Dual Functions for the *Schizosaccharomyces pombe* Inositol Kinase Ipk1 in Nuclear mRNA Export and Polarized Cell Growth†‡

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Received 21 August 2008/Accepted 23 November 2008

Inositol polyphosphates (IPs) constitute an emerging class of signaling molecules that regulate multiple cellular activities including chromatin remodeling and transcription, mRNA export, telomere length regulation, RNA editing, exocytosis, ciliary beating and length maintenance, and translation (8, 21, 32, 48, 51, 56, 59, 70–72). IP production is initiated with the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C (PLC), producing diacylglycerol and soluble inositol 1,4,5-trisphosphate (IP3). IP3 is sequentially phosphorylated by the coordinated actions of specific kinases to produce more highly phosphorylated IP molecules, including inositol 1,3,4,5-tetrakisphosphate (IP4), inositol 1,3,4,5,6-pentakisphosphate (IP5), and inositol 1,3,4,5,6,7-hexakisphosphate (IP6) in eukaryotic cells. Previous studies have shown that IP6 is required for efficient nuclear mRNA export in the budding yeast *Saccharomyces cerevisiae*. Here, we report the first functional analysis of *ipk1* in *Schizosaccharomyces pombe*. *S. pombe* Ipk1 (SpIpk1) is unique among Ipk1 orthologues in that it harbors a novel amino (N)-terminal domain with coiled-coil structural motifs similar to those of BAR (Bin-amphiphysin-Rvs) domain proteins. Mutants with *ipk1* deleted (*ipk1Δ*) had mRNA export defects as well as pleiotropic defects in polarized growth, cell morphology, endocytosis, and cell separation. The SpIpk1 catalytic carboxy-terminal domain was required to rescue these defects, and the mRNA export block was genetically linked to *SpDhp5* function and, likely, *IP6* production. However, the overexpression of the N-terminal domain alone also inhibited these functions in wild-type cells. This revealed a distinct noncatalytic function for the N-terminal domain. To test for connections with other inositol polyphosphates, we also analyzed whether the loss of *asp1* function, encoding an IP6 kinase downstream of Ipk1, had an effect on *ipk1Δ* cells. The *asp1Δ* mutant alone did not block mRNA export, and its cell morphology, polarized growth, and endocytosis defects were less severe than those of *ipk1Δ* cells. Moreover, *ipk1Δ asp1Δ* double mutants had altered inositol polyphosphate levels distinct from those of the *ipk1Δ* mutant. This suggested novel roles for *asp1* upstream of *ipk1*. We propose that *Ip6* production is a key signaling linchpin for regulating multiple essential cellular processes.

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† Supplemental material for this article may be found at http://ec.asm.org/.

‡ Published ahead of print on 1 December 2008.
are essential for mRNA export in *S. cerevisiae* are the DEAD box protein Dbp5 and its IP₆-bound activator Gle1 (1, 19, 52, 58, 62, 67). Gle1/IP₆ activation of Dbp5 at the NPC cytoplasmic face results in a nucleotide-dependent switch in Dbp5 and triggers changes in mRPN protein composition, thus providing directionality to the export process (61). Interestingly, the *IPK1* gene was first discovered in an *S. cerevisiae* genetic screen aimed at studying Gle1 function (71). Global IP production is also required for efficient mRNA export in mammalian cells (15). To date, *S. cerevisiae* has been the primary model system used to study the mechanism of IP₆ function in mRNA export. Whether metazoans or other fungi also specifically require IP₆ production for mRNA export has not been directly tested.

In addition to direct protein binding targets for IP₆ function, IP₆ is also the substitute for downstream IP₆ kinases and is inherently required for the production of IP₇ pyrophosphates (1). SpAsp1 and its *S. cerevisiae* orthologue, Vip1, have recently been defined as IP₆ and IP₇ kinases, with Vip1 acting as a 1/3-kinase contributing to the synthesis of 1/3-PP-IP₅ and 1/3,5-(PP₂)-IP₆ (31, 41). One *S. cerevisiae* IP₆ target has been defined, the Pho80-Pho85-Pho81 cyclin-CDK-CKI system required for nutrient homeostasis (29, 30). However, even if this regulation is conserved in *S. pombe*, it does not account for the phenotypes observed in *S. pombe asp1Δ* cells. Notably, *S. pombe* asp1Δ cells are defective in cell morphology, polarized growth, and endocytosis, and asp1Δ cells are synthetically lethal with mutations in genes encoding components of the Arp2/3 complex and actin (14).

*S. pombe* cells grow in a polarized fashion. Immediately after cell division, the daughter cells initially grow in a monopolar manner from the cell end that existed before division. Subsequently, cells initiate growth from the new end and resume bipolar growth until mitosis (38). The actin cytoskeleton is critical for such polarized growth, and cytoskeleton perturbations result in round, swollen cells. Actin is organized at the growing surfaces of the cell as cortical patches, which function in membrane growth and endocytosis, and along the long axis of the cell as actin cables, which function as tracks for the delivery of secretory vesicles to growing cell ends (9, 17, 42). Actin patches are delocalized during mitosis and concentrated around the medial septum during cytokinesis (33). Dynamic actin assembly (and disassembly) is essential for the assembly, maintenance, and closure of the contractile actomyosin ring and cytokinesis (44). A functional actin cytoskeleton is also required for the proper trafficking of secretory cargoes during cytokinesis (17). For example, secretory vesicles containing Enc1 and Agn1 endoglucanases are delivered to the septum region by the exocyst complex, allowing the digestion of the division septum and the surrounding cell wall and the final physical separation of the daughter cells (10, 34, 66).

