Dual Functions for the \textit{Schizosaccharomyces pombe} Inositol Kinase Ipk1 in Nuclear mRNA Export and Polarized Cell Growth$^\dagger$

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The inositol 1,3,4,5,6-pentakisphosphate (IP$_5$) 2-kinase (Ipk1) catalyzes the production of inositol hexakisphosphate (IP$_6$) in eukaryotic cells. Previous studies have shown that IP$_6$ is required for efficient nuclear mRNA export in the budding yeast \textit{Saccharomyces cerevisiae}. Here, we report the first functional analysis of \textit{ipk1} in \textit{Schizosaccharomyces pombe}. \textit{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 \textit{ipk1} deleted (\textit{ipk1}$^\Delta$) 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, IP$_6$ 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 \textit{asp1} function, encoding an IP$_6$ kinase downstream of Ipk1, had an effect on \textit{ipk1}$^\Delta$ cells. The \textit{asp1}$^\Delta$ mutant alone did not block mRNA export, and its cell morphology, polarized growth, and endocytosis defects were less severe than those of \textit{ipk1}$^\Delta$ cells. Moreover, \textit{ipk1}$^\Delta$ \textit{asp1}$^\Delta$ double mutants had altered inositol polyphosphate levels distinct from those of the \textit{ipk1}$^\Delta$ mutant. This suggested novel roles for \textit{asp1} upstream of \textit{ipk1}$^\Delta$. We propose that IP$_6$ production is a key signaling linchpin for regulating multiple essential cellular processes.

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 (PIP$_2$) by phospholipase C (PLC), producing diacylglycerol and soluble inositol 1,4,5-trisphosphate (IP$_3$). IP$_3$ is sequentially phosphorylated by the coordinated actions of specific kinases to produce more highly phosphorylated IP molecules, including inositol 1,3,4,5-tetrakisphosphate (IP$_4$), inositol 1,3,4,5,6-pentakisphosphate (IP$_5$), inositol hexakisphosphate (IP$_6$), and inositol pyrophosphate isomers (e.g., PP-IP$_4$ and IP$_2$) (2, 24, 31, 41, 43, 55, 71). The perturbation of IP synthesis pathways is linked to defects in nutrient homeostasis in fungi (30, 41, 43) and vertebrates (23) all apparently converge on a single IP$_6$ 2-kinase, Ipk1 (64). Ipk1 enzymes have highly conserved putative catalytic site motifs and display functional cross-species complementation (25, 50, 64) (see Fig. S1 in the supplemental material). However, conservation at the overall protein sequence level is relatively low (~11%), suggesting potential functional and/or regulatory diversification in different organisms. The most striking difference in structural regions is observed in \textit{Schizosaccharomyces pombe} Ipk1 (SpIpk1), wherein a distinctive N-terminal domain exists with coiled-coil structural motifs similar to those of BAR (Bin-amphiphysin-Rvs) domain proteins (25). In the mammalian amphiphysin and \textit{S. cerevisiae} Rvs161/167 proteins, such BAR domains are dimerization, membrane-binding, and membrane curvature-sensing modules (45). Functional analysis of the SpIpk1 N-terminal domain has not been reported, and this domain might mediate specialized cellular roles of the protein.

Several recent studies have made key insights into defining the cellular targets for IP$_6$. The human RNA-editing enzyme ADAR2 and the \textit{S. cerevisiae} RNA-editing enzyme ADAT1 both require IP$_6$ binding for protein function (32). The efficient nuclear export of mRNA also specifically requires the Ipk1-catalyzed production of IP$_6$ (71). mRNAs are exported as large ribonucleoprotein (mRNP) complexes in a unidirectional manner through nuclear pore complexes (NPCs), embedded in the nuclear envelope (28). The targeting of export-competent mRNPs to NPCs is dependent on the essential mRNA export receptor dimer Mex67-Mtr2 in the budding yeast \textit{S. cerevisiae} and TAP/NXF1-p15/NXT1 in metazoan cells (18, 26, 54); however, Mex67 is not essential in \textit{S. pombe} (69). Two factors that

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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 mRNPs protein composition, thus providing directionality to the mRNA 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 (13). 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 for mRNA export has not been directly tested.

