Asp1, a Conserved 1/3 Inositol Polyphosphate Kinase, Regulates the Dimorphic Switch in *Schizosaccharomyces pombe*[▽]

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The ability to undergo dramatic morphological changes in response to extrinsic cues is conserved in fungi. We have used the model yeast *Schizosaccharomyces pombe* to determine which intracellular signal regulates the dimorphic switch from the single-cell yeast form to the filamentous invasive growth form. The *S. pombe* Asp1 protein, a member of the conserved Vip1 1/3 inositol polyphosphate kinase family, is a key regulator of the morphological switch via the cAMP protein kinase A (PKA) pathway. Lack of a functional Asp1 kinase domain abolishes invasive growth which is monopolar, while an increase in Asp1-generated inositol pyrophosphates (PP) increases the cellular response. Remarkably, the Asp1 kinase activity encoded by the N-terminal part of the protein is regulated negatively by the C-terminal domain of Asp1, which has homology to acid histidine phosphatases. Thus, the fine tuning of the cellular response to environmental cues is modulated by the same protein. As the *Saccharomyces cerevisiae* Asp1 ortholog is also required for the dimorphic switch in this yeast, we propose that Vip1 family members have a general role in regulating fungal dimorphism.

Eucaryotic cells are able to define and maintain a particular cellular organization and thus cellular morphology by executing programs modulated by internal and external signals. For example, signals generated within a cell are required for the selection of the growth zone after cytokinesis in the fission yeast *Schizosaccharomyces pombe* or the emergence of the bud in *Saccharomyces cerevisiae* (37, 44, 81). Cellular morphogenesis is also subject to regulation by a wide variety of external signals, such as growth factors, temperature, hormones, nutrient limitation, and cell-cell or cell-substrate contact (13, 34, 66, 75, 81). Both types of signals will lead to the selection of growth zones accompanied by the reorganization of the cytoskeleton.

The ability to alter the growth form in response to environmental conditions is an important virulence-associated trait of pathogenic fungi which helps the pathogen to spread in and survive the host's defense system (7, 32). Alteration of the growth form in response to extrinsic signals is not limited to pathogenic fungi but is also found in the model yeasts *S. cerevisiae* and *S. pombe*, in which it appears to represent a foraging response (1, 24).

The regulation of polarized growth and the definition of growth zones have been studied extensively with the fission yeast *S. pombe*. In this cylindrically shaped organism, cell wall biosynthesis is restricted to one or both cell ends in a cell cycle-regulated manner and to the septum during cytokinesis (38). This mode of growth requires the actin cytoskeleton to direct growth and the microtubule cytoskeleton to define the growth sites (60). In interphase cells, microtubules are organized in antiparallel bundles that are aligned along the long

axis of the cell and grow from their plus ends toward the cell tips. Upon contact with the cell end, microtubule growth will first pause and then undergo a catastrophic event and microtubule shrinkage (21). This dynamic behavior of the microtubule plus end is regulated by a disparate, conserved, microtubule plus end group of proteins, called the +TIPs. The +TIP complex containing the EB1 family member Mal3 is required for the delivery of the Tea1-Tea4 complex to the cell tip (6, 11, 27, 45, 77). The latter complex docks at the cell end and recruits proteins required for actin nucleation (46, 76). Thus, the intricate cross talk between the actin and the microtubule cytoskeleton at specific intracellular locations is necessary for cell cycle-dependent polarized growth of the fission yeast cell.

The intense analysis of polarized growth control in single-celled *S. pombe* makes this yeast an attractive organism for the identification of key regulatory components of the dimorphic switch. *S. pombe* multicellular invasive growth has been observed for specific strains under specific conditions, such as nitrogen and ammonium limitation and the presence of excess iron (1, 19, 50, 61).

Here, we have identified an evolutionarily conserved key regulator of the *S. pombe* dimorphic switch, the Asp1 protein. Asp1 belongs to the highly conserved family of Vip1 1/3 inositol polyphosphate kinases, which is one of two families that can generate inositol pyrophosphates (PP) (17, 23, 42, 54). The inositol polyphosphate kinase IP6K family, of which the *S. cerevisiae* Kcs1 protein is a member, is the "classical" family that can phosphorylate inositol hexakisphosphate (IP₆) (70, 71). These enzymes generate a specific PP-IP₅ (IP₇), which has the pyrophosphate at position 5 of the inositol ring (20, 54). The Vip1 family kinase activity was unmasked in an *S. cerevisiae* strain with *KCS1* and *DDP1* deleted (54, 83). The latter gene encodes a nudix hydrolase (14, 68). The mammalian and *S. cerevisiae* Vip1 proteins phosphorylate the 1/3 position of the inositol ring, generating 1/3 diphosphoinositol pentakisphosphate (42). Both enzyme families collaborate to generate IP₈ (17, 23, 42, 54, 57).

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TABLE 1. Strains used in this study

