+ progeny (hereafter referred to as *asp1*-Δ*1*) were recovered from these diploids and the disruption confirmed by Southern blot analysis (data not shown). Thus, *S. pombe asp1*$^+$ is not an essential gene.

To determine whether *VIP1* was an essential gene, a disruption of one genomic copy of *VIP1* was created in a diploid strain (see materials and methods). Precise replacement of one copy of *VIP1* by a *VIP1::HIS3* disruption fragment was confirmed by PCR (data not shown). We note that the disruption did not remove the hypothetical 152-amino-acid N-terminal extension. Two independent *vip1*-Δ*1::HIS3*/*VIP1* heterozygotes were sporulated and tetrads dissected. In each case, four spores, two His$^+$ and two His$^-$, germinated and formed colonies, indicating that the region of *VIP1* that was removed by this disruption is not essential for viability in *S. cerevisiae*. Haploid *vip1*-Δ*1::HIS3* cells are morphologically indistinguishable from wild-type cells and are capable of growing at all temperatures tested (16°–36°).

***asp1*-Δ*1* demonstrates a temperature-sensitive morphology defect:** Although *asp1*$^+$ was not an essential gene, *asp1*-Δ*1* cells exhibited slow growth and morphological defects at 19° and 36°, although the defects were

Figure 1.—Analysis of *asp1*$^+$. (A) The *arp3*-c1 strain was transformed with empty vector, *arp3*$^+$ or *asp1*$^+$. Transformants that were isolated at 29° were struck to selective plates and incubated at 19° for 5 days. (B) Alignment of *S. pombe* Asp1p (lines 1) with *S. cerevisiae* Vip1p (lines 2), a human homolog (accession no. AB002375; lines 3), and a *C. elegans* homolog (accession no. U88173; lines 4). The Asp1 protein sequence was deduced from the DNA sequence of the relevant portion of *S. pombe* cosmid c962. Vip1p, encoded by ORF YLR410w, is the closest relative of Asp1p in *S. cerevisiae*. Identical amino acids are indicated by vertical dashes. The asterisks denote the positions of stop codons. The 152 N-terminal amino acids of Vip1p, predicted of ORF YLR410w, were not included in the alignment. The human and *C. elegans* proteins have much longer C termini, which also are not included in this alignment and not related to one another. Amino acids that are identical between Asp1p and at least one other protein, as well as amino acids identical among the other three proteins, are shaded.

A



B

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1  ----------------------MIQNASHLTSIDTESS-TRTASPVSSIVTPTKRNVVGICAMDAKARSKPCRNILNRIIAEG--EFEAI   65
2  ---------------------MDAEQTNMGTNSVPTSSASSRKSSTSHPKPRLPKVGKIGVCAMDAKVLSKPMRHILNRLIEHG--EFETV   68
3  MWSLTASEGESTTAHFFLGAGDEGLGTRGIGMRPEESD-SELLEDEEDEVPPEPQIIVGICAMTKKSKSKPMTQILERLCRFD--YLTVV   87
4  ----------------------------MAHKGTESK-------EQ--IWP-YKITIGICAMNRKATSKPMRAIMKKIIDFYGQWVDSF   51

1  VFGDNMILDEAVENWPACDYLICFYSSGFPLKKAEKYVELRKPFCVNDVVFQELLWDRRLVLNILDAIRVSTPQRLICSRDGGPKINKVL  155
2  IFGDKVILDERIENWPTCDFLISFFSSGFPLDKAIKYVKLRKPFIINDLIMQKILWDRRLCLQVLEAYNVPTPPRLEISRDGGPRANEEL  158
3  ILGEDVILNEPVENWPSCHCLISFHSKGFPLDKAVAYSKLRNPFLINDLAMQYYIQDRREVYRILQEEGIDLPRYAVLNRD--P------  169
4  IFPEQVIINEPVENWPLCHCLVSFHSTEFPLEKAIAYVKLRNPYVINNLDRQYDLLDRRTVFKILSDNGIEHPRHGYVIRGR-P------  134

1  EEKLRRKFGIEITEVPTPEVKMLDEDTLSVDGKIIKKPYVEKPVYGEDHNIYIYFPKSVGGGGRKLFRKVANKSSDYDPDLCAPRTEGSF  245
2  RAKLR-EHGVEVKPVEEPEWKMVDDDTLEVDGKTMTKPFVEKPVDGEDHNIYIYYHSKNGGGGRRLFRKVGNKSSEFDPTLVHPRTEGSY  247
3  ---AR----------PEECNLIEGEDQVEVNGAVFPKPFVEKPVSAEDHNVYIYYPSSAGGGSQRLFRKIGSRSSVYSP-ESSVRKTGSY  245
4  ----N----------EPDTELVEHPDHIEVNGEVFNKPFVEKPISSEDHNVYIYYPSSVGGGSQRLFRKINNRSSWYSP-KSEVRKEGSY  209

1  IYEEFMNVDNAEDVKVYTVGPHYSHAETRKSPVVDGIVRRNPHGKEIRFITNLSEEEKNMASKISIAFEQPVCGFDLLRVSGQSYVIDVN  335
2  IYEQFMDTDNFEDVKAYTIGENFCHAETRKSPVVDGIVRRNTHGKEVRYITELSDEEKTIAGKVSKAFSQMICGFDLLRVSGKSYVIDVN  337
3  IYEEFMPTDG-TDVKVYTVGPDYAHAEARKSPALDGKVERDSEGKEIRYPVMLTAMEKLVARKVCVAFKQTVCGFDLLRANGHSFVCDVN  334
4  IYEEFIPADG-TDVKVYAVGPFYAHAEARKAPGLDGKVERDSDGKEVRYPVILSNKEKQIAKKIVLAFGQTVCGFDLLRANGKSYVCDVN  298

1  GWSFVKDNNDYYDNAARILKQMFHVAERHRRNRVPSVQEVLNPP---------------PRESEAWRLKSLVGVLRHADRTPKQKFKFSF  410
2  GFSFVKDNKAYYDSCANILRSTFIEAKKKMDMEKKNLPIIREE-----------------KE-QKWVFKGLAIIIRHADRTPKQKFKHSF  409
3  GFSFVKNSMKYYDDCAKILGNTIMRELAPQFQIPWSIPTEAEDI-----------PIVPTTSGTMMELRCVIAIIRHGDRTPKQKMKMEV  413
4  GFSFVKTSTKYYEDTAKILGNQIVRHYAKSKN--WRVPSDMPQFPILDLGLGDDPPMITTPSGKLAELRCVVAVIRHGDRTPKQKMKLIV  386