We speculated that an interspecies comparison of Ipk1 between *S. cerevisiae* and *S. pombe*, two phylogenetically distant yeasts, would allow the dissection of functional conservation and divergence in the soluble IP pathway. To test this, we used a combined genetic and cell biological approach to investigate SpIpk1 function. In addition to conserved defects in mRNA export, a loss of *ipk1Δ* function resulted in pleiotropic defects in cell morphology, polarized growth, endocytosis, and cell separation. By analyzing *ipk1Δ asp1Δ* double mutants, we gained unique insights into the independent roles of the respective kinases in IP production and cell physiology.

**MATERIALS AND METHODS**

*S. pombe* strain construction, media, and genetic methods. *S. pombe* strains were grown in YE medium or Edinburgh minimal medium (EMM) with the appropriate supplements as previously described (40). Strains were constructed by a PCR-based gene disruption strategy and tetrad dissection (see below). Crosses were performed on glutamate medium (EMM lacking ammonium chloride and containing 10 mM glutamate). DNA transformations were done by the lithium acetate method (27). For the regulated expression of genes by the nmt1 promoter, cells were grown in EMM either lacking thiamine to allow expression or with the addition of 10 μM thiamine to repress expression (36). For the generation of the *ipk1Δ* strain, the *ipk1Δ* open reading frame (ORF) was completely deleted by PCR-based one-step homologous recombination as previously described (3), using *ura4* as a selectable marker. *ura4* was amplified by PCR from plasmid pKg355 using a forward oligonucleotide primer (spipk1-D-F) (see Table S1 in the supplemental material) corresponding to 80 bp upstream of the ATG start codon and a reverse primer (spipk1-D-R) (see Table S1 in the supplemental material) corresponding to 80 bp downstream of the TAA stop codon. Deletion of *ipk1Δ* and *asp1Δ* genes was confirmed by PCR using primers *ipk1Δ F* and *ipk1Δ R* (see Table S1).

For generation of the *ipk1Δ asp1Δ* strain, SWY2559 was crossed with KG956 (*hΔ1 asp1Δ:ura4 32 dileu1-32 ade6-M20*) and sporulated, and double mutants were identified by tetrad analysis and confirmed by PCR.

**Gene cloning and deletion constructs.** To clone *ipk1Δ*, specific cDNA was amplified by PCR with oligonucleotide primers *spipk1-NdeI-F* and *spipk1-BamHI-R* and cloned into pREP1 in the NdeI-SmaI sites of pREP1, replacing the *ura4* gene with an *aspl* fragment to create *ipk1Δ* strain pSW2023. For cloning of the *ipk1Δ* C terminus (*ipk1Δ C-term*) under the nmt1 promoter, the *ipk1Δ C-term* sequence was PCR amplified from pSW3019 with vector primers spipk1-BamHI and spipk1-HindIII and cloned in pREP1, replacing the nmt1 promoter with the *ipk1Δ C-term* sequence and the 5′-kb *ipk1Δ* sequence upstream of the *nmt1* ORF and the 3′-kb *ipk1Δ* sequence downstream of the *nmt1* ORF, resulting in pSW3021. For cloning of the *ipk1Δ* ORF, the *ipk1Δ* sequence was amplified by PCR using oligonucleotide primers *spipk1-NdeI-F* and *spipk1-BamHI-R* and cloned in pREP1, replacing the nmt1 promoter with the *ipk1Δ ORF* and the *ipk1Δ* sequence upstream of the *nmt1* ORF. The resulting *ipk1Δ* and *asp1Δ* strains were constructed in *S. pombe* as described above. A *S. pombe* strain with the *ipk1Δ ORF* and the *asp1Δ* sequence inserted in the *nmt1* ORF is synthetically lethal. The *ipk1Δ ORF* insertion results in pleiotropic defects, and the *asp1Δ* allele was synthetically lethal with mutations in genes encoding components of the Arp2/3 complex and actin (14).

**Analysis of levels of cellular [*H]*inositol-labeled IP.** The soluble-IP profiles of *S. pombe* cells were determined according to a protocol previously described for *S. cerevisiae* cells (71). Briefly, *S. pombe* cells were grown in EMM containing 20 μCi [*H]inositol (Perkin-Elmer) to mid-logarithmic phase. Cells (1 ml) were harvested, washed in H₂O, and resuspended in 100 μl of 0.5 N HCl. Soluble IPs were extracted by adding 372 μl of chloroform-methanol (1:2, vol/vol) and 100 μl of glass beads. The mixture was vortexed at maximum speed for 2 min, followed by the addition of 125 μl of chloroform and 125 μl of 2 M potassium chloride and another 2 min of vortexing. The lysates were clarified by a spin at 13,000 × g for
5 min, and the supernatant was recovered. Samples were analyzed by high-performance liquid chromatography (HPLC), with the IPs resolved by use of a Whatman Partispher strong-anion-exchange column (4.6 by 125 mm) and a linear gradient from 10 mM to 1 M ammonium phosphate (pH 3.5) over 25 min, followed by elution with 1 M ammonium phosphate for 20 min.