Name	Genotype	Source
UFY851	<i>h⁻ mal3-1 ade6-M210 leu1-32 ura4-D6 Ch¹⁶[ade6-M216]</i>	U. Fleig
UFY1156	<i>h⁻ asp1Δ::Kan^r his3-D1 ade6-M216 leu1-32 ura4-D18</i>	This study
UFY1511	<i>h⁺ asp1^{D333A}/Kan^r his3-D1 ade6-M210 leu1-32 ura4-D18</i>	This study
UFY1579	<i>h⁺ asp1^{H397A}/Kan^r his3-D1 ade6-M210 leu1-32 ura4-D18</i>	This study
UFY1639	<i>h⁺ asp1^{H397A}/Kan^r arp3-c1 his3⁻ ade6-M216 leu1-32 ura4⁻</i>	This study
UFY1722	<i>h⁻ asp1^{H397A}/Kan^r sop2-1 ade6-M210 leu1-32 ura4-D18</i>	This study
UFY1640	<i>h⁻ asp1^{H397A}/Kan^r act1-48 leu1-32 ura4-D18 lys1-131</i>	This study
UFY1731	<i>h⁺ asp1^{H397A}/Kan^r sty1Δ::ura4⁺ ade6-M210 leu1-32 ura4-D18</i>	This study
UFY1729	<i>h⁻ aps1Δ::his3⁺ his3-D1 ade6-M210 leu1-32 ura4-D18</i>	This study
UFY1725	<i>h⁺ asp1^{H397A}/Kan^r pmk1Δ::ura4⁺ leu1⁻ ura4⁻</i>	This study
UFY1580	<i>h⁺ asp1^{D333A}-GFP/ura4⁺ his3-D1 ade6-M210 leu1-32 ura4-D18</i>	This study
UFY1521	<i>h⁻ asp1^{H397A}-GFP/ura4⁺ his3-D1 ade6-M216 leu1-32 ura4-D18</i>	This study
UFY1771	<i>h⁻ asp1-pkGFP/ura4⁺ his3-D1 ade6-M210 leu1-32 ura4-D18</i>	This study
UFY1158	<i>h⁻ mal3Δ::his3⁺ his3⁻ ade6-M210 leu1-32 ura4-D18</i>	This study
UFY1641	<i>h⁻ asp1^{H397A}/Kan^r mal3Δ::his3⁺ his3⁻ ade6-M210 leu1-32 ura4-D18</i>	This study
UFY1724	<i>h⁻ asp1^{H397A}/Kan^r tea2Δ::his3⁺ his3-D1 ade6-M210 leu1-32 ura4-D18</i>	This study
UFY1774	<i>h⁻ asp1^{H397A}/Kan^r tea1Δ::ura4⁺ ade6-M210 leu1-32 ura4-D18</i>	This study
UFY1723	<i>h⁻ asp1^{H397A}/Kan^r peg1-1 his3-D1 leu1-32 ura4-D18</i>	This study
UFY1765	<i>h⁻ asp1^{H397A}/Kan^r gpa2Δ::ura4⁺ his3-D1 ade6-M210 leu1⁻ ura4-D18</i>	This study
UFY1766	<i>h⁺ asp1^{H397A}/Kan^r cyr1Δ::ura4⁺ his3-D1 ade6-M216 leu1⁻ ura4-D18</i>	This study
UFY1767	<i>h⁺ asp1^{H397A}/Kan^r cdc8-110 his3-D1 ade6-M219 leu1-32 ura4-D18</i>	This study
UFY1780	<i>MATa/MATα ura3-52/ura3-52 vip1Δ::Kan^r/VIP1</i>	This study
UFY1781	<i>MATa ura3-52 vip1Δ::Kan^r</i>	This study
UFY1788	<i>MATa/MATα ura3-52/ura3-52 kcs1Δ::Kan^r/KCS1</i>	This study
KG425	<i>h⁻ his3-D1 ade6-M210 leu1-32 ura4-D18</i>	K. Gould
KG460	<i>h⁺ arp3-c1 his3-237 leu1-32 ura4-D18</i>	K. Gould
KG480	<i>h⁻ sop2-1 ade6-M210 leu1-32 ura4-D18</i>	K. Gould
KG41010	<i>h⁻ act1-48 leu1-32 ura4-D18 lys-131</i>	K. Gould
YUG94	<i>MATa ura3-52</i>	J. H. Hegemann
CGX31	<i>MATa/MATα ura3-52/ura3-52</i>	J. H. Hegemann
IH1563	<i>h⁻ peg1-1 leu1-32 ura4-D18</i>	I. Hagan
	<i>h⁻ tea2Δ::his3⁺ his3-D1 ade6-M210 leu1-32 ura4-D18</i>	J. McIntosh
TP319-31A	<i>h⁺ sty1Δ::ura4⁺ leu1-32 ura4-D18</i>	J. Millar
PN1687	<i>h⁻ pmk1Δ::ura4⁺ leu1⁻ ura4⁻</i>	T. Toda
FY7092	<i>h⁻ tea1Δ::ura4⁺ ura4-D18</i>	F. Verde
FY7461	<i>h⁹⁰ gpa2Δ::ura4⁺ leu1⁻ ura4-D18</i>	YGRC
FY7728	<i>h⁺ cyr1Δ::ura4⁺ ade6-M216 leu1⁻ ura4-D18</i>	YGRC
FY12017	<i>h⁺ spk1Δ::ura4⁺ leu1⁻ ura4⁻</i>	YGRC
	<i>h⁹⁰ cdc8-110 leu1-32 ura4-D18</i>	YGRC

Two modes of action have been described for the high-energy moiety containing inositol pyrophosphates. First, these molecules can phosphorylate proteins by a nonenzymatic transfer of a phosphate group to specific prephosphorylated serine residues (2, 8, 69). Second, inositol pyrophosphates can regulate protein function by reversible binding to the *S. cerevisiae* Pho80-Pho85-Pho81 complex (39, 40). This cyclin-cyclin-dependent kinase complex is inactivated by inositol pyrophosphates generated by Vip1 when cells are starved of inorganic phosphate (39, 41, 42).

Regulation of phosphate metabolism in *S. cerevisiae* is one of the few roles specifically attributed to a Vip1 kinase. Further information about the cellular function of this family came from the identification of the *S. pombe* Vip1 family member Asp1 as a regulator of the actin nucleator Arp2/3 complex (22). The 106-kDa Asp1 cytoplasmic protein, which probably exists as a dimer *in vivo*, acts as a multicopy suppressor of *arp3-c1* mutants (22). Loss of Asp1 results in abnormal cell morphology, defects in polarized growth, and aberrant cortical actin cytoskeleton organization (22).

The Vip1 family proteins have a dual domain structure which consists of an N-terminal “rimK”/ATP-grasp superfamily

domain found in certain inositol signaling kinases and a C-terminal part with homology to histidine acid phosphatases present in phytase enzymes (28, 53, 54). The N-terminal domain is required and sufficient for Vip1 family kinase activity, and an Asp1 variant with a mutation in a catalytic residue of the kinase domain is unable to suppress mutants of the Arp2/3 complex (17, 23, 54). To date, no function has been described for the C-terminal phosphatase domain, and this domain appears to be catalytically inactive (17, 23, 54).

Here we describe a new and conserved role for Vip1 kinases in regulating the dimorphic switch in yeasts. Asp1 kinase activity is essential for cell-cell and cell-substrate adhesion and the ability of *S. pombe* cells to grow invasively. Interestingly, Asp1 kinase activity is counteracted by the putative phosphatase domain of this protein, a finding that allows us to describe for the first time a function for the C-terminal part of Vip1 proteins.

MATERIALS AND METHODS

Strains and media. All strains used are listed in Table 1. New strains were obtained by crossing the appropriate strains and subjecting them to tetrad/random spore analysis or transformation. *S. pombe* strains were grown in rich

media (YE5S) or minimal media (MM) with supplements (52). To repress or derepress, the *nut1*⁺ promoter cells were grown in MM plus 5 μ g/ml thiamine or for 18 h at 30°C in thiamine-less MM, respectively. *S. cerevisiae* strains (Σ 1278b background) were grown in YPD or SLAHD (25, 74). Gene deletions were done by PCR-based gene targeting (4) using the kanamycin resistance (Kan^r) cassette and plating on 100 mg/ml G418 YE5S, or the *his3*⁺ selection marker. 8-Br-cAMP was added to YE5S plates. Unless otherwise indicated, experiments were carried out at 30°C. Sporulation and tetrad analysis of diploid *S. cerevisiae* strains confirmed the heterozygous deletion of *VIP1* or *KCS1*.