1  TSDPFVKLLQGH----TEEVILRNE-QLNSVLAATNLATELKCED-----------IN-------------------KLKQLRLALETKK  465
2  TSPIFISLLKGH----KEEVVIRNVNDLKIVLQALRIALDEKAGN-----------PA-------------------KIKVLANALEKKL  465
3  KHPRFFALFEKHGGYKTGKLKLKRPEQLQEVLDITRLLLAELEKE-----------PGGE-----------IEEKTGKLEQLKSVLEMYG  481
4  TDQRFFALFEKYDGYKKHEIKMKKPNQLMEVLELARALVIEKQRDRHQILEKLREGTGEEEIHKSEHDLEVCEEEMKKWEQMRTVLEMYG  476

1  DLPGTKVQLKPAYSP-------EGK-------LLKLQLIIKWGGEFTHSARYQSKDLGEQFHKDL---------------------YIMNR  521
2  NFPGTKIQLKEVLNK-------ENE--------VEKVQFILKWGGEPTHSAKYQATELGEQMRQDF---------------------DLLNK  521
3  HFSGINRKVQLTYYPHGVKASNEGQDPQRETLAPSLLLVLKWGGELTPAGRVQAEELGRAFRCMYPGGQGDYA-----GFPGCGLLRLHS  566
4  HFSGINRKVQMKYLKERETKTSDEE-LRRE--GPALLLILKWGGELTTAGNMQAEALGRLFRTLYPGIRRTDGKSSPEDTQGLGFLRLHS  563

1  DCLKDVEIYTSSERRVSASAEIFAMAFLEQETIPS------DLLKVRKDLLD-DSNAAKDTMDKVKKHLKSLLRVGDTARKEFTWPENMP  604
2  SILQNIKIFSSSERRVLHTAQYWTRALFGADELGS------DEISIRKDLLD-DSNAAKDLMDKVKKKLKPLLREGKEAPPQFAWPSKMP  604
3  TFRHDLKIYASDEGRVQMTAAAFAKGLLALEGELTPILVQMVKSANMNGLLDSDGDSLSSCQHRVKARLHHILQQDAPFGPE-DYDQLAP  655
4  TYRHDLKIYASDEGRVQTTAAAFAKGLLALEGELTPILMQMVKSANTDGLLD-DDCQARLYQTELKRYLHKALQADRDFTPQ-DYLELNP  651

1  KPCEVMQQVVQLMKYHRAVMRENFIILGPEVEQVQSRWCCN----------ENPALFRERWEKLFSEFCD-----SEKADPSKVSELYDT  679
2  EPYLVIKRVVELMNYHKKIMDNNFAKK--DVNSMQTRWCTS----------EDPSLFKERWDKLFKEFNN-----AEKVDPSKISELYDT  677
3  TRSTSLLNSMTIIQNPVKVCDQVFALIENLTHQIRERMQDPRSVDLQLYHSETLELMLQRWSKLERDFRQK----SGRYDISKIPDIYDC  741
4  NGLRAITAAMEFIKNPRKMCHEIAGYVEKMCGVIVEYSQTRPTG-STLYLQESMDLAQRRWNKELREFRRKNKHGEVEFDISKIPDIYDN  740

1  LKYDALHNRQFLERIFTP--------YQYLKLPQSPS-LIAKEPP-------------------QRTDSN-------GNLVGMTGANTNH  734
2  MKYDALHNRQFLENIFDPGL-P---NEAIADELGSHS-LVDRYPINVLAKNNFKIIDSHSMNNSGKNSSNS-V----GSLGWVLESGKTS  757
3  VKYDVQHNGSLGLQGTAELLRL---SKALADVVIPQEYGISREEKLEIAVGFCLPLLRKILLDLQRTHEDESV----NKLHPLYSRGVLS  824
4  IKYDMEHNPDLCINNEVEFERMYVCVKNMADIVVPQEYGIKTENKMVIAQRVCTPLLRKIRNDLHRCLENKESEETQTRLDPRASQGIAT  830

1  TER-P----------LEKLYELYDLAKVLFD--FVSPQEYGIEPKEKLEIGLLTSVPLLRQIIHDIKEAR--DSDHASIRMYFTKESHIY  809
2  TARNPKSSSQFDEPRFMQLRELYKLAKVLFD--FICPKEYGISDAEKLDIGLLTSLPLAKQILNDIGDMK--NRETPACVAYFTKESHIY  843
3  PGR-HVRTR----LYFTSESHVHSLLSVFRYGGLLDETQDAQWQRALDYLSAISELNYMTQIVIMLYEDN--TQDPLSE-ERFHVELHFS  906
4  PFR-HVRTR----LYFTSESHIHTLMNLIRYG-NLCSVDDKKWQRAMNFLSGVTEFNYMTQVVLMVYEDSRKENDEADTGPRFHIEILFS  914

1  TLLNCILESG---------------LPMKLPR----NQIP-----ELDYLTQIC----------------------FELFERTN---PS  849
2  TLLNILYESG---------------IPMRIAR----NALP-----ELDYLSQIT----------------------FELYESTD---AS  883
3  PGVKGVEEEGSAPAGCGFRPASSENEEMKTNQGSMENLCPGKASDEPDRALQTSPQPPEGPGLPRRSPLIRNRKAGSMEVLSETSSSRPG  996
4  PGLYPCFLTE-------------K-ERIYETRFN-LSTNP-----KPATSSRSSG--------------RESRDTNDSASSSTEGRRPS  969

1  GNKEFS-VR----ITLS----PGC---YAQCPLDMNLDAKHCISVSPRRS---LTRHLDLQ-------QFITKTEDLCNSVHLP--KRFI  915
2  GQKSHS-IR----LKMS----PGC---HTQDPLDVQLDDRHYISCIPKIS---LTKHLDMD-------YVQQKLRNKFTRVIMP--PKFT  949
3  GYRLFSSSRPPTEMKQSGLGFEGCSMVPTIYPLETLHNALSLRQVSEFLSRV-CQRHTDAQA------QASAALFDSMHSSQASDNPFSP 1079
4  IEKVVTVVTP---TQLS---TPSV----TNDDLSISSNAESTAAESTGLVNTTTKTHNDSEDDLNDVESVNLVALDELNNTTKASDIIFH 1049