Transmission electron microscopy. *S. pombe* cells were grown in YE medium to early log phase, fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), postfixed in 1% OsO4, dehydrated through an ethanol series, equilibrated in propylene oxide, and then embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined using a Hitachi H-800 electron microscope.

In situ hybridization and indirect immunofluorescence. *S. pombe* cells were grown in YE medium or EMM to early log phase at 30°C. Additionally, the EMM-grown cells were shifted to cell growth at 36°C for 90 min. Cells were fixed for 10 min and processed as previously described (22, 68). Cells were incubated overnight with a digoxigenin-dUTP-labeled oligo(dT) probe and were detected with fluorescein-labeled antidigoxigenin Fab (1:25; Boehringer). DNA was stained with 0.1 μg/ml DAPI (4′,6′-diamidino-2-phenylindole), and samples were mounted for imaging in 90% glycerol and 1 mg/ml p-phenylenediamine (pH 8.0; Sigma-Aldrich). Images were acquired using a microscope (BX50, Olympus) with a UPlanF1 100x objective (Olympus) and a camera (CoolSNAP HQ; Photometrics). Within each experiment, all images were collected and scaled identically using MetaVue, version 4.6 (Molecular Devices), or Image-Pro Express (Media Cybernetics) and processed with Photoshop 9.0 software (Adobe).

Cytochemistry and microscopy. Septa were visualized by staining ethanol-fixed cells with 1 mg/ml methyl blue solution (Sigma-Aldrich). To visualize DNA, cells were stained with 0.1 μg/ml DAPI. Strains expressing green fluorescent protein-tagged proteins were grown in liquid YE medium and visualized and photographed live. All images were acquired and processed as described above.

F-actin and FM4-64 staining. For F-actin staining, cells were grown to early log phase, fixed by adding formaldehyde to a final concentration of 3.7% for 10 min, and suspended in 0.1 M potassium phosphate buffer (pH 6.5). Cells were fixed in 3.7% formaldehyde again for 45 min, washed twice in phosphate-buffered saline, and stained with rhodamine-phalloidin (Molecular Probes) on ice for 30 min.

FM4-64 staining was performed as described previously by Feoktistova et al. (14). Briefly, cells were grown in YE medium at 30°C to mid-log phase, concentrated 100-fold by centrifugation, and suspended in fresh YE medium. FM4-64 (Molecular Probes) was added to a final concentration of 16 μM, and cells were incubated on ice for 15 min. Cells were then washed and suspended in YE medium and incubated at 30°C. Aliquots of cells were collected at 15-min intervals, mounted onto slides, and visualized immediately. Images were collected using Image-Pro Express (Media Cybernetics) and processed with Photoshop 9.0 software (Adobe).

RESULTS

*ipk1*Δ *S. pombe* cells are defective in nuclear mRNA export, cell morphology, polarized growth, and cell separation. We cloned full-length *S. pombe* ipk1Δ based on the previously published sequence information (25); additionally, we generated an *ipk1*Δ strain. The *ipk1*Δ cells were viable at 30°C, temperature sensitive at 36°C, and cold sensitive at 18°C (Fig. 1B and see Fig. 8A). To test the metabolic effect of the *ipk1*Δ deletion on IP production, we compared the levels of soluble IPs in extracts isolated from *ipk1*Δ cells to those of wild-type cells. Cells were labeled to steady state with [3H]inositol, and extracted IPs were separated and analyzed by HPLC. In wild-type cells, IP3 was most abundant, with markedly lower levels of IP5, and minimal detection of other IPs (see Fig. 7C). In contrast, IP3 was absent in extracts from *ipk1*Δ cells, and IP5, IP4, IP6, and PP-IP4 levels were distinctly elevated (see Fig. 7C). Others have shown that increased upstream IP levels are an established indicator of inhibited IP6 production in *S. cerevisiae*, *Drosophila melanogaster* S2 cells, and zebrafish embryos (25, 50, 53, 71). Thus, our results indicate that the deletion of *ipk1*Δ results in a loss of IP6 production and a general perturbation of the IP synthesis pathway.

We also examined if the expression of *ScIPK1* or *ZlIPK1* could restore IP6 production in *ipk1*Δ cells. Both *ScIPK1* and *ZlIPK1* expressions rescued IP6 production in *ipk1*Δ cells (see Fig. S3 in the supplemental material), suggesting enzymatic complementation across species by the IP2 kinase enzymes. Next, we analyzed the *ipk1*Δ cells for mRNA export defects using oligo(dT) in situ hybridization for the subcellular distribution of poly(A)⁺ RNA. The *ipk1*Δ cells showed an accumulation of poly(A)⁺ RNA in the nucleus at a growth temperature of 30°C (Fig. 1A). Thus, we conclude that IP6 production is required for efficient mRNA export in *S. pombe*.