Generation of *asp1* mutant strains and plasmids *pasp1*¹⁻³⁶⁴ and *pasp1*³⁶⁵⁻⁹²⁰. A pBSK-based 4,292-bp-long DNA fragment containing wild-type *asp1*⁺, *asp1*^{D333A}, or *asp1*^{H397A} followed by the Kan^r cassette was transformed into strain KG425, thereby replacing the endogenous *asp1*⁺ open reading frame (ORF) and generating *asp1*⁺/Kan^r, *asp1*^{D333A}/Kan^r, and *asp1*^{H397A}/Kan^r strains (*asp1*^{D333A} and *asp1*^{H397A} DNA were a gift from John D. York, Duke University). Correct integration was verified by PCR and sequence analysis. To obtain endogenous *asp1*-*gfp* fusion variants, the Kan^r cassette of the appropriate strain was exchanged with the *gfp/ura4*⁺ cassette. For construction of *pasp1*¹⁻³⁶⁴ and *pasp1*³⁶⁵⁻⁹²⁰, a PCR fragment containing the first 1,092 bp and the last 1,671 bp of the *asp1*⁺ ORF, respectively, was cloned into NotI- and XhoI-cut pJR2-3XL (51). Expression of plasmid-encoded *asp1* variants was approximately 20-fold higher than that of endogenous *asp1*⁺. For expression of the Asp1 kinase domain in *vip1* Δ /*VIP1* cells, *asp1*¹⁻³⁶⁴ was cloned into a *CEN* plasmid under the control of the *MET25* promoter.

Protein analysis. Protein extracts were prepared as described previously (35). Blots were probed with anti-green fluorescent protein (anti-GFP) antibody (polyclonal rabbit; Invitrogen) followed by alkaline phosphatase (AP)-conjugated secondary antibody (affinity-purified goat anti-rabbit IgG [Fc]; Promega, Madison, WI) and detected with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) as the substrate.

Invasive growth assays. A total of 5 μ l containing 10⁵ cells or 1 μ l containing 10⁶ cells of logarithmically grown overnight (o/n) cultures were spotted on 2% YE5S agar plates or 0.3% and 2% YE5S agar plates, respectively, and incubated. Adhesive and invasive growth assays have been described (61). As the number of invasively growing colonies per strain was dependent on the incubation temperature and the position of the cell spot on the agar plate, we always used an incubation temperature of 30°C and ensured that only cell spots plated on the same position on an agar plate were compared with each other. Quantification of invasive growth was done by determining the number of invasive colonies per strain in at least 3 different experiments.

Enzymatic cell wall degradation assay. An assay to determine the sensitivity to enzymatic cell wall degradation has been described (58).

Microscopy. Cells were plated and grown o/n (surface growth) or spotted and grown for 20 days (invasive growth) on YE5S agar plates. Two-millimeter-thick agar blocks were cut out and placed in a temperature-controlled microscope chamber at 30°C (Bioptechs Inc., Butler, PA). Differential interference contrast (DIC) images were obtained with a Zeiss Axiovert200 fluorescence microscope (Carl Zeiss, Jena, Germany) coupled to a charge-coupled-device camera (ORCA-ER; Hamamatsu, Bridgewater, NJ) and Openlab imaging software (Improvision, Coventry, United Kingdom). Photomicrographs of invasively growing or surface colonies were taken with a Zeiss Axioskop.

RESULTS

The Asp1 kinase regulates cell-cell adhesion. We isolated Asp1 as a multicopy suppressor of the thiabendazole (TBZ) hypersensitivity of a *mal3* mutant strain, as overexpression of an Asp1 variant increased the tolerance towards TBZ (Fig. 1). During our experimental analysis of Asp1 variants, we noticed that expression of certain Asp1 mutants led to a severe flocculation phenotype, implying that Asp1 is involved in cell-cell adhesion. We used strains that expressed wild-type Asp1 or endogenous variants with a single point mutation in either the Asp1 kinase domain (Asp1^{D333A}, Asp1 kinase dead) or the Asp1 phosphatase domain (Asp1^{H397A}, Asp1 kinase only) (Fig. 2A). The D333 amino acid has been identified as a key catalytic residue for Asp1 kinase activity, while H397 was classified as a highly conserved histidine residue of the putative acid phosphatase domain (54). Both Asp1 variants were expressed

FIG. 1. Asp1 kinase activity increases resistance towards TBZ. (A) Diagrammatic representation of wild-type Asp1 (top) and the Asp1 variant that rescues the *mal3* mutant TBZ phenotype. Note that the conserved histidine residue H807 is not present in Asp1¹⁻⁷⁹⁴. (B) Serial dilution patch test of a *mal3* mutant strain expressing *asp1*¹⁻⁷⁹⁴ or *mal3*⁺. Cells were spotted on plasmid-selective plates containing 8 μ g/ml TBZ and incubated for 7 days at 24°C. (C) Wild-type-, *asp1*^{D333A}-, or *asp1*^{H397A}-expressing cells were grown on YE5S containing 9 μ g/ml TBZ for 5 days at 24°C.

from the endogenous *asp1*⁺ promoter and were generated by replacing the wild-type copy of the *asp1*⁺ ORF (see Materials and Methods). As shown by Western blot analysis, the Asp1^{D333A} and Asp1^{H397A} proteins are present in amounts similar to that of the wild-type Asp1 protein (Fig. 2B).

The strain expressing the Asp1 kinase-only variant Asp1^{H397A} flocculated strongly when the cells were grown in rich medium, and this cell-cell adhesion was dependent on the presence of Ca²⁺ and galactose residues present in cell wall glycoproteins (Fig. 2C; data not shown). Flocculation was seen most prominently when cells reached stationary phase, implying that it is cell density dependent. The incubation temperature or the growth medium used did not alter the flocculation phenotype (data not shown). Flocculation was observed always for the Asp1^{H397A}-expressing strain and rarely for the isogenic wild-type strain, but not for the Asp1^{D333A} (Asp1 kinase-dead)-expressing strain or a strain in which the entire *asp1*⁺ gene had been deleted (*asp1* Δ strain) (Fig. 2C).

Our results indicated that the presence of a functional Asp1 kinase domain was required for cell-cell adhesion. We therefore tested and found that plasmid-borne overexpression of Asp1^{H397A} induced flocculation in an *asp1* Δ strain (Fig. 2D). In addition, overexpression of the Asp1 variant Asp1¹⁻³⁶⁴, which contains solely the Asp1 kinase domain (Fig. 2A), resulted in flocculating *asp1* Δ mutant cells (Fig. 2D).

If increased amounts of Asp1-generated inositol pyrophosphates lead to cell-cell adhesion and flocculation, then increasing the intracellular level of these molecules by elimination of a protein that degrades them should give rise to flocculation in wild-type cells. It has been shown that disruption of the *S. pombe* *aps1*⁺ ORF, which encodes a nudix hydrolase, results in a 3-fold increase in the intracellular concentration of IP₇ (33).