1  PVNIN*                                                                                      921
2  PVNITSPNLSFQKR-------KTRRKSVSVEKLKRPASSGSSSSTSVNKTLD*                                      994
3  PRTLHSPPLQLQQRSEKPPWLETRFCHVGQAGLELLTSSDLPASASQSAGIT.....................................1349
4  HKKLFVFLLHARRY------FPYRFKHHTAQLLTGMSGGGVHMQNRLISTDV.....................................1406
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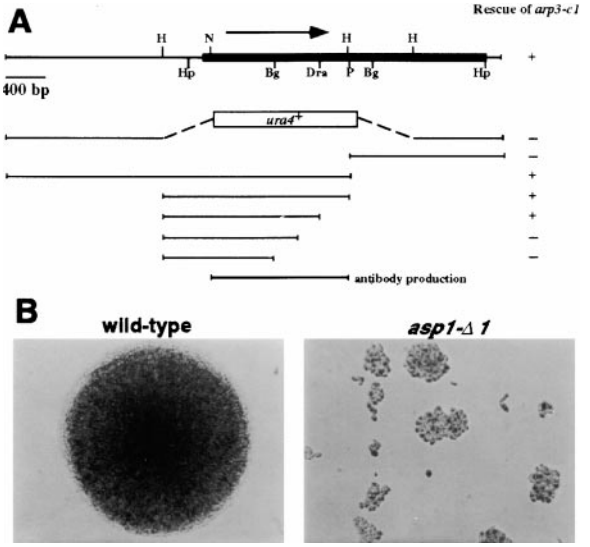

Figure 2.—*asp1* deletion. (A) Restriction map of the *asp1* genomic locus. The open reading frame is denoted by a solid box and the direction of transcription is indicated with an arrow. Relevant restriction sites are indicated. H, *Hin*dIII; Bg, *Bgl*II; Hp, *Hpa*I; Dra, *Dra*I; P, *Pst*I; N, *Nco*I. Below the map are diagrammed the *asp1* gene disruption construct as well as partial deletions of the *asp1* genomic clone with their ability to rescue growth of *arp3*-c1 indicated. The fragment used for bacterial expression and antibody production is shown. (B) Colony formation of wild-type and *asp1*-Δ*1* cells at 36° on YE agar after 3 days of growth.

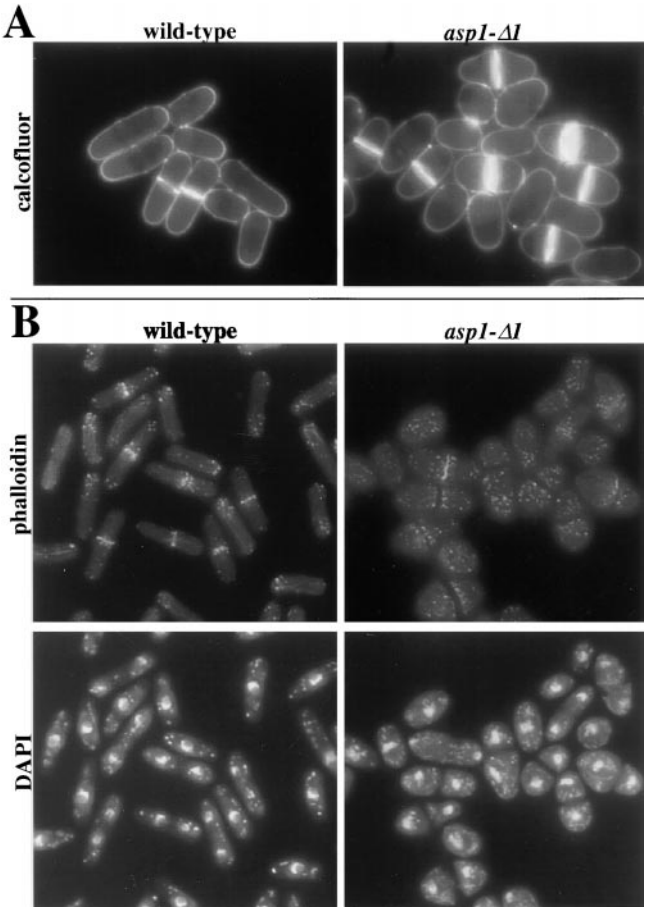

Figure 3.—Phenotype of *asp1*-Δ*1* cells. (A) Wild-type and *asp1*-Δ*1* cells were grown at 36°, collected, and stained with calcofluor. (B) Wild-type or *asp1*-Δ*1* cells were grown at 36°, fixed with formaldehyde, and stained with rhodamine-conjugated phalloidin and DAPI.

more severe at 36°. At 36°, *asp1*-Δ*1* cells were rounded, swollen, and lysed frequently. Colony size of the *asp1*-Δ*1* strain after 3 days of incubation at 36° on rich media plates was significantly smaller than wild-type cells plated in parallel (Figure 2B).

To examine the *asp1*-Δ*1* phenotype more carefully, wild-type and *asp1*-Δ*1* cells were grown in liquid at 36° and stained with the cell-wall stain calcofluor (Figure 3A). The *asp1*-Δ*1* cells were shorter and rounder than wild-type-cells, and 27% of the *asp1*-Δ*1* cells contained a septum in comparison to 13% of wild-type cells. The septa in *asp1*-Δ*1* cells were also thicker than normal. This phenotype was not obvious until the culture had grown for at least 10 hr at the nonpermissive temperature (data not shown). Although lysis of the *asp1*-Δ*1* cells in liquid media was observed, this phenotype was more obvious on solid media. To quantitate the lysis phenotype, *asp1*-Δ*1* cells were plated on rich medium as a sparse lawn, grown overnight at 36°, and then washed from the plates and stained with calcofluor. Whereas no cell lysis was observed in a preparation of wild-type cells prepared in parallel, ~7% of *asp1*-Δ*1* cells were lysed as judged by the ability of calcofluor to stain evenly throughout the cell interior. This lysis phenotype explains the small colony size of *asp1*-Δ*1* cells in comparison to wild-type cells grown for the same length of time. We examined *vip1::HIS3*$^+$ cells for similar defects in morphology at elevated and lowered temperatures but were unable to discern any.

Actin organization in *asp1*-Δ*1*: Because changes in morphology might reflect altered actin and/or microtubule organization, we examined F-actin structures in the *asp1*-Δ*1* strain using rhodamine-conjugated phalloidin and microtubule structures with antibodies to tubulin. Whereas microtubule organization appeared normal (data not shown), F-actin structures were delocalized. In *asp1*-Δ*1* cells grown at 36°, F-actin patches were diffusely distributed around the cortex of the cell, whereas they generally cluster to cell ends and to the septa of wild-type cells (Figure 3B). However, there was no apparent defect in the formation of medial F-actin rings. Nuclear morphology also appeared normal.