Although ipk1Δ is not essential, *ipk1*Δ cells exhibited morphological and cell separation defects. The *ipk1*Δ cells were rounder than wild-type cells, and the cell morphology defects were exacerbated by growth at an elevated temperature (36°C) (Fig. 1B and see Fig. 8A). We also observed an increased number of binucleate-septated cells in a nonsynchronous exponentially growing cell population (Fig. 1C and see Fig. 8A and B). To further assess the cell separation defect in *ipk1*Δ cells, the morphology of the septum region was examined by thin-section transmission electron microscopy. The formation and organization of septa in wild-type and *ipk1*Δ cells were normal (Fig. 1D), with the three-layer septum structure (a clear primary septum surrounded by two darker secondary septa) apparent in both cell types. However, there were distinctions in the apparent degradations of the primary septum. Compared to wild-type cells, where the primary septum is degraded centripetally from the cortex to the septum midpoint, in *ipk1*Δ cells, degradation of the primary septum material appeared asymmetric, with the daughter cells remaining attached by remnants of the cell wall at one end of the division plane (Fig. 1D). This finding indicates that *ipk1*Δ cells are defective in the dissolution of both the septum and the cell wall that surrounds the septum. We speculate that *ipk1*Δ cells fail to disassemble the division septa, leading to an accumulation of binucleate cells with a medial division septum. Such cell shape and separation defects have not been reported for *ipk1*Δ *S. cerevisiae* cells, suggesting that roles for Ipk1 in these cellular processes are specific to *S. pombe*.

Defects in morphology and cell separation in *ipk1*Δ cells might reflect a perturbation of the polarized growth. We first examined the organization of the F-actin cytoskeleton by staining exponentially growing cells with rhodamine-conjugated phalloidin. In 70% of wild-type cells at 30°C, actin was organized in cortical patches at both cell ends and in thin cables running along the long axis of the cell (Fig. 1E and see Fig. 8C). In contrast, only 41% of *ipk1*Δ cells at 30°C showed bipolar cortical patches, whereas 22% displayed disorganized actin structures and actin patches delocalized throughout the cell body (Fig. 1E and see Fig. 8C). After shifting to 36°C for 6 h, *ipk1*Δ cells were swollen and round, with 36% of the cells exhibiting delocalized cortical actin patches (see Fig. 8C). At 36°C, only 2% of the wild-type cells showed an altered actin distribution. This delocalization of F-actin in *ipk1*Δ cells was at the expense of its normal bipolar and monopolar distributions (see Fig. 8C). Compared to the wild type, we also observed an increase in the medial ring localization of F-actin in *ipk1*Δ cells at both growth temperatures (see Fig. 8C). These
results indicate that ipk1<sup>+</sup> plays a critical role in the organization of cortical actin patches, whereas it is dispensable for the formation of medial actin rings.

The SpIpk1 C-terminal domain is sufficient to rescue mRNA export, cell separation, and polarized growth defects in ipk1<sup>Δ</sup> <i>S. pombe</i> cells. We previously reported that the purified recombinant C-terminal domain of SpIpk1 has substrate selectivity and catalytic efficiency similar to those of ScIpk1 despite sharing only 24% sequence identity (25). The most striking difference between the ScIpk1 and SpIpk1 proteins is the unique N-terminal region of the <i>S. pombe</i> protein (see Fig. S1 in the supplemental material). To identify the relevant protein activity responsible for the defects exhibited by ipk1<sup>Δ</sup> <i>S. pombe</i> cells, we constructed a series of plasmids expressing ipk1<sup>Δ</sup> deletion mutants under the control of the nmt1 promoter (Fig. 2). As complementation controls, plasmids harboring either
the full-length \textit{ipk1} under the control of the \textit{nm1} promoter or a genomic fragment of \textit{ipk1}, with the endogenous promoter (\textit{ipk1} gDNA) were used. The mRNA export, cell separation, and polarized growth phenotypes were assayed after induction for overexpression by the \textit{nm1} promoter. The results from these analyses are summarized in Fig. 2. The \textit{ipk1} cells exhibited nuclear poly(A)\textsuperscript{-}RNA accumulation in 59\% of the cells (Fig. 3C, D, and U). The overexpression of \textit{ipk1}, \textit{ipk1} gDNA, and \textit{ipk1} C-term suppressed the mRNA export defect of \textit{ipk1} cells (Fig. 2 and 3) and restored IP\textsubscript{6} production (see Fig. S2 in the supplemental material). However, the level of rescue of the mRNA export defect by full-length \textit{ipk1} was partial. Of note, in wild-type cells, the overexpression of \textit{ipk1} resulted in a weak mRNA export defect, with nuclear poly(A)\textsuperscript{-}RNA accumulation in 21\% of the cells (Fig. 3E, F, and U). Most clearly, the overexpression of \textit{ipk1} N-term did not rescue the \textit{ipk1} mRNA export defect (Fig. 3S, T, and U), and IP\textsubscript{6} production was not restored (see Fig. S2 in the supplemental material). In addition, 41\% of wild-type cells overexpressing \textit{ipk1} N-term showed nuclear poly(A)\textsuperscript{-}RNA accumulation (Fig. 3Q, R, and U). Together, we conclude that the loss of the \textit{Ipk1} catalytic domain is the critical defect linked to the mRNA export perturbation in \textit{ipk1} cells. For wild-type cells, there are also dominant negative effects on mRNA export from overexpressing either \textit{ipk1} or \textit{ipk1} N-term.