FIG. 2. Expression of the Asp1 kinase-only variant Asp1^{H397A} leads to flocculation. (A) Diagrammatic representation of Asp1 variants. The Asp1 kinase domain and the putative histidine acid-phosphatase domain are indicated by black and gray boxes, respectively. The changed amino acid in the Asp1 kinase-dead variant (Asp1^{D333A}) and the Asp1 kinase-only variant (Asp1^{H397A}) is indicated. (B) Western blot analysis of strains expressing GFP-tagged endogenous versions of the indicated Asp1 variants. Similar amounts of protein extracts were resolved by SDS-PAGE and probed with an anti-GFP antibody. Asp1-Pk1-GFP runs at 143 kDa (expected, 137 kDa), while the two mutant Asp1-GFP variants run at 137 kDa (expected, 133 kDa). Control, wild-type strain without GFP-tagged *asp1*⁺. (C) The indicated strains were grown to stationary phase in rich medium at 30°C. (D) The *asp1Δ* strain transformed with a vector control or plasmids overexpressing *asp1*^{H397A}, *asp1*¹⁻³⁶⁴ or *asp1*⁺ were grown in plasmid-selective minimal medium at 30°C. (E) The *aps1*⁺-deleted strain was grown to stationary phase in rich medium at 30°C.

We therefore tested if an *aps1Δ* strain showed flocculation and found that this was the case (Fig. 2E).

Increased flocculation was observed only when the Asp1 kinase domain was overexpressed or when cells expressed Asp1^{H397A}, in which a highly conserved histidine residue of the putative acid phosphatase domain has been mutated (Fig. 2C and D). This finding implies that (i) the phosphatase domain has a biological function, although no enzymatic activity has been reported, and (ii) the presence of a wild-type phosphatase domain exerts a negative effect on the kinase domain function either directly or indirectly.

The Asp1 kinase activity is required for invasive, pseudohypha-like growth. Although the input signals are different for morphogenic events like flocculation, invasive growth, and filamentation, these processes all require adhesion to a substrate (30, 80). We therefore tested if Asp1 also played a role in the adhesion of cells to surfaces such as agar and in invasive, filamentous growth (29). To this end wild-type, *asp1*^{H397A}, *asp1*^{D333A}, and *asp1Δ* cells were spotted on rich media. After 20 days of incubation at 30°C (Fig. 3A, top), we tested if cells could adhere to the agar by rinsing them with water. We found that the wild-type and *asp1*^{H397A} strains were able to adhere to the agar, although the Asp1 kinase-only mutant showed significantly increased adhesion compared to the *asp1*⁺ strain (Fig. 3A, middle). In contrast, the *asp1*^{D333A} and the *asp1Δ* strains showed no adhesion to the agar. These data imply that fission yeast cells are able to adhere to a substrate and that this adhesion requires Asp1 kinase activity.

We next tested invasion into the agar by vigorously rubbing cells off the agar (29). Only the *asp1*^{H397A} strain showed massive invasion into the agar (Fig. 3A, bottom). The wild-type

strain was also able to invade a solid substratum, although at a much-reduced frequency compared to the *asp1*^{H397A} strain. The latter gave rise to approximately 30 times more invasively growing colonies of the type shown in Fig. 3B (Fig. 3F). Invasion into the agar was first observed after 8 days of incubation at 30°C and occurred at the edges of the cell spots possibly because by that time nutrients were available only at the edge of the colony. Microscopic analysis of invasively growing cells revealed that they formed long filament-like structures, which grew away from the colony and consisted of chains of cells separated by septa (Fig. 3B). We named these structures pseudohyphae. Incubating *S. cerevisiae* cells on low concentrations of agar (0.3%) triggers a form of growth observed in biofilms, namely, the formation of a confluent mat of cells (64). We tested the growth behavior of the *S. pombe* strains under such conditions and found that wild-type cells did not form mats of cells but instead showed massively invasive growth into the semisolid agar (Fig. 3C, bottom). The *asp1*^{H397A} strain showed an even stronger invasive growth phenotype than the wild-type strain (Fig. 3C). The center of the colonies consisted of invasively growing cells, while long feather-like structures arose from the edges of the colonies. Again, Asp1 kinase activity was required for invasive growth on 0.3% agar plates, as the *asp1*^{D333A} and the *asp1Δ* strains were unable to grow invasively, even under such favorable conditions (Fig. 3C).

Our data suggest that (i) Asp1 kinase activity and therefore, presumably, specific IP₇ isomers or IP₈ regulate pseudohypha-like, invasive growth and that (ii) increased Asp1 kinase activity or loss of the negative influence of the C-terminal phosphatase domain results in a stronger invasive growth phenotype.

FIG. 3. Asp1 kinase activity is needed for pseudohypha-like invasive growth. (A) A total of 10^5 wild-type, *asp1^{H397A}*, *asp1^{D333A}*, and *asp1Δ* cells were spotted on rich medium and incubated for 20 days at 30°C (top), and the plates were rinsed with water (middle), and the remaining cells were rubbed off (bottom). (B) Photomicrographs of invasively growing colonies show pseudohyphae that grow away from the colony and consist of chains of cells (inset). White bar, 20 μm; black bar, 10 μm. (C) Growth on semisolid 0.3% agar. A total of 10^6 wild-type, *asp1^{H397A}*, *asp1^{D333A}*, or *asp1Δ* cells were spotted on rich medium with 2% agar (top) or 0.3% agar, pH 7 (bottom), and incubated for 13 days at 30°C. (D) *asp1Δ* cells overexpressing *asp1^{H397A}* (with thiamine) or *asp1¹⁻³⁶⁴* (without thiamine) were grown on plasmid-selective minimal medium for 20 days at 30°C before rinsing and rubbing off the plates. (E) The *aps1Δ* strain was treated as described in the legend to panel A. (F) Number of invasive colonies formed by the indicated strains were counted and set to 1 for the *asp1^{H397A}* strain. **, $P < 0.01$, or *, $P < 0.05$ for *asp1^{H397A}* or *aps1Δ* strains, respectively, compared to wild-type strain as determined using Welch test. (G) Wild-type cells were grown on YE5S with or without NaF for 20 days, and the number of invasive colonies was determined. *, $P < 0.05$ for 1 mM NaF compared to 5 mM NaF as determined using Welch test. (H) *asp1^{H397A}* cells overexpressing *asp1^{D333A}* or *asp1³⁶⁵⁻⁹²⁰* were grown on plasmid-selective minimal medium for 20 days at 30°C and photographed before and after rinsing the plates.

To test if increased Asp1 kinase activity was responsible for increasing the ability of cells to grow in a pseudohypha-like, invasive manner, we (i) overexpressed the Asp1¹⁻³⁶⁴ and Asp1^{H397A} variants in the *asp1Δ* strain and (ii) assayed invasive growth in the *aps1Δ* strain or in the presence of NaF, which has been shown to inhibit the activity of nudix hydrolases (67).

Overexpression of Asp1^{H397A} or Asp1¹⁻³⁶⁴ in the *asp1Δ* strain strongly increased the number of invasively growing cells compared to the vector control (Fig. 3D). In addition, loss of the nudix hydrolase Aps1 or growth on NaF-containing medium also led to increased invasive growth phenotypes (Fig. 3E, F, and G).