Polarized growth in *asp1*-Δ*1*: To determine whether Asp1p was important for the establishment of polarized growth in addition to its maintenance, we starved *asp1*-Δ*1* cells and wild-type cells of nitrogen for 18 hr at 36°. This treatment causes *S. pombe* cells to become round, short, and arrested in G1 (reviewed in Forsburg and Nurse 1991). *asp1*-Δ*1* cells responded normally to this treatment (Figure 4 and data not shown). The cells were then allowed to resume growth in YE media at 36°, and cells were photographed until wild-type cells began to

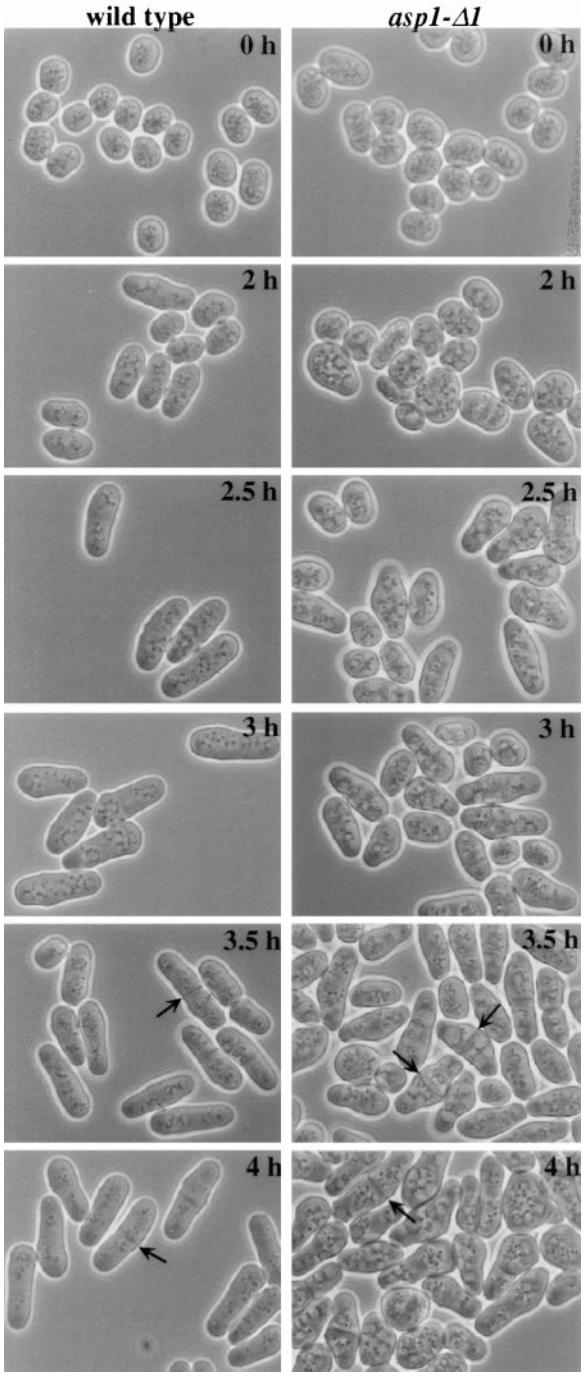

Figure 4.—Polarized growth in *asp1*-Δ*1* cells. Following 18 hr of nitrogen deprivation at 36°, wild-type or *asp1*-Δ*1* cells were released into YE medium at 36° and phase images were taken at the indicated times. Small arrows indicate the positions of septa.

septate and divide at 4 hr (Figure 4). The *asp1*-Δ*1* cells were unable to polarize growth normally and remained rounder and wider throughout the time course. The increased width of *asp1*-Δ*1* cells probably explains their difficulty undergoing septation because more time, septal material, and cell-wall material would be needed to form the primary and secondary septa. Consistent with this scenario, we observed that the wider cells lysed frequently following their attempt to divide (data not shown). These data suggest that Asp1p, although not essential, is important for establishing and maintaining the normal rod-shaped morphology of *S. pombe*.

***asp1*-Δ*1* is osmosensitive, hypersensitive to Ca^+ and low pH, and defective in endocytosis:** Because of the lysis phenotype we observed in the *asp1*-Δ*1* strain described above, we wished to determine whether membrane and/or cell-wall function was compromised in the absence of $asp1^+$ function. First, we examined cell-wall integrity by measuring the sensitivity of *asp1*-Δ*1* cells to digestion with β-glucanase and resistance to heat shock. In both cases, *asp1*-Δ*1* cells behaved identically to wild-type cells, indicating that the cell wall of *asp1*-Δ*1* cells was not defective in structure or function (data not shown). Next, we examined the sensitivity of *asp1*-Δ*1* cells to agents that affect osmolarity or membrane dynamics. We found that the morphological and lysis defects of *asp1*-Δ*1* cells at 36° were rescued by the addition of 1.2 m sorbitol to the YE medium (Figure 5A). Similarly, the inclusion of 0.8 m KCl in the medium rescued the growth defects of *asp1*-Δ*1* cells at 36°, although at 1 m or 1.2 m, KCl exacerbated the growth defects of the *asp1*-Δ*1* cells. Additional NaCl was also inhibitory to *asp1*-Δ*1* cell growth. However, the most dramatic hypersensitivity we observed was to additional Ca^{2+} in the medium (Figure 5B). Growth of *asp1*-Δ*1* cells at 36° was greatly inhibited by the inclusion of 80 mm $CaCl_2$ and abolished at 120 mm $CaCl_2$. Even at 32°, the addition of 160 mm $CaCl_2$ strongly inhibited growth of *asp1*-Δ*1* cells, whereas another divalent cation, Mg^{2+}, had no effect on *asp1*-Δ*1* growth (Figure 5B). Growth at lowered pH (pH 4.5) was also inhibited in comparison to wild-type cells (Figure 5B). In sum, *asp1*-Δ*1* cells were hypersensitive to perturbations of the medium, indicating defects in osmoregulation and also sensitivities to particular ions.

Because many mutants in actin cytoskeletal proteins, including *S. cerevisiae* Arp2p, exhibit defects in endocytosis (Moreau *et al.* 1997), we chose to examine this process in *asp1*-Δ*1* cells. As a measure of endocytosis, we compared the ability of *asp1*-Δ*1* cells to take up and deliver the fluorescent dye FM 4-64 relative to wild-type cells. FM 4-64 is a marker of fluid-phase endocytosis that stains the vacuolar membrane but not the vacuolar compartment (Vida and Emr 1995). FM 4-64 bound wild-type and *asp1*-Δ*1* cells in a speckled pattern at 4° but was not taken up into the cell (data not shown). As the temperature was increased to 36°, the *asp1*-Δ*1* cells were noticeably slower in delivering the dye to internal membranes. This was determined qualitatively by examining the appearance of stained circles, representing vacuolar membranes, within wild-type and *asp1*-Δ*1* cells (Figure 6). Vacuolar membranes were intensely stained in wild-type cells by 10 min of incubation with the dye. The same degree of staining was observed after only 30 min in *asp1*-Δ*1* cells, and this delay was reproducible in three separate experiments. Interestingly, the delay