In addition to direct protein binding targets for IP₆, IP₆ is also the substrate 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-IP₄ and 1/3,5-(PP)₂-IP₄ (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 asp1*Δ cells. Notably, *S. pombe asp1*Δ 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 cytokinesis 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 also 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 Eng1 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 Ipkl 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 SpIpkl function. In addition to conserved defects in mRNA export, a loss of *ipkl*Δ function resulted in pleiotropic defects in cell morphology, polarized growth, endocytosis, and cell separation. By analyzing *ipkl1Δ asp1*Δ 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 *nml1* 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 *ipkl1*Δ strain, the *ipkl1*Δ 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 pKG358 using a forward oligonucleotide primer (spipk1Δ-F) and primer *nml1R* (see Table S1 in the supplemental material) corresponding to 80 bp downstream of the TAA stop codon and a reverse primer (spipk1Δ-R) (see Table S1 in the supplemental material) corresponding to 80 bp downstream of the TAA stop codon. For each primer, amplified products were cloned into the *S. pombe* vector pREP1 and transformed into the *S. pombe* diploid strain, and stable integrants were selected. The deletion of one copy of *ipkl1*Δ in strain SWY2558 was confirmed by PCR. For generating *ipkl1Δ* haploid strains SWY2559 and SWY2560, SWY2558 was sporulated, and tetrads were dissected. Strain SWY2559 (*h- ipkl1::ura4* leu-32 ura4-D18 ade6-M210) was used in this study.

For the generation of the *ipkl1 Δ asp1Δ* strain, SWY2559 was crossed with KGY956 (*h- asp1::ura4* leu-32 ura4-D18 ade6-M210) and sporulated, and double mutants were identified by tetrad analysis and confirmed by PCR.

**Gene cloning and deletion constructs.** To clone *ipkl1*Δ, specific cDNA was amplified by PCR with oligonucleotide primers *spipk1-NdeI-F* and *spipk1-3*C-term from a *S. cerevisiae* full-length cDNA library (Invitrogen)—derived total cDNA preparation from *S. pombe* wild-type cells. The PCR product was cloned into the EcoRI site of pBluescript SK (+) (Stratagene), resulting in pSW3019. The *S. pombe* strain SWY2509 was cloned into vectors pREP1 and pREP81GFP (5, 11, 36), placing *ipkl1* under the control of 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).

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**Analysis of levels of cellular [3H]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/ml [3H]inositol (Perkin-Elmer) to mid-logarithmic phase. Cells (5 ml) were harvested, washed in H₂O₃, 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 0.5 N NaCl. The mixture was vortexed at maximum speed for 2 min, followed by 100 μl of 0.5 N NaCl. The mixture was allowed to separate. By analyzing *ipkl1Δ asp1Δ* double mutants, we gained unique insights into the independent roles of the respective kinases in IP production and cell physiology.
RESULTS

ipk1Δ S. pombe cells are defective in nuclear mRNA export, cell morphology, polarized growth, and cell separation. We cloned full-length S. pombe ipk1+ based on the previously published sequence information (25); additionally, we generated an ipk1Δ strain. The ipk1Δ cells were viable at 30°C, temperature sensitive at 36°C, and cold sensitive at 18°C (Fig. 1B and see Fig. 7A and 8A). To test the metabolic effect of the ipk1Δ deletion on IP production, we compared the levels of soluble IPs in extracts isolated from ipk1Δ cells to those of wild-type cells. Cells were labeled to steady state with [3H]inositol, and extracted IPs were separated and analyzed by performance liquid chromatography (HPLC), with the IPs resolved by use of a Whatman Partisphere 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 in cacodylate buffer, 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 an 100-fold by centrifugation, and suspended in fresh YE medium. FM4-64 was stained with 0.1 μg/ml methyl blue solution (Sigma-Aldrich). To visualize DNA, 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 and scaled identically using MetaVue, version 4.6 (Morphos) and a camera (CoolSNAP HQ; Photometrics). Within each experiment, all images were collected and processed as described above.

Cytofluorometry. Septa were visualized by staining ethanol-fixed cells with 1 mg/ml methylene blue solution (Sigma-Aldrich). To visualize DNA, cells were 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 100× 1.30-numerical-aperture oil immersion objective (Olympus) and a camera (CoolSNAP HQ; Photometrics). Within each experiment, all images were collected and stained identicaly using MetaVue, version 4.6 (Molecular Devices), or Image-Pro Express (Media Cybernetics) and processed with Photoshop 9.0 software (Adobe).