We also examined the effects of overexpressing the \textit{ipk1} deletions on cell separation and polarized growth in both wild-type and \textit{ipk1} cells. The overexpression of the \textit{ipk1}\textsuperscript{\textminus}, \textit{ipk1} C-term, \textit{ipk1} \textsc{\textminus}RVS (Rvs homology), or \textit{ipk1} \textsc{\textminus}CC (coiled-coil) constructs in wild-type cells did not significantly alter the ratios between binucleate-septated and mononucleate-unseptated cells (Fig. 4A to M). Similarly, wild-type vector-only cells and those overexpressing \textit{ipk1} or \textit{ipk1} C-term did not display any significant difference in F-actin distribution (Fig. 5A to F and U). Strikingly, the overexpression of \textit{ipk1} gDNA, \textit{ipk1}\textsuperscript{\textminus}, or \textit{ipk1} C-term suppressed both cell separation and polarized growth defects exhibited by \textit{ipk1} cells (Fig. 4N and 5I to P and U). There were differences in the cell separation defect levels between \textit{ipk1} cells grown in minimal medium and those grown in rich medium (see Fig. S4 in the supplemental material). The \textit{ipk1} cells grown in rich medium had a significant defect, with 30\% and 47\% of the cells being binucleate-septated at 30\°C and 36\°C, respectively. However, the \textit{ipk1} cells grown in minimal medium had a more modest defect, with 16\% and 19\% of the cells being binucleate-septated at 30\°C and 36\°C, respectively. Overall, we conclude that the \textit{SpIpk1} C-terminal domain, and potentially IP\textsubscript{6} production as well as the proper maintenance of other IP levels, is required for correct cell separation and polarized growth.

Next, we analyzed the effects of the \textit{Ipk1} N-terminal domain. A high percentage of wild-type cells overexpressing \textit{ipk1} N-term were multinucleated and multiseptated (Fig. 4C, F, and M). In contrast, the \textit{ipk1} cells overexpressing \textit{ipk1} N-term exhibited an increase in the number of binucleate-septated cells only (Fig. 4N). Additionally, we observed that \textit{ipk1} N-term overexpression specifically perturbs septum cleavage, cell separation, and polarized growth.

Specific rescue of the mRNA export defect in \textit{ipk1} cells by overexpressing \textit{dbp5}\textsuperscript{\textplus}. Given that the \textit{SpIpk1} C-terminal domain is sufficient to rescue both the mRNA export and cell separation defects in \textit{ipk1} cells, we speculated that the cell separation defects might be an indirect effect due to a lack of an IP\textsubscript{6}-dependent export of an mRNA(s) encoding proteins involved in cell separation. In \textit{S. cerevisiae}, the IP\textsubscript{6} target in the mRNA export pathway has been pinpointed to Gle1, a cofactor for Dbp5, and \textit{DBP5} overexpression specifically suppresses the mRNA export defect of an \textit{S. cerevisiae} \textit{ipk1} \textsc{\textminus}ap42\Delta mutant (1). Thus, we tested whether \textit{S. pombe} \textit{dbp5}\textsuperscript{\textplus} overexpression suppresses the mRNA export defect of \textit{ipk1} \textit{S. pombe} cells. Strikingly, the overexpression of \textit{dbp5}\textsuperscript{\textplus} fully rescued the mRNA export defect in \textit{ipk1} cells, whereas its overexpression in wild-type cells had no effect (Fig. 6). This suggests that the mRNA export defect of the \textit{ipk1} \textit{S. pombe} cells is specifically linked to SpDbp5 function, similar to what is known for \textit{S. cerevisiae} (1). In sharp contrast, the cell separation and polarized growth defects in \textit{ipk1} cells were not rescued by \textit{dbp5}\textsuperscript{\textplus} overexpression (Fig. 4N and 5B, T, and U). We conclude that the role of \textit{SpIpk1}/IP\textsubscript{6}, in cell separation and polarized growth is independent of its role in mRNA export.

Elevated levels of PP-IP\textsubscript{6} production in \textit{ipk1} cells are linked to Asp1 function. A loss of the IP\textsubscript{6} kinase activity in \textit{aspl} cells results in cell morphology, cell separation, and polarized growth defects that are similar to those observed here in \textit{ipk1} cells (14, 41). Thus, we reasoned that these \textit{ipk1} phenotypes might be due to the indirect effect of a loss of IP\textsubscript{6}. 