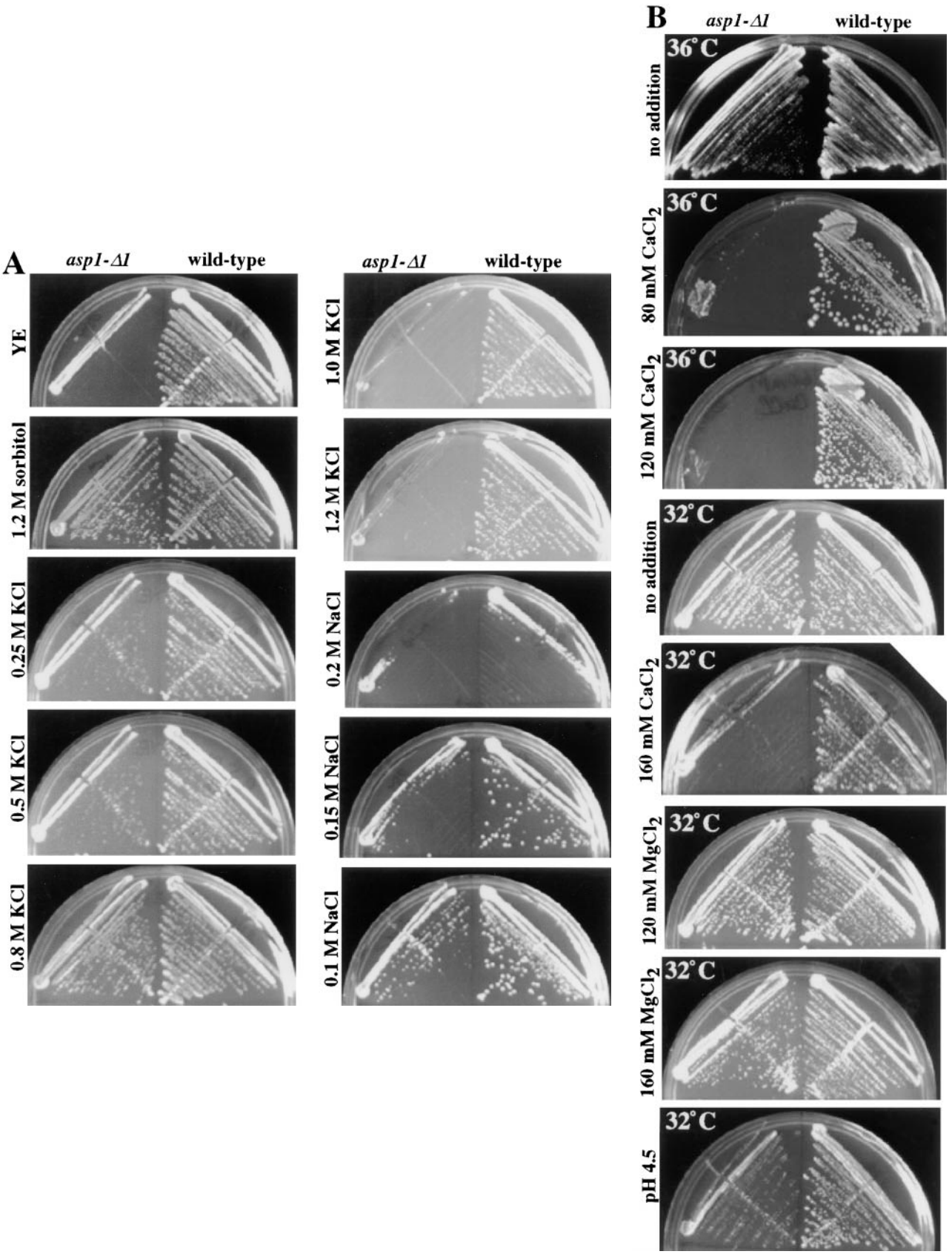

Figure 5.—*asp1-Δ1* cells are sensitive to media composition. Wild-type or *asp1-Δ1* cells were streaked onto YE plates that contained the components indicated and incubated at 36° (A) or at the indicated temperatures (B) for 3 days.