RESULTS

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We also examined if the expression of ScIPK1 or Zlipk1 could restore IPα production in ipk1Δ cells. Both ScIPK1 and Zlipk1 expressions rescued IPα production in ipk1Δ cells (see S3 in the supplemental material), suggesting enzymatic complementation across species by the IPα 2-kinase enzymes. Next, we analyzed the ipk1Δ cells for mRNA export defects using oligo(dT) in situ hybridization for the subcellular distribution of poly(A)⁺ RNA. The ipk1Δ cells showed an accumulation of poly(A)⁺ RNA in the nucleus at a growth temperature of 30°C (Fig. 1A). Thus, we conclude that IPα production is required for efficient mRNA export in S. pombe.

Although ipk1Δ is not essential, ipk1Δ cells exhibited morphological and cell separation defects. The ipk1Δ 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 ipk1Δ 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 ipk1Δ 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 ipk1Δ 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 ipk1Δ cells are defective in the dissolution of both the septum and the cell wall that surrounds the septum. We speculate that ipk1Δ 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 ipk1Δ S. cerevisiae cells, suggesting that roles for Ipk1 in these cellular processes are specific to S. pombe.

Defects in morphology and cell separation in ipk1Δ 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 as 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 ipk1Δ 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 ipk1Δ cells are defective in the dissolution of both the septum and the cell wall that surrounds the septum. We speculate that ipk1Δ 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 ipk1Δ S. cerevisiae cells, suggesting that roles for Ipk1 in these cellular processes are specific to S. pombe.
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.

**FIG. 1.** *ipk1*<sup>+</sup> is required for nuclear mRNA export, polarized growth, and morphology. (A) mRNA export is inhibited in *ipk1*Δ cells. In situ hybridization with a digoxigenin-coupled oligo(dT) probe was conducted in wild-type and *ipk1*Δ cells grown in YE medium at 30°C. Poly(A)<sup>+</sup> RNA localization was visualized by indirect immunofluorescence microscopy with a fluorescein isothiocyanate-coupled anti-digoxigenin antibody (left), and nuclei were visualized with coincident DAPI staining (right). (B) *ipk1*Δ cells developed a swollen, rounded cell shape at 36°C. Presented are the differential interference contrast (DIC) images of wild-type and *ipk1*Δ cells grown in YE medium at 36°C for 6 h. (C) *ipk1*Δ cells are defective in cell separation. Shown are wild-type and *ipk1*Δ cells grown in YE medium at 30°C, ethanol fixed, and then stained with methyl blue and DAPI to reveal division septum and DNA, respectively. (D) *ipk1*Δ cells formed normal septa but exhibited defects in cell wall digestion and cleavage. Wild-type and *ipk1*Δ cells were grown at 30°C, fixed, and processed for transmission electron microscopic analysis as described in Materials and Methods. Presented are representative images of wild-type cells with a division septum (left) and *ipk1*Δ cells in the early (middle) and late (right) stages of septum digestion. Black arrowheads (right) point to the undigested cell wall materials. (E) *ipk1*Δ cells are defective in polarized growth. After growth at 30°C, wild-type (left) and *ipk1*Δ (right) cells were stained with rhodamine-phalloidin and DAPI for actin cytoskeleton organization and DNA, respectively.