![Diagram](image-url)
FIG. 3. Overexpression of *ipk1* C-term is sufficient to restore mRNA export in *ipk1Δ* cells, whereas *ipk1* N-term has an inhibitory effect in both wild-type and *ipk1Δ* cells. Wild-type (A, B, E, F, I, M, N, O, and R) and *ipk1Δ* (C, D, G, H, K, L, O, P, S, and T) cells were transformed with the empty vector (A to D) or the plasmid constructs carrying *ipk1Δ* (E to H), *ipk1* gDNA with *ipk1Δ* under the transcriptional control of its own promoter (I to L), *ipk1* C-term (M to P), and *ipk1* N-term (Q to T); grown in EMM at 30°C for 18 h; and shifted to cell growth at 36°C for 90 min. The subcellular distribution of poly(A)^+ RNA was visualized by in situ hybridization with oligo(dT) (columns 1 and 3). DNA was visualized by subsequent DAPI staining (columns 2 and 4). (U) Bar graph quantifying nuclear poly(A)^+ RNA distribution in wild-type and *ipk1Δ* cells harboring different plasmids (n > 200 cells).
To test this, we directly compared the ipk1Δ mutant, the asp1Δ mutant, and an ipk1Δ asp1Δ double mutant for growth in rich medium. As shown in Fig. 7A, wild-type and ipk1Δ asp1Δ cells showed similar levels of growth at 23°C, 29°C, 32°C, and 36°C. The growth of ipk1Δ asp1Δ cells was slightly compromised at 18°C. In comparison, the asp1Δ cells displayed modest temperature sensitivity at 36°C. The ipk1Δ cells showed a similar level of temperature sensitivity at 36°C; however, it was severely cold sensitive at 18°C (Fig. 7A). The expression of wild-type ipk1Δ or asp1Δ in the respective mutants resulted in a partial or complete rescue of the temperature- and/or cold-sensitive growth defects (Fig. 7B). However, there were relative differences in the levels of growth defects between rich and minimal media, potentially reflecting differential effects of culture media on growth. Overall, the ipk1Δ cells had the most severe growth perturbations. This is not unexpected, because ipk1Δ cells fail to produce both IP3 and IP4 isomers. However, the difference in the growth characteristics between the ipk1Δ asp1Δ double mutant and the ipk1Δ single mutant is surprising.

To directly examine the effects on the IP metabolic pathways, we compared the IP profiles of the mutant strains. Following steady-state radiolabeling with [3H]inositol, lysates from equivalent total cell numbers were prepared, and total soluble IPs were resolved by HPLC. As shown in Fig. 7C, IP3 and IP6 peaks were detected in wild-type cells. The ipk1Δ cells had elevated levels of all upstream IPs (e.g., IP3, IP4, IP5, and PP-IP4) and did not have the IP6 peak. In contrast, only the level of IP6 was elevated in the asp1Δ cells compared to those of the wild type. Interestingly, although the ipk1Δ asp1Δ cells had elevated levels of IP3, IP4, and IP6, the relative level of the ratio of PP-IP4 to IP5 was significantly lower than that in ipk1Δ samples. In ipk1Δ asp1Δ cells, the PP-IP4-to-IP5 ratio was -0.09, whereas in ipk1Δ cells, it was ~0.43. This indicates that Asp1 has an IP5 kinase activity that contributes to PP-IP4 synthesis in the ipk1Δ cells. Others reported previously that ScVip1, the Asp1 orthologue, can produce PP-IP4 in vitro (41). As some PP-IP4 is still present in the ipk1Δ asp1Δ strain, there must be an additional kinase(s) responsible for this synthesis. Taken together, the elevated PP-IP4 level in the ipk1Δ asp1Δ cells might be responsible for the mutant’s more severe cold-sensitive growth defect, with the more modest level of PP-IP4 accumulation in the ipk1Δ asp1Δ mutant having a lesser effect.

Comparison of roles of SpIpk1 and Asp1 in mRNA export, cell morphology, cell separation, polarized growth, and endocytosis. To further dissect how the cellular defects were linked to specific perturbations in IP production for the ipk1Δ, asp1Δ, and ipk1Δ asp1Δ mutants, we compared the relative defects in mRNA export, polarized growth, and cell separation. In situ hybridization for poly(A)+ RNA showed that the asp1Δ cells did not accumulate poly(A)+ RNA in the nucleus (see Fig. S5 in the supplemental material). In contrast, the ipk1Δ asp1Δ cells accumulated poly(A)+ RNA in the nucleus at a level similar to that for the ipk1Δ cells (see Fig. S5 in the supplemental material). Because the common IP perturbation between the ipk1Δ and ipk1Δ asp1Δ mutants (Fig. 7C) is the loss of IP6, we conclude that proper IP6 production is the most critical effector of mRNA export in S. pombe.