Taken together, these data strongly imply that the inositol pyrophosphate molecules generated by the Asp1 kinase act as regulators/signaling molecules for invasive pseudohyphal growth. The absence of the C-terminal Asp1 domain or muta-

tion of a highly conserved histidine residue in the putative phosphatase domain leads to an increase in cell-cell and cell-substrate adhesion (Fig. 2 and 3). Thus, the putative phosphatase domain appears to have a negative effect on kinase function. Overexpression of the phosphatase-only Asp1 variant Asp1³⁶⁵⁻⁹²⁰ in the Asp1^{H397A} strain indeed decreased the ability of the strain to adhere to the agar and grow invasively (Fig. 3H and data not shown).

Growth mode of invasively growing cells is predominantly monopolar. Invasive colonies of the *asp1^{H397A}* strain consisted of a densely grown center from which long chains of cells irradiated (Fig. 3B). As the growth mode of such *S. pombe* cells had not been analyzed previously, we imaged invasively growing cells using live cell microscopy (example shown in Fig. 4A). Wild-type and Asp1^{H397A}-expressing cells were mostly viable and grew at similar rates, with generation times of 2.1 and 2 h,

FIG. 4. Live cell analysis of invasively growing *S. pombe* cells. (A) Agar blocks containing invasively growing cells were placed in a temperature-controlled microscope chamber. Growth of cells growing at the end of pseudohyphae was monitored for up to 9 h at 30°C. The *asp1*^{H397A} cell marked with a box was monitored for 7.5 h. The progeny produced by cells growing away from the colony is shown by photomicrographs and depicted diagrammatically. Numbers indicate minutes, arrows the orientation of growth, and the asterisks a septum that did not resolve during the analysis. Bar, 5 μ m. (B) Wild-type or *asp1*^{H397A} cells were monitored as described in the legend to panel A and growth, nongrowth, or lysis of cells at the very end or within pseudohyphae was counted. (C) Example of morphological alterations in invasively growing wild-type cells. Bar, 5 μ m. (D) Cells of the indicated genotypes were treated with zymolyase for 90 min, and decrease of the optical density at 600 nm (OD_{600}) was measured to assess cell lysis. (E) Percentage of cleaved or noncleaved septa of wild-type or *asp1*^{H397A} cells that were monitored as described in the legend to panel A.

respectively (Fig. 4B). Few major morphological defects, such as round or branched cells, were observed; however, cells adjusted their cell shape to externally present constraints, such as other cells lying in their path of growth (example shown in Fig. 4C).

Growth of the pseudohyphae was not restricted to the end-most cells (1st cell, Fig. 4B), and most of the cells analyzed in a pseudohyphal structure were able to divide (Fig. 4B). Wild-type cells that either showed no growth or lysed during the analysis were present mostly within the long chains of cells. In contrast, the vast majority of nongrowing or lysing *Asp1*^{H397A} cells were

the end-most cells (Fig. 4B). The latter phenotype might be caused by changes in the cell wall composition of the *Asp1*^{H397A} strain, as this strain is hypersensitive to digestion by the beta-1,3-glucan laminaripentaohydrolase containing zymolyase (Fig. 4D). Furthermore, strong overexpression of the *Asp1*^{H397A} protein leads to cell lysis (54). Absence of *Asp1* kinase activity leads to resistance to digestion by zymolyase (Fig. 4D).

Pseudohyphal growth is facilitated by defects in cytokinesis (3). Indeed, we found that 18.5% of *Asp1*^{H397A}-expressing cells and 8% of wild-type cells formed septa that did not resolve during the course of the experiment (Fig. 4A and E).

FIG. 5. Analysis of the growth pattern of invasively growing cells. (A) Cells of the indicated strain or genotype were pregrown invasively (YE5S for 20 days at 30°C) or on the agar surface (on YE5S for 16 h at 30°C), and the growth pattern was then determined using time lapse microscopy at 30°C. Invasive growth: wild-type strain, $n = 36$; *asp1^{H397A}* strain, $n = 57$. Surface growth: wild-type strain, $n = 26$; *asp1^{H397A}* strain, $n = 61$; *asp1^{D333A}* strain, $n = 55$. **, $P < 0.01$ for wild-type and *asp1^{H397A}* strains invasively grown compared to surface grown and for *asp1^{D333A}* strain compared to wild-type strain as determined using χ^2 test. (B) Cell size at division of cells of the indicated strains grown in or on agar. Invasive growth: wild-type strain, $n = 16$; *asp1^{H397A}* strain, $n = 15$. Surface growth: wild-type strain, $n = 30$; *asp1^{H397A}* strain, $n = 42$; *asp1^{D333A}* strain, $n = 10$. (C) Selection of growth site of invasively and surface-grown strains of the indicated strain or genotype. Top row of cells denotes “parental” cells, and the growth pattern of these cells up to cell division is indicated by arrows. Bottom row of cells depicts the progeny of the upper cells and their mode of growth following cytokinesis. Numbers given are cells in that particular category. (D) Following cytokinesis, the growth zones of the end-most cell and other cells in the pseudohyphal structure were determined.

Wild-type cells that grow in liquid or on an agar surface initiate growth after cytokinesis by growing in a monopolar fashion using the old end (the end present before cell division) and then undergo bipolar growth at a transition point named NETO in G₂ phase (49). This mode of growth was dramatically altered in invasively growing cells, in which 58% of wild-type and 70.2% of the *Asp1^{H397A}* cells grew in an exclusively monopolar manner (Fig. 5A). As only 3.8% of the wild-type and 18% of the *Asp1^{H397A}* cells showed exclusive monopolar growth when grown on an agar surface, we conclude that invasive growth leads to an alteration of the normal growth pattern (Fig. 5A). Interestingly, 83.6% of surface-grown *asp1^{D333A}* cells (*Asp1* kinase-dead cells) showed monopolar growth, implying that *Asp1* kinase activity is required for the switch from mono- to bipolar growth (Fig. 5A and C). As NETO can occur only after cells have attained a minimum size, we measured the cell length at division of invasively and surface-grown cells (Fig. 5B). The invasively growing wild-type and *Asp1^{H397A}* cells had cell lengths of $11.4 \pm 1.7 \mu\text{m}$ and

$11.9 \pm 2.1 \mu\text{m}$, respectively, cell lengths significantly longer than those of the same strains grown on the agar surface (Fig. 5B). Our data imply that invasive growth leads to an alteration of the normal bipolar pattern and results in predominantly monopolar growth.

Next, we analyzed growth zone selection of invasively growing cells. The majority of invasively growing cells defined the old end as the growth zone after cytokinesis (Fig. 5C, 70% of wild-type and 63% of *Asp1^{H397A}* cells). Interestingly, all analyzed end-most cells of a pseudohyphal structure (1st cell, Fig. 5D) initiated growth at the old end. As this end is farthest away from the colony, it is possible that this growth zone is environmentally determined.