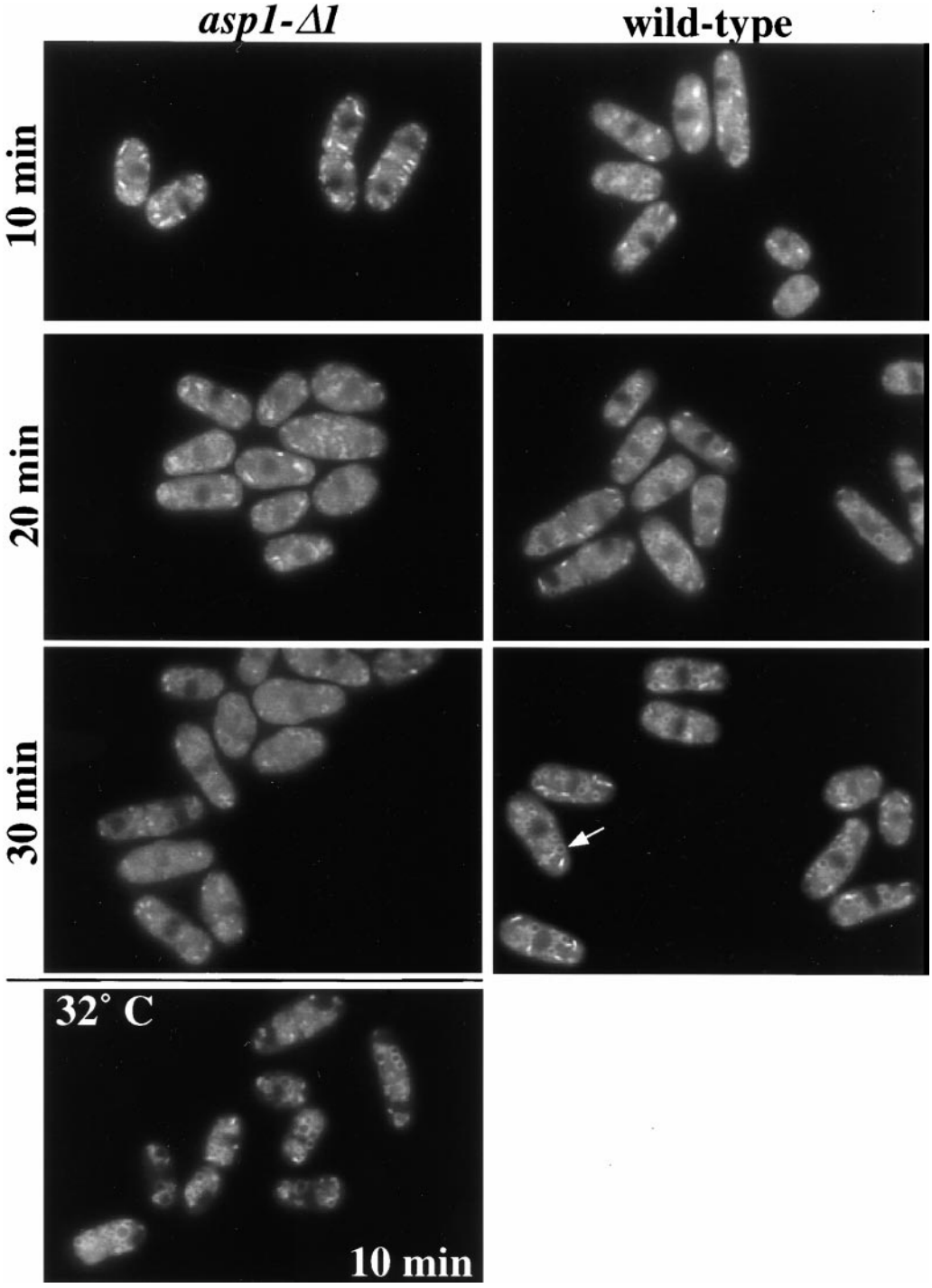

Figure 6.—Endocytosis is slower in *asp1*-Δ*1* cells. Wild-type or *asp1*-Δ*1* cells were incubated at 36° after treatment with the fluorescent dye FM 4-64 for 10, 20, or 30 min. A representative image of *asp1*-Δ*1* cells after incubation for 10 min at 32° is also presented. Note the appearance of prominent circles of staining appearing during the time course, an example of which is indicated by the arrow.

correlated with the morphological defects in *asp1*-Δ*1* cells as it was observed only at 36°. At 32°, intense vacuolar membrane staining in *asp1*-Δ*1* cells was observed after just 10 min of incubation.

The *asp1*-Δ*1* and *vip1*Δ mutations show genetic interactions with mutations that affect the actin cytoskeleton: Because overexpression of *asp1*$^+$ rescued *arp3*-c1 and the *asp1*-Δ*1* mutant strains had defects similar to actin cytoskeletal mutants, we tested the *asp1*-Δ*1* mutant for genetic interactions with *arp3*-c1 and other actin cytoskeletal mutants. *asp*-Δ*1* was crossed to the cold-sensitive mutants *arp3*-c1, *sop2*-1, and *act1*-48. Tetrads were dissected from the crosses and spores were allowed to germinate at 29°, a permissive temperature for all single-mutant strains. Colonies were then replica plated to score for growth at 19°, and to selective plates to score for Ura+ colonies. In all cases, tetratypes resulted in three viable colonies, nonparental ditypes gave rise to two viable colonies, and four viable colonies were observed for parental ditypes. In the *sop2*-1 cross, 11 tetrads

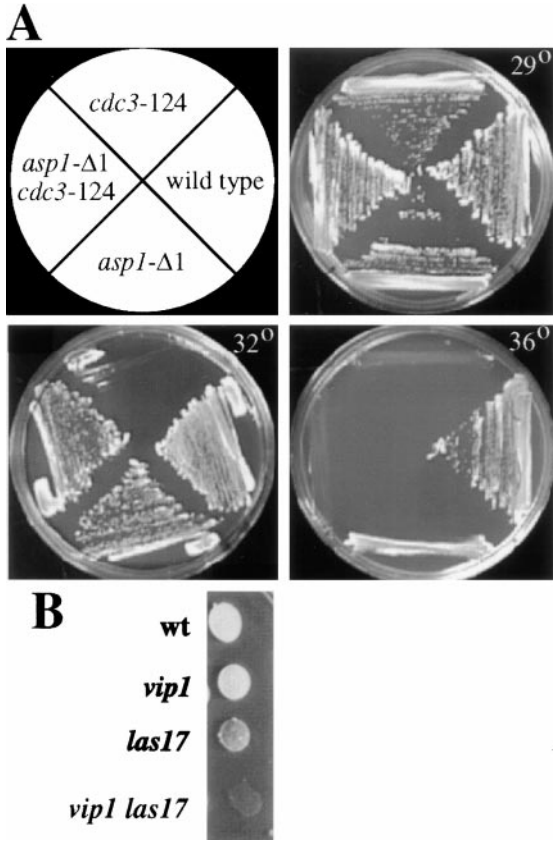

Figure 7.—*asp1-Δ1* and *vip1Δ* strains interact with actin cytoskeletal mutants. (A) *asp1-Δ1* rescues the *cdc3*-124 temperature-sensitive phenotype. Wild-type, *asp1-Δ1*, *cdc3*-124, or *asp1-Δ1 cdc3*-124 strains were streaked to YE agar. Plates were incubated at 29°, 32°, or 36°. (B) Negative genetic interaction between *vip1* disruption strain and *las17/bee1* deletion strain. Cells from a representative tetratype resulting from a cross between the *las17Δ* and *vip1* disruption strains were grown in YPD, and their cell numbers determined. Equal numbers of cells were spotted onto synthetic medium containing glucose and all nutritional supplements at 25°.

were examined; 7 were tetratypes and 2 were parental ditypes. In the *arp3*-c1 cross, 16 tetrads were examined; 11 were tetratypes, 2 were nonparental ditypes, and 3 were parental ditypes. In the *act1*-48 cross, 18 tetrads were examined; 9 were tetratypes, 6 were nonparental ditypes, and 3 were parental ditypes. Thus, we were able to conclude that the *asp1-Δ1* mutation was synthetically lethal with the *sop2*-1, *act1*-48, and *arp3*-c1 mutations.

Because the *sop2*-1 and *arp3*-c1 mutations suppress the profilin-defective strain *cdc3*-124 at 32°, we tested whether *asp1-Δ1* might also suppress *cdc3*-124. Indeed, we found that the double mutant *asp1.d cdc3*-124 could grow at 32° whereas the single *cdc3*-124 mutant could not (Figure 7A). Because the *asp1-Δ1* mutation had negative interactions with the *sop2*-1, *act1*-48, and *arp3*-c1 mutations that affect actin patches but not the actin ring (Balasubramanian *et al.* 1996; McCollum *et al.* 1996; McCollum *et al.* 1999), but rescued the actin ring mutant *cdc3-124*, it seemed possible that the *asp1-Δ1* mutation caused promotion of actin ring formation to the detriment of actin patches. This was not found to be the case, however, because the *asp1-Δ1* mutation did not rescue or display any interactions with the actin ring mutant *cdc12-112*. Thus, like *sop2*-1 and *arp3*-c1, the *asp1-Δ1* mutation specifically rescued the *cdc3-124* profilin mutation.

In *S. cerevisiae*, *LAS17/BEE1* encodes a nonessential protein that is important for cortical actin function (Li 1997; Karpova *et al.* 1998) and endocytosis (Naqvi *et al.* 1998). Hence, we examined whether there was any genetic interaction between the *las17Δ* strain (Naqvi *et al.* 1998) and the *vip1* disruption strain. Although double mutants were obtained, they grew more slowly than either single mutant alone (Figure 7B); this impaired growth was more evident on synthetic complete medium than on rich medium. This genetic interaction is consistent with a role for Vip1p in cortical actin function.