Interestingly, the ipk1Δ and ipk1Δ asp1Δ mutants had comparable perturbations in the levels of binucleate-septated cells
at both 30°C and 36°C (Fig. 8A and B). The cell separation defect in each mutant was exacerbated to a similar extent at 36°C, with ~50% of the cells being binucleate-septated (compared to less than 20% in the wild-type cell population). Again, the asp1 mutant exhibited a more modest cell separation defect, with ~30% of the cells being binucleate-septated at 36°C (Fig. 8A and B). We also observed that both the ipk1Δ and ipk1Δ asp1Δ cells developed a rounded shape, which was
more pronounced at 36°C (Fig. 8A). The asp1Δ mutant had only a subtle cell shape defect (Fig. 8A).

The ipk1Δ and ipk1Δ asp1Δ mutants also had comparable perturbations in polarized growth at both 30°C and 36°C (Fig. 8C). In contrast, asp1Δ cells exhibited a modest polarized growth defect, with only 19% of the cells displaying delocalized F-actin patches (Fig. 8C). Because defects in the actin cytoskeleton result in perturbations of endocytosis (17, 39), we measured endocytosis with a qualitative assay. The amphiphilic fluorescent dye FM4-64 enters cells through endocytosis and is transported to the vacuolar membrane (17, 65). As shown in Fig. 9, at 4°C in wild-type, ipk1Δ, asp1Δ, and ipk1Δ asp1Δ cells, FM4-64 localized in a speckled pattern, indicating that dye uptake was normal. Within 30 min of incubation at 30°C, vacuolar membranes were intensely stained in wild-type and asp1Δ cells (Fig. 9). In contrast, a similar level of staining in the ipk1Δ and ipk1Δ asp1Δ cells was not observed until after 1 h (Fig. 9). These results suggest that endocytosis is more strongly inhibited in the ipk1Δ and ipk1Δ asp1Δ cells than in the asp1Δ cells. We conclude that the more severe defects in cell separation, cell morphology, polarized growth, and endocytosis found in the ipk1Δ mutant are due to the loss of both IP6 and IP7.

**DISCUSSION**

Here, we report the first analysis of cellular functions for ipk1Δ− in *S. pombe*. This work directly complements and extends prior analyses of *S. cerevisiae* in several important ways. We find that SpIpk1 is required for mRNA export and is genetically linked to SpDbp5 function. More strikingly, the ipk1Δ mutants have pleiotropic defects in cell morphology, polarized growth, endocytosis, and cell separation. These defects are potentially due to the loss of production of both IP6 and IP7. In addition, increased PP-IP4 levels from Asp1 kinase activity are correlated with cold-sensitive ipk1Δ cell growth. However, the noncatalytic unique N-terminal SpIpk1 domain is also required. Taken together, the phenotypes of the ipk1Δ and asp1Δ mutants delineate multiple roles for SpIpk1 function and IP6 production and highlight the cellular consequences of perturbing IP flux.

This work provides direct evidence implicating IP6 production as being required for mRNA export in an organism other than *S. cerevisiae*. Our data suggest that the steps and factors mediating the highly intricate export process are conserved between *S. cerevisiae* and *S. pombe* and potentially across all eukaryotes. Based on the complementation of ipk1Δ *S. pombe* cells by *dbp5* Δ overexpression (Fig. 6), we predict that SpGlc1 will be an *S. pombe* IP6 target for the activation of SpDbp5, similar to the mechanism in *S. cerevisiae* (1, 67). Of note, as with Kcs1 in *S. cerevisiae* (46), mRNA export in *S. pombe* is not dependent on asp1Δ function or, presumably, IP7 production. It is intriguing that the overexpression of ipk1Δ results in a modest mRNA export defect in wild-type cells. Thus, the SpIpk1 protein might compete for an essential mRNA export factor(s). These findings are similar to the reported effects of an overproduction of SpMex67 (69). We predict that the noncatalytic, unique N-terminal domain of SpIpk1 is mediating specialized cellular functions (see below).

Previous studies of *S. pombe* have revealed roles for asp1Δ function in endocytosis and the actin cytoskeleton (14). As SpIpk1 activity is upstream of Asp1 and inherently required for all IP6 production, it is not unexpected that ipk1Δ cells show similar defects. IP7 might regulate Arp2/3 complexes that participate in actin cytoskeleton and cellular morphology (14, 41). The fusion of the exocyst complex to the plasma membranes for the release of secretory vesicles requires Arp2/3 complex-mediated actin assembly (6). Thus, a functional actin cytoskeleton is critical for polarized membrane growth, protein secretion, and endocytosis (15, 17, 39), and the endocytic and morphological defects in the ipk1Δ and asp1Δ cells are potentially indirect effects of perturbations in the actin cytoskeleton.

A direct role for IP6 and/or IP7 in vesicular trafficking is also possible. It is known that IP6 and IP7 both can modulate vesicular trafficking in fungi and mammalian cells (12, 20, 21, 49), and IP6 promotes dynamin-mediated endocytosis in pancreatic β cells (20). Additionally, a recent report suggested that IP7 is required for full exocytic capacity in insulin-secreting pancreatic β cells (21). Interestingly, in ipk1Δ cells, the septum appears normally, but the septum and its surrounding cell wall are not cleaved completely, resulting in an accumulation of septated cells. The overexpression of *dbp5*Δ does not suppress the cell separation defect in ipk1Δ cells (Fig. 4N), suggesting that the phenotype is not related to mRNA export or SpDbp5 function. ipk1Δ cells might be defective in the trafficking and secretion of hydrolytic enzymes or their release at the medial region.