Microtubule and actin cytoskeleton in invasive growth. Proper polarized growth of surface- and/or liquid-grown *S. pombe* cells is dependent on functional actin and microtubule cytoskeletons (15, 44, 60). To determine the impact of these two cytoskeletal structures on invasive, pseudohyphal growth, we assayed the ability of a number of actin and microtubule

FIG. 6. An altered microtubule or actin cytoskeleton increases the invasive growth of *asp1^{H397A}*. (A) The wild-type strain and strains with the indicated genotypes were grown on YE5S for 20 days at 30°C (top), and the cells were washed/rubbed off (bottom). (B) Photomicrographs of invasively growing *asp1^{H397A} arp3-c1* cells. Bar, 10 μ m. (C) The indicated strains were grown at 30°C or 24°C as described in the legend to panel A, and the number of invasive colonies was counted. Invasiveness of *asp1^{H397A}* was set to 100%. At least 3 independent experiments were carried out. *, $P < 0.05$ for *mal3Δ* strain compared to wild-type strain as determined using Welch test. **, $P < 0.01$ for *asp1^{H397A}* or *tea1Δ* strains compared to wild-type strain and for *mal3Δ asp1^{H397A}*, *tea1Δ asp1^{H397A}*, *act1-48 asp1^{H397A}*, *sop2-1 asp1^{H397A}*, *arp3-c1 asp1^{H397A}*, *asp1^{H397A} + LatB*, and *cdc8-110 asp1^{H397A}* strains compared to *asp1^{H397A}* strain as determined using Welch test.

cytoskeletal mutants to grow invasively. We tested invasive growth of *mal3*, *tea1*, and *tea2* deletion strains (the *mal3Δ*, *tea1Δ*, *tea2Δ* strains) and the temperature-sensitive *peg1-1* strain at the permissive temperature (6, 10, 12, 27). The Tea2 kinesin protein is required for the transport of the kelch repeat protein Tea1 to the cell ends, where morphogenesis is controlled via the Tea1-Tea4 complex (9, 45, 77). Mal3 and Peg1 are components of the microtubule + TIP complex that regulates microtubule dynamics (6, 12, 27). Mal3 is also re-

quired for Tea1 deposition at the cell end, while Peg1 is not (9, 12, 27).

The *mal3Δ* and *tea1Δ* strains showed a higher number of agar-invading colonies than the wild-type strain, while the *peg1-1* mutant strain had a frequency of agar invasion similar to that of the wild-type strain (Fig. 6A and C and 7A). The *tea2Δ* strain did not show invasive growth after 20 days of incubation; however, a *tea2Δ* strain can grow invasively after prolonged incubation (Fig. 6C) (19). Double mutants carrying *mal3Δ*, *tea1Δ*, *tea2Δ*, *peg1-1*, or

FIG. 7. Photomicrographs of invasive growth assays. (A and B) Wild-type strain and strains with the indicated genotypes were grown for 20 days at 30°C or 24°C on YE5S (top), and the cells were washed/rubbed off (bottom).

FIG. 8. Asp1 function in invasive growth requires the cAMP PKA pathway. (A) Strains with the indicated genotypes were grown for 20 days at 30°C on YE5S (top), and the cells were washed/rubbed off (bottom). + to +++, ability, from weakest to strongest, respectively, to adhere to the agar. + represents wild-type adhesion (compare to Fig. 3A). (B) *spk1Δ* cells transformed with a vector control or plasmids expressing *asp1^{H397A}* or *asp1¹⁻³⁶⁴* were incubated on plasmid-selective agar for 20 days at 30°C and treated as described in the legend to panel A. (C) Strains with the indicated genotypes were grown and treated as described for panel A. (D) The indicated strains were grown for 13 days at 30°C on YE5S with or without 1 mM cAMP, and the cell spots were rinsed or were rinsed and rubbed off. (E) Photomicrographs of the invasively growing wild-type and *asp1^{H397A}* colonies shown in panel D.

asp1^{H397A} showed more invasively growing colonies than the single *asp1^{H397A}* mutant strain (Fig. 6A and C). However, the colonies of the double mutant strains were, in general, smaller than those of the *asp1^{H397A}* single mutant strain.

We then analyzed the impact of the actin cytoskeleton on invasive growth and found that cold-sensitive *act1* (encodes actin), *sop2* (encodes a subunit of the Arp2/3 complex), and *arp3* (encodes the Arp3 protein) mutants and the temperature-sensitive *cdc8* (encodes tropomyosin) mutant were unable to grow invasively under permissive growth conditions (Fig. 6A and C and 7B) (5, 16, 47, 48). The *asp1^{H397A} act1-48* double mutant strain showed no invasive growth (Fig. 6C). The *asp1^{H397A} sop2-1* strain was able to grow invasively, albeit at a reduced rate comparable to that of the *asp1^{H397A}* strain grown in the presence of the actin polymerization inhibitor latrunculin B (Fig. 6C). Surprisingly, the *asp1^{H397A} arp3-c1* strain had an increased number of invasively growing cells compared to the *asp1^{H397A}* single mutant strain (Fig. 6A and C). Microscopic analysis revealed that the *asp1^{H397A} arp3-c1* strain showed an increased cell separation defect, which results in very neat chains of invasively growing cells (Fig. 6B). This type of pseudohyphal growth might facilitate invasion into the agar.

Asp1-mediated invasive growth is dependent on the cyclic AMP (cAMP) protein kinase A (PKA) pathway. Environmental changes are detected and adapted to via a number of kinase signaling pathways (18). These include mitogen-activated protein (MAP) kinase pathways, such as the pheromone-responsive pathway, the Sty1/Spc1 stress response pathway, the Pmk1/Spm1 cell-integrity pathway, and the cAMP-dependent PKA pathway (26, 31, 78, 79, 84). To analyze which of these pathways were required for Asp1-mediated invasive growth, we made double mutants of *asp1^{H397A}* and *pmk1Δ* (component of cell integrity pathway) or *sty1Δ* (component of stress response pathway). The double mutants could still grow invasively, thus indicating that these pathways are not required for Asp1-induced invasive growth (Fig. 8A). Next, as the *asp1^{H397A}* strain and a *spk1* deletion strain (pheromone response pathway) could not mate, we transformed the *spk1Δ* strain with plasmids expressing Asp1^{H397A} or Asp1¹⁻³⁶⁴. We found that production of these proteins could induce increased invasive growth (Fig. 8B). However, double mutants of *asp1^{H397A}* and null alleles of genes encoding components of the protein kinase A pathway were unable to grow invasively, as shown for the *asp1^{H397A} gpa2Δ* and *asp1^{H397A} cyr1Δ* strains (Fig. 8C). *gpa2⁺* codes for