The **SpIpk1 C-terminal domain is sufficient to rescue mRNA export, cell separation, and polarized growth defects in *ipk1*Δ S. pombe 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 *S. pombe* protein (see Fig. S1 in the supplemental material). To identify the relevant protein activity responsible for the defects exhibited by *ipk1*Δ *S. pombe* cells, we constructed a series of plasmids expressing *ipk1*<sup>+</sup> deletion mutants under the control of the *nmt1* promoter (Fig. 2). As complementation controls, plasmids harboring either
the full-length ipk1Δ under the control of the nmt1 promoter or a genomic fragment of ipk1+, with the endogenous promoter (ipk1+ gDNA) were used. The mRNA export, cell separation, and polarized growth phenotypes were assayed after induction for overexpression by the nmt1 promoter. The results from these analyses are summarized in Fig. 2. The ipk1Δ cells exhibited nuclear poly(A)+ RNA accumulation in 59% of the cells (Fig. 3C, D, and U). The overexpression of ipk1+, ipk1- gDNA, and ipk1 C-term suppressed the mRNA export defect of ipk1Δ cells (Fig. 2 and 3) and restored IP6 production (see Fig. S2 in the supplemental material). However, the level of rescue of the mRNA export defect by full-length ipk1+ was partial. Of note, in wild-type cells, the overexpression of ipk1+ resulted in a weak mRNA export defect, with nuclear poly(A)+ accumulation in 21% of the cells (Fig. 3E, F, and U). Most clearly, the overexpression of ipk1 N-term did not rescue the ipk1Δ mRNA export defect (Fig. 3S, T, and U), and IP6 production was not restored (see Fig. S2 in the supplemental material). In addition, 41% of wild-type cells overexpressing ipk1 N-term showed nuclear poly(A)+ accumulation (Fig. 3Q, R, and U). Together, we conclude that the loss of the Ipk1 catalytic domain is the critical defect linked to the mRNA export perturbation in ipk1Δ cells. For wild-type cells, there are also dominant negative effects on mRNA export from overexpressing either ipk1+ or ipk1 N-term.

We also examined the effects of overexpressing the ipk1+ deletions on cell separation and polarized growth in both wild-type and ipk1Δ cells. The overexpression of the ipk1+, ipk1 C-term, ipk1 ΔRVS (Rvs homology), or ipk1 ΔCC (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 ipk1+ or ipk1 C-term did not display any significant difference in F-actin distribution (Fig. 5A to F and U). Strikingly, the overexpression of ipk1+ gDNA, ipk1+, or ipk1 C-term suppressed both cell separation and polarized growth defects exhibited by ipk1Δ cells (Fig. 4N and S1 to P and U). There were differences in the cell separation defect levels between ipk1Δ cells grown in minimal medium and those grown in rich medium (see Fig. S4 in the supplemental material). The ipk1Δ 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 ipk1Δ 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 SpIpk1 C-terminal domain, and potentially IP6 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 Ipk1 N-terminal domain. A high percentage of wild-type cells overexpressing ipk1 N-term were multinucleated and multiseptated (Fig. 4C, F, and M). In contrast, the ipk1Δ cells overexpressing ipk1 N-term exhibited an increase in the number of binucleate-septated cells only (Fig. 4N). Additionally, we observed that ipk1 N-term overexpression perturbed polarized growth. Thirty-nine percent of wild-type cells overexpressing ipk1 N-term had de-localized cortical actin patches, whereas only 3% of wild-type vector-only cells showed an altered F-actin distribution (Fig. 5A, B, G, and H). Together, these data suggest that ipk1 N-term overexpression specifically perturbs septum cleavage, cell separation, and polarized growth.

Specific rescue of the mRNA export defect in ipk1Δ cells by overexpressing dbp5+. Given that the SpIpk1 C-terminal domain is sufficient to rescue both the mRNA export and cell separation defects in ipk1Δ cells, we speculated that the cell separation defects might be an indirect effect due to a lack of an IP6-dependent export of an mRNA(s) encoding proteins involved in cell separation. In S. cerevisiae, the IP6 target in the mRNA export pathway has been pinpointed to Gle1, a cofactor for Dbp5, and DBP5 overexpression specifically suppresses the mRNA export defect of an S. cerevisiae ipk1Δ nup42Δ mutant (1). Thus, we tested whether S. pombe dbp5+ overexpression suppresses the mRNA export defect of ipk1Δ S. pombe cells. Strikingly, the overexpression of dbp5+ fully rescued the mRNA export defect in ipk1Δ cells, whereas its overexpression in wild-type cells had no effect (Fig. 6). This suggests that the mRNA export defect in the ipk1Δ S. pombe cells is specifically linked to SpDbp5 function, similar to what is known for S. cerevisiae (1). In sharp contrast, the cell separation and polarized growth defects in ipk1Δ cells were not rescued by dbp5+ overexpression (Fig. 4N and 5B, T, and U). We conclude that the role of SpIpk1/Ip6 in cell separation and polarized growth is independent of its role in mRNA export.

Elevated levels of PP-IP6 production in ipk1Δ cells are linked to Asp1 function. A loss of the Ip6 kinase activity in asp1Δ cells results in cell morphology, cell separation, and polarized growth defects that are similar to those observed here in ipk1Δ cells (14, 41). Thus, we reasoned that these ipk1Δ phenotypes might be due to the indirect effect of a loss of IP6.