The ipk1Δ mutant phenotypes presumably result from the
combined effects of the loss of IP₆ and IP₇ production and the accumulation of upstream IPs. As such, IP₆ and IP₇ could have nonoverlapping independent functions that mediate distinct events during cell separation. For example, the membrane fusion step of the vesicles might be perturbed in the absence of IP₆, whereas the Arp2/3 complex mediating actin organization is defective in the absence of IP₇. Consistent with this hypothesis, the ipk₁/H9004 mutant phenotypes are consistently more severe than the asp₁/H9004 mutant phenotypes. Alternatively, the more severe ipk₁/H9004 phenotypes could reflect a role for an additional IP₆ kinase that partially compensates for the absence of Asp1 and allows some IP₇ production in the asp₁/H9004 mutant. In S. cerevisiae, Kcs1 also acts as an IP₆ kinase (47). A Kcs1 orthologue in S. pombe has not been fully characterized. With regard to the IP synthesis pathway, it is intriguing that ipk₁/H9004 mutants are more severely cold sensitive than the ipk₁/H9004 asp₁/H9004 double mutant. Our results suggest that Asp1-dependent elevated PP-IP₄ levels in ipk₁/H9004 cells might be responsible. However, physiological targets and functions for such a PP-IP₄ molecule are unknown. Future analysis of the ipk₁/H9004 and asp₁/H9004 mutants might reveal such targets.

Several pieces of evidence implicate a role for the noncatalytic N-terminal domain of SpIpk1 in cell function. The mRNA export, temperature-sensitive growth, and morphological defects exhibited by ipk₁/H9004 cells are rescued by overexpressing the ipk₁/H11001 catalytic C-terminal domain but not the N-terminal do-
main. Moreover, the overexpression of the SpIpk1 N-terminal domain in wild-type cells perturbs mRNA export, cell separation, and polarized growth. A high percentage of the ipk1 N-term-overexpressing wild-type cells are multinucleate multiseptate (Fig. 4). This ipk1 N-term phenotype is similar to those of mutants with a loss or reduction of glucanases (e.g., ace2, septin genes, and mid2) (7, 34, 57, 60). In these mutants, septa also form normally; however, cells are defective in cell separation, with a chain of cells connected by septa upon subsequent rounds of nuclear division. We speculate that the N-terminal domain serves as a protein-protein-interacting module, and when overexpressed, it sequesters a critical cellular factor that mediates IP synthesis pathway functions. One potential candidate for such interactions could be SpIpk1 itself, with the truncated N-terminal domain alone heterodimerizing to inhibit IP production and possibly block effective substrate exchange among different IP kinases. Additionally, the coiled-coil BAR homology domain might act as a membrane-binding and curvature-sensing module to localize SpIpk1 in distinct cellular microenvironments. Interestingly, SpIpk1 is

FIG. 8. Cells without ipk1* and/or asp1* function(s) are defective in cell separation and polarized growth. Wild-type, ipk1Δ, asp1Δ, and ipk1Δ asp1Δ cells were grown in YE medium at 30°C, and aliquots were shifted to 36°C for 6 h. (A) To analyze cell separation, cells were fixed in ethanol and stained with methyl blue and DAPI. Representative images (rows 2 and 4) along with their respective DIC images (rows 1 and 3) are presented. (B) Quantification of the number of septa and nuclei in cells from A (n > 300 cells). (C) Bar graph showing F-actin distribution in cells from A (n > 300 cells). Ring, actin localized to medial septum; monopolar, actin only at one end; bipolar, actin at both ends; delocalized, actin patches throughout the cell.
localized predominantly in the cytoplasm (http://cgl.riken.go.jp) (35; our unpublished data for an ectopically expressed, N-terminal green fluorescent protein-tagged fusion protein). Such subcellular localization is consistent with evidence for a role for SpIPK1 in the secretory pathway (19) (Fig. 9). Endocytosis is inhibited in ipk1Δ cells. Wild-type, ipk1Δ, asp1Δ, and ipk1Δ asp1Δ cells were treated with the fluorescent dye FM4-64 on ice for 15 min (left), washed and suspended in YE medium, and incubated at 30°C. Images of FM4-64 distribution within cells were digitally acquired at 15-min intervals, and the time required for the vacuolar membranes to become fully fluorescent was noted for each strain (right).

ACKNOWLEDGMENTS

We are indebted to Kathy Gould for critical input and guidance throughout the project and to Anna Feoktistova for generous assistance with strain generation. We thank Gary Olson and Virginia Winnfrey for assistance and expertise with the electron microscopy experiments; Elizabeth Tran, Laura Terry, Timothy Bolger, and Li-En Jao for comments on the manuscript; and Srinivas Venkatram and members of the Wente and Gould laboratories for discussions.

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