***Asp1p* does not associate stably with the Arp2/3 complex:** To begin exploring the biochemical basis of the genetic interactions we observed, polyclonal antibodies against Asp1p were generated. Immunoblot analysis of *S. pombe* lysates demonstrated that these antibodies specifically recognized an ~110-kD protein that corresponds to the predicted molecular mass of Asp1p (Figure 8A, lane 2). This protein was not detected in lysates prepared from the *asp1-Δ1* mutant (Figure 8A, lane 1) and was increased in abundance in wild-type cells carrying high-copy plasmid-borne *asp1*$^+$ (Figure 8A, lane 3). In *asp1-Δ1* cells expressing the *asp1*$^+$ ORF under control of the thiamine-repressible *nmt1* promoter, the ~110-kD protein was present in small amounts when the promoter was repressed (Figure 8A, lane 4) and in large amounts when the promoter became induced (Figure 8A, lane 5). Thus, anti-Asp1p antibodies specifically recognize Asp1p in *S. pombe* cell lysates. We noted in these experiments that overproduction of Asp1p did not affect growth or morphology of wild-type cells (data not shown).

In glycerol gradient analysis, Arp3p sediments as one member of a high molecular weight complex (McCollum *et al.* 1996). To determine whether Asp1p might associate with Arp3p in the same high molecular weight complex, lysates were prepared from wild-type cells and subjected to glycerol gradient centrifugation. Fractions collected from gradients were resolved by SDS-PAGE, blotted to membranes, then probed with either anti-Arp3p or anti-Asp1p antibodies. Arp3p and Asp1p did not cosediment (Figure 8B). Consistent with previous results (McCollum *et al.* 1996), Arp3p sedimented as a member of a high molecular weight complex, peaking in fraction 7 of the gradient. In contrast, Asp1p peaked in fraction 12 of the gradient. Because Arp3p and Asp1p also failed to coimmunoprecipitate (data not shown), we conclude that Asp1p does not stably associate with the Arp2/3 complex. Interestingly, the sedimentation peak of Asp1p was deeper in the gradient that would

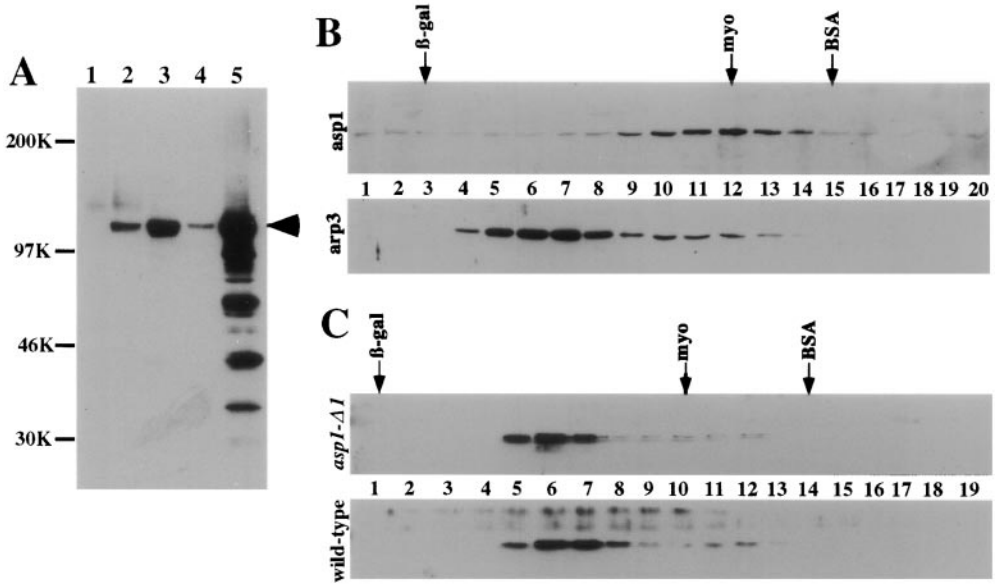

Figure 8.—Asp1p does not associate stably with the Arp2/3 complex. (A) Characterization of anti-Asp1p antibody. Lysates were prepared from the following strains: *asp1-Δ1* (lane 1); wild type (lane 2); *asp1-Δ1* carrying on the multicopy plasmid pUR19asp1 (lane 3); *asp1-Δ1* carrying pREP1asp1 grown in the presence of thiamine, promoter repressed (lane 4); or *asp1-Δ1* carrying pREPasp1 grown in the absence of thiamine, promoter induced (lane 5). Lysates were resolved by SDS-PAGE, then immunoblotted with anti-Asp1p serum. (B) Asp1p is not present in the Arp3p high molecular weight complex. Lysates prepared from wild-type cells grown at 32° were subjected to glycerol gradient sedimentation. Fractions of the gradient were immunoblotted using anti-Asp1p antibodies (top) or anti-Arp3p antibodies (bottom). (C) The sedimentation profile of Arp3p is not affected by loss of Asp1p function. Lysates prepared from *asp1-Δ1* or wild-type cells, both grown at 36° for 18 hr, were subjected to glycerol gradient sedimentation. Fractions were resolved by SDS-PAGE and immunoblotted using anti-Arp3p antibodies. In both B and C, fractions were collected from the bottom of the gradient (fraction 1), and fraction numbers are indicated between the panels. The peak fractions of the molecular weight standards β-galactosidase (β-gal, 464 kD), myosin (myo, 200 kD), and bovine serum albumin (BSA, 69 kD) in gradients prepared and centrifuged in parallel are indicated above the panels.

be expected for monomeric Asp1p. Hence, Asp1p might exist as a dimer or in a complex with another protein(s).

We also entertained the possibility that Asp1p might be important for the stabilization of the Arp2/3 complex. To test this notion, glycerol gradient analysis was performed on lysates prepared from *asp1-Δ1* or wild-type cells grown at 36°. The Arp3p sedimentation profile from *asp1-Δ1* cells was indistinguishable from wild-type cells (Figure 8C), indicating that the morphological defects of *asp1-Δ1* cells are not attributable to the destabilization of the Arp2/3 complex.

Finally, we examined the intracellular distribution of Asp1p by indirect immunofluorescence. Affinity-purified antibodies did not detect a specific localization for Asp1p, although it appeared to be excluded from the nucleus (data not shown). Similarly, a GFP-Asp1p fusion protein that is able to rescue the *arp3*-c1 mutation was diffusely distributed throughout the cytoplasm and excluded from the nucleus (data not shown). These localization data are also consistent with our conclusion that Asp1p does not stably interact with the Arp2/3 complex.