**FIG. 2.** Ipk1 kinase activity is critical for mRNA export, cell separation, and polarized growth. Shown are schematic diagrams of the ipk1+ ORF schematic. Presented is a summary of the results obtained from the overexpression of the constructs in wild-type and ipk1Δ cells shown in Fig. 3 [subcellular distribution of poly(A)+ RNA], Fig. 4 (cell separation), and Fig. 5 (polarized growth). ND, not determined.
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, J, 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 ipk1C-term (E to H), ipk1 gDNA with ipk1C-term under the transcriptional control of its own promoter (I to L), ipk1 N-term (M to P), and ipk1 C-term (M to P); 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).
empty vector, and plasmids expressing ipk1 overexpression were analyzed as described above. Presented is a bar graph as a control. Others represent percent multinucleate (at least one nucleus), multiseptum (more than one septum) cells (see Fig. S5 in the supplemental material). In contrast, 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 strain 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.

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 ipk1 or asp1+ in the respective mutants resulted in a partial to 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 unanticipated, because ipk1Δ cells fail to produce both IP2 and IP3 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, IP5 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 IP6 was significantly lower than that in ipk1Δ samples. In ipk1Δ asp1Δ cells, the PP-IP4-to-IP6 ratio was ~0.09, whereas in ipk1Δ cells, it was ~0.43. This indicates that Asp1 has an IP2 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 strain, there might be an additional kinase(s) responsible for this synthesis. Taken together, the elevated PP-IP4 level in the ipk1Δ strain 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.
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 asp1Δ 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 fluorochrom dyes 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 support 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 IPα and IPγ.

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 IPα and IPγ. 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 IPα production and highlight the cellular consequences of perturbing IP flux.

This work provides direct evidence implicating IPα 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 dbp5Δ overexpression (Fig. 6), we predict that SpGle1 will be an S. pombe IPγ 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, IPγ 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 IPγ production, it is not unexpected that ipk1Δ cells show similar defects. IPγ 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 IPα and/or IPγ in vesicular trafficking is also possible. It is known that IPα and IPγ both can modulate vesicular trafficking in fungi and mammalian cells (12, 20, 21, 49), and IPγ promotes dynamin-mediated endocytosis in pancreatic β cells (20). Additionally, a recent report suggested that IPγ is required for full exocytic capacity in insulin-secreting pancreatic β cells (21). Interestingly, in ipk1Δ cells, the septum assemblies normally, but the septum and its surrounding cell wall are not cleaved completely, resulting in an accumulation of septated cells. The overexpression of dbp5Δ 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-

FIG. 7. ipk1Δ cells exhibit both distinct and shared growth phenotypes compared with those of asp1Δ cells. (A) ipk1Δ cells have both cold-sensitive and temperature-sensitive growth defects. Wild-type, ipk1Δ, asp1Δ, and ipk1Δ asp1Δ cells were spotted onto YE medium in fivefold serial dilutions and grown at the temperatures shown. (B) ipk1Δ overexpression rescues the growth defects of ipk1Δ cells. Wild-type, ipk1Δ, asp1Δ, and ipk1Δ asp1Δ cells containing the indicated plasmids were grown in EMM lacking Leu and with 10 μM thiamine (to repress expression) and spotted onto EMM lacking Leu. (C) Deletion of the ipk1Δ and asp1Δ genes perturbs soluble IP levels. The strains in A were grown in minimal medium containing [³H]inositol. Soluble IPs were extracted and separated by Partisphere strong-anion-exchange HPLC. Presented are the IP profiles obtained from the same total cell number from each strain. Labels indicate IP elution positions.
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
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). The nuclear envelope, the medial cortex region where acto-myosin ring assembles, and the cytosolic membrane components involved in secretory cargo formation represent potential membrane microenvironments for SpIpk1 function. We propose that the unique N-terminal domain of SpIpk1 allows the spatially restricted production of IP₆ for roles in mRNA export, endocytosis, polarized cell growth, and cell separation.

In summary, our studies show that alterations in SpIpk1 function lead to defects in mRNA export and polarized growth and morphology and demonstrate that IP₆ acts as a critical regulator of these cellular processes. This places proper IP₆ function as a general mediator for a variety of cellular signaling processes involved in secretory cargo formation represent potential membrane microenvironments for