FIG. 9. *S. cerevisiae* Vip1 is required for invasive growth. (A) A total of 10^5 haploid wild-type and *vip1Δ* cells were spotted on YPD plates and grown for 9 days at 30°C (top). Plates were rinsed, and the remaining cells rubbed off (bottom). (B) A total of 10^5 diploid wild-type and *vip1Δ/VIP1* cells were incubated on SLAHD plates for 14 days at 30°C and treated as described in the legend to panel A. (C) Photomicrographs of diploid wild-type, *vip1Δ/VIP1*, and *vip1Δ/VIP1* cells expressing *S. pombe* Asp1^{1–364}. Cells were grown on SLAHD agar for 5 days. Bar, 20 μm. (D) A diploid *kcs1Δ/KCS1* strain was analyzed as described in the legend to panel B.

part of the heterotrimeric G protein, while *cyr1⁺* encodes the adenylate cyclase (31). These findings imply that the cAMP pathway is required for invasive growth. To determine if cAMP PKA acts upstream or downstream of Asp1 kinase function, we grew wild-type, *asp1^{H397A}*, and *asp1^{D333A}* strains on medium containing cAMP (Fig. 8D and E). We found that in the presence of cAMP, wild-type and *asp1^{H397A}* strains showed increased invasive growth compared to growth of these strains on cAMP-less medium, but the *asp1^{D333A}* strain was still unable to grow invasively. We conclude that the cAMP PKA pathway acts upstream of Asp1 kinase function (1, 31).

The Vip1 family also regulates invasive pseudohyphal growth in *S. cerevisiae*. To determine if the Vip1 family function, with respect to invasive growth, was conserved, we generated *S. cerevisiae* haploid and diploid strains in which the *VIP1* gene was deleted and tested these strains in invasive growth assays (25, 65). The haploid *vip1Δ* strain showed a moderately reduced ability to grow invasively compared to the isogenic wild-type strain, implying that Vip1 activity contributes to but is not essential for haploid invasive growth (Fig. 9A). Next, we tested the requirement of Vip1 in diploid pseudohyphal growth. As a homozygous *vip1Δ/vip1Δ* strain showed growth defects on our test medium, we assayed the ability of the heterozygous *vip1Δ/VIP1* strain to grow in an invasive fashion on the appropriate SLAHD medium (25). Loss of one *VIP1* copy and thus a presumably 50% reduction in Vip1 protein levels severely reduced the ability of this strain to grow in a pseudohyphal manner (Fig. 9B and C). Expression of the *S. pombe* Asp1^{1–364} variant in this strain restored the ability to grow invasively (Fig. 9C). Thus, the *S. pombe* Asp1 and *S. cerevisiae* Vip1 proteins

FIG. 10. Model for the regulation of invasive growth by Asp1. (A) Increased Asp1 kinase activity leads to increased invasive growth phenotype. (B) Asp1 kinase activity is regulated negatively by the Asp1 phosphatase domain. The latter domain might have an enzymatic activity that uses the Asp1 kinase substrate. Alternatively, it might regulate downstream targets of Asp1 kinase. Active Asp1 kinase leads to the production of IP₇ (or IP₈), which regulates a number of pathways required for invasive growth.

are functional orthologs, and the role of the Vip1 family in dimorphic switching is conserved.

To determine if members of the IP6K family regulate dimorphic switching, we generated a homozygous *kcs1Δ/kcs1Δ* strain (70, 71). However, this strain could not be tested in our assay, due to massive growth defects (data not shown). We therefore assayed the ability of a heterozygous *kcs1Δ/KCS1* strain to make pseudohyphae. Formation of pseudohyphae was comparable to that of the wild-type strain (Fig. 9D).

DISCUSSION

Vip1 kinases regulate fungal dimorphism. We have used *S. pombe* to identify a key regulator of the switch from the single-cell yeast form to an invasive pseudohyphal growth form. Our analysis has shown that the kinase activity of the Vip1 member Asp1 is essential for the alternative growth form in *S. pombe*. Similarly, Vip1 plays a role in haploid invasive and diploid pseudohyphal growth of *S. cerevisiae* cells. As *S. pombe* cells and diploid and haploid *S. cerevisiae* cells change to an invasive filamentous type of growth in response to different environmental cues, our data suggest that Vip1-generated inositol pyrophosphates could *per se* play a role in fungal dimorphism (1, 24). Furthermore, as an increase in Asp1-generated inositol pyrophosphates increases the ability to switch to and grow in an invasive growth mode, we propose that these inositol molecules act as “second messengers” (Fig. 10).

Higher inositol phosphates have been discussed as sensors/transmitters for environmental conditions and have been shown to be upregulated in response to hyperosmotic stress and to mediate chemotaxis in *Dictyostelium* (43, 59). One of the very few studies that have described such a role specifically for members of the Vip1 family is the regulation of phosphate metabolism. In response to phosphate starvation, the *S. cerevisiae* Pho80-Pho85-Pho81 complex is inactivated via Vip1-generated IP₇ molecules, leading to activation of the Pho4 transcription factor (39, 41). Here, we have identified a new function of inositol pyrophosphates generated by the

Vip1 family as being part of the signaling network that leads to an altered growth mode in response to external cues.

Members of the Vip1 family generate a specific IP₇ isomer, namely, 1/3 diphosphoinositol pentakisphosphate, and phosphorylate the IP₇ isomer generated by members of the IP6K family to IP₈ (17, 23, 42, 54, 57). We were unable to determine if Vip1-generated IP₇ and/or Vip1- and IP6K-generated IP₈ was required for pseudohyphal growth, as the *S. pombe* IP6K member SPCC970.08 is essential for growth (36) and the *S. cerevisiae* *kcs1Δ/kcs1Δ* diploid strain showed massive growth defects. However, in contrast to the diploid *vip1Δ/VIP1* strain which has a haploinsufficient phenotype, the diploid *S. cerevisiae* strain heterozygous for *KCS1* generated pseudohyphae similar to those of the wild-type strain. This implies that a presumably 50% reduction in Kcs1-generated IP₇ does not affect the dimorphic transition and might suggest that the IP₇ molecules generated by Vip1 kinases regulate the dimorphic switch. In this context, it has been shown recently that activation of the *S. cerevisiae* Pho4 transcription factor by Vip1-regulated inhibition of Pho85 kinase function results in the downregulation of cellular Kcs1 activity by the generation of antisense *KCS1* RNAs (56). As the Kcs1 and Vip1 kinases both use IP₆ as a substrate and Kcs1 is the major inositol polyphosphate kinase, such a regulation might enable Vip1 to utilize more of its substrate (54, 56, 83). Thus, reduction of Kcs1 protein levels in our diploid heterozygous strain could result in more IP₆ substrate that can be utilized by Vip1.