DISCUSSION

The Arp2/3 complex is critical for the function of the actin cytoskeleton in all eukaryotes (reviewed in Machesky and Gould 1999). In this report, we identified a gene, which we have termed *asp1*$^+$, that interacts with mutants in components of this complex in *S. pombe* cells. *asp1*$^+$ was identified on the basis of its ability to rescue the cold-sensitive *arp3*-c1 mutant when overexpressed. Our analysis of *asp1*$^+$ indicated that it is important, but not essential, for normal cortical actin function.

The deletion of *asp1*$^+$ produces viable cells that have disorganized actin patches and morphological abnormalities at elevated or lowered temperatures (36° or 19°, respectively). *asp1-Δ1* cells were rounded or otherwise misshapen at these temperatures and had difficulty undergoing cell division as measured by their enhanced septation index. *asp1-Δ1* cells also lysed at high rates. By analyzing the phenotypes of *asp1-Δ1* cells that were synchronized in G_1 by nitrogen starvation and then released into rich medium, we were able to attribute the lysis phenotype to their difficulty in undergoing cell division as we did not observe cells lysing at any other stage of the cell cycle. Of the cells that lysed, the width of the cell at the time of septation was greater than that of wild-type cells. Perhaps the extra time and/or materials required to form a wider septum and/or new cell wall was insufficient in these circumstances, and cell lysis resulted. Another conclusion drawn from the nitrogen starvation and release experiment was that Asp1p is important for the establishment of the normal rod shape of *S. pombe* cells. After return to rich media following rounding induced by nitrogen starvation, a significant fraction of *asp1-Δ1* cells resumed growth as spherical or pear-shaped cells and did not adopt the typical cylindrical shape. All of these observed alterations in cell shape could have been due to defects in

the actin cytoskeleton, the cell wall, or the microtubule cytoskeleton (reviewed in Nurse 1994). We found that the cell wall of the *asp1-Δ1* strain was no more sensitive to lysing enzymes than a wild-type strain, and that the microtubule cytoskeleton of *asp1-Δ1* cells appeared normal (data not shown). However, the actin cytoskeleton of *asp1-Δ1* cells was disorganized, and thus a defective actin cytoskeleton is most likely responsible for the alterations in *asp1-Δ1* cell morphology.

Other mutants defective in cortical actin function, including the *S. cerevisiae* Arp2 mutant, exhibit defects in the process of endocytosis (Moreau *et al.* 1997). These observations prompted us to examine this process qualitatively in *asp1-Δ1* cells compared to wild-type cells. We used the uptake of FM 4-64 as a measure of endocytosis. This dye is taken up by fluid-phase endocytosis and stains the vacuolar membrane rather than the vacuolar compartment (Vida and Emr 1995). In *asp1-Δ1* cells at 36°, the uptake of FM 4-64 was slower than in wild-type cells, indicating a defect in endocytosis in this strain.

An observation supporting the proposed role of Asp1p in cortical actin function was that *asp1-Δ1* cells were very sensitive to additional salts, particularly $CaCl_2$, in the medium. Sensitivity to ionic conditions has been observed previously for actin mutants in *S. cerevisiae* (Wertman *et al.* 1992). Further, as discussed in Jackson and Heath (1993) and Janmey (1994), it is well-known that Ca^{2+} regulates the behavior of the actin cytoskeleton. Ca^{2+} ions can bind actin and alter its properties (Bertazzon *et al.* 1990; Miki 1990). They also can interact with and modulate the activity of a number of actin-binding proteins (Vandekerckhove 1990). Thus, as a protein required for normal actin function, it is not unexpected that Asp1p function might lead to a difference in the effect of Ca^{2+} on the actin cytoskeleton. Alternatively, an actin cytoskeleton defective due to loss of Asp1p might render cells more permeable and thus more sensitive to ionic conditions.

In addition to the morphological defects of the *asp1-Δ1* strain, our conclusion that Asp1p is important for the function of the actin cytoskeleton rests heavily on genetic interactions between *asp1-Δ1* and mutations affecting actin cytoskeleton function. The *asp1-Δ1* strain exhibited synthetic lethal interactions with mutations in the Arp2/Arp3 complex, *arp3*-c1 and *sop2*-1, as well as the actin mutant *act1*-48. Interestingly, the Arp2/Arp3 complex seems to be important for actin patch function but not for actin ring function, and the *act1-48* mutant is defective in actin patch but not actin ring formation (McCollum *et al.* 1999). Like the *arp3-c1* and *sop2*-1 mutations, the *asp1-Δ1* mutation specifically suppressed the *cdc3*-124 mutation at 32°. These data support the conclusion the Asp1p may play a role in the function of actin patches, perhaps in conjunction with the Arp2/Arp3 complex. Despite these strong genetic interactions, we must point out that Asp1p might be influencing the function of the actin cytoskeleton indirectly because we have no evidence that Asp1p interacts physically with the Arp2/3 complex, actin, or profilin. Further, antibodies to Asp1p do not stain any specific structure within the cell that we have been able to detect; Asp1p appears to be diffusely distributed throughout the cytoplasm (data not shown). Thus, to understand the mechanism whereby Asp1p influences the actin cytoskeleton, it will be important in the future to identify proteins with which it interacts directly. It might also be informative to learn where Asp1p homologs are located in larger eukaryotic cells where more detail can be visualized.

The *asp1*$^+$ gene encodes a protein with predicted molecular mass of 106 kD. It does not contain motifs of known function, although it does contain a region predicted to form a coiled-coil domain. Although we have not tested this directly, Asp1p might oligomerize through this domain, because we found that Asp1p sediments at a position in glycerol gradients consistent with the size of a dimer rather than the size of a monomer. Although its sequence provides no clues as to its function, Asp1p is a highly conserved protein. In higher eukaryotes, homologs of *asp1*$^+$ were identified as uncharacterized human and *C. elegans* ORFs. We also identified ORF YLR410w in the *S. cerevisiae* genome database because it is predicted to encode a protein that is 55% identical to Asp1p if the predicted N-terminal extension of the *S. cerevisiae* protein is ignored. We termed this ORF *VIP1*. Unlike the deletion of *asp1*$^+$, the disruption of *VIP1* caused no discernible phenotype. Because we do not detect other proteins closely related to Vip1p in the *S. cerevisiae* genome database that might perform a redundant function, we predict that Vip1p plays a less important role in the cortical actin cytoskeleton of *S. cerevisiae*, than Asp1p does in *S. pombe*. However, Vip1p most likely contributes to cortical actin function in *S. cerevisiae*, because the *vip1* disruption mutant displayed a negative genetic interaction with a deletion mutant of *las17/bee1*. Las17p/Bee1p is important for cortical actin function (Li 1997; Karpova *et al.* 1998), particularly endocytosis (Naqvi *et al.* 1998), and interacts with another protein important for endocytosis and actin function, End5p (Naqvi *et al.* 1998). It will be interesting to determine whether Asp1p shows physical or genetic interactions with the homologs of these proteins in *S. pombe*.

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