Autoinhibition of Asp1 kinase activity? The present analysis implies that certain environmental conditions induce an up-regulation of Asp1 kinase activity via the PKA pathway. How this regulation is achieved at the molecular level is at present unclear. However, it has been proposed that the C-terminal part of Vip1 kinases, which includes a seemingly enzymatically inactive histidine acid phosphatase domain, might act as a regulatory domain (73). Our data strongly suggest that this is the case, as the presence of the wild-type Asp1 phosphatase domain has a negative impact on Asp1 function. In all our assays, we find that the phenotype of the wild-type strain is in between those of strains expressing Asp1 kinase-only variants (Asp1^{H397A} or Asp1¹⁻³⁶⁴) and strains expressing the Asp1 kinase-dead variant (*asp1*^{D333A}) or an *asp1Δ* strain. (i) The *asp1*^{H397A} strain is more resistant to TBZ than the wild-type strain, which is more TBZ resistant than the *asp1*^{D333A} strain (Fig. 1C). Similar data were obtained for the resistance to digestion by zymolyase. The *asp1*^{D333A} and *asp1Δ* strains are more resistant to digestion by the enzyme than the wild-type strain, which is more resistant than the *asp1*^{H397A} strain. (ii) The *asp1*^{H397A} strain shows an increased ability to grow in an invasive, pseudohyphal manner compared to the wild-type strain, while the *asp1*^{D333A} strain and the *asp1Δ* strain are unable to grow invasively. (iii) Overexpression of the Asp1 kinase-only variants (Asp1¹⁻³⁶⁴ and Asp1^{H397A}) gives rise to massive flocculation of an *asp1Δ* strain, while overexpression of wild-type *asp1*⁺ does not.

These data imply that the C-terminal part of Asp1, which encompasses a putative histidine acid phosphatase domain, negatively regulates the Asp1 kinase function. How can this be brought about? Such a regulation could be mediated by influencing Asp1 kinase activity either directly or by modulating downstream effectors of the Asp1-mediated inositol pyrophos-

phate regulation. For example, the phosphatase domain could regulate the amount of Asp1-generated inositol pyrophosphates directly by competing with the Asp1 kinase for the substrate. However, it must be pointed out that no enzymatic activity has as yet been described for the Asp1 phosphatase domain (17, 23, 54). Alternatively, the C-terminal part of Asp1 could influence a pathway that is regulated in the opposite way by Asp1 kinase activity. As all scored phenotypes of the *asp1Δ* strain and the *asp1*^{D333A} strain are very similar if not identical, we conclude that the putative phosphatase domain has a function that comes into play only in the presence of a wild-type kinase domain. Interestingly, *in vitro* analysis of human and yeast Vip1 kinase activities suggests that the kinase activity might be regulated directly by the phosphatase domain. Full-length human and yeast Vip1 proteins have significantly less specific activity than variants, which consist only of the kinase domain (23). These findings fit with our observation that plasmid-borne strong overexpression of *asp1*^{D333A} or *asp1*³⁶⁵⁻⁹²⁰ (an Asp1 variant that consists only of the C-terminal phosphatase domain) massively reduces the ability of the *asp1*^{H397A} strain to adhere to the agar and grow invasively. *trans* inhibition by massive overexpression of the inhibitory domain has been shown for a number of proteins regulated by autoinhibition (63).

Upstream regulators of Asp1 kinase function. A number of MAP kinase pathways detect alterations in their surroundings, leading to cellular adaptation. By testing several kinase signaling pathways, we found that Asp1 kinase-mediated pseudohyphal invasive growth requires a functional cAMP PKA pathway. Mutations in this pathway abolish this type of growth, while exogenous cAMP enhances pseudohyphal growth in strains with a wild-type Asp1 kinase domain. Exogenous cAMP does not lead to invasive growth of the *asp1*^{D333A} strain, indicating that cAMP regulates Asp1 kinase function and not vice versa. Both the *S. pombe* Asp1 and *S. cerevisiae* Vip1 proteins have multiple phosphorylation sites, and *S. cerevisiae* Vip1 is a target of Tpk1 and Tpk3, which represent two of the catalytic cAMP PKA catalytic subunits (62, 72, 82). Thus, we speculate that activation or enhancement of the kinase activity of Vip1 family members could be modulated directly by PKA.

Growth pattern of invasively growing *S. pombe* cells and the role of the cytoskeleton. Filamentous invasive growth has been described for *S. pombe* (1, 19, 61), but we have analyzed for the first time the growth pattern of such cells. Invasive growth of wild-type and *asp1*^{H397A} cells is a variation on the same theme that is a combination of different properties, such as cell-cell and cell-substrate adhesion, defects in cell separation, the ability to alter cell shape in response to environmental constraints, and an alteration of the normal growth pattern. A wild-type actin cytoskeleton is needed for this type of growth, as strains harboring mutations in genes coding for the actin cytoskeletal components Arp2/3, formin, tropomyosin, and actin are unable to grow invasively (19; our data).

The role of the interphase microtubule cytoskeleton and associated proteins in invasive growth is less clear-cut. We and others found that invasive pseudohyphal growth did not depend on the presence of the Tea1-Tea4 cell end marker complex or the microtubule regulators Mal3 and Peg1 (19; our data). Indeed in the absence of, for example, the Tea1 protein, the *asp1*^{H397A} strain showed an increased number of invasively

growing colonies. However, these colonies were, in general, smaller than those of the *asp1*^{H397A} single mutant strain, possibly due to the aberrant microtubule cytoskeleton which led to an alteration of the cylindrical cell shape and bent or lemon-shaped cells (data not shown). A cylindrical cell form probably facilitates the formation of straight chains of cells required for efficient invasive pseudohyphal growth. Thus, a wild-type microtubule cytoskeleton and the Tea1-Tea4 complex are not essential for invasive pseudohyphal growth, similar to what has been described for mating *S. pombe* cells, in which growth is redirected by external pheromones (55). In both systems, extrinsic signals regulate/modify the normal internal polarization program that requires the microtubule-based delivery of the Tea1-Tea4 complex: mating *S. pombe* cells turn into the pheromone-induced shmooing growth mode (55), while invasively growing cells show an alteration of the normal growth pattern, as they do not initiate NETO and grow predominantly monopolarly. Interestingly, the absence of Tea1 led to a faster switch to pheromone-induced growth, indicating that factors that regulate the intrinsic growth mode need to be dismantled before extrinsically controlled growth can commence (55). We speculate that this might also be the case for the switch from the single-celled growth form to the pseudohyphal growth form, and thus, a defective microtubule cytoskeleton might aid in the switch. However, as the vast majority of wild-type and *asp1*^{H397A} invasively growing cells were cylindrical, microtubules and associated proteins must play some role during invasive growth. Indeed, our preliminary analysis of Tea1-GFP localization in invasively growing cells indicates that Tea1 is present at one cell tip, with no preference for the growing or nongrowing tip.

Taken together, these data suggest that extrinsic signals modify the normal internal microtubule-dependent polarization program leading to the switch from bipolar single-celled growth to monopolar pseudohyphal growth. If Asp1 kinase is involved in growth zone definition in invasively growing cells is as yet unclear. However, Asp1 kinase function is needed for NETO in vegetatively growing cells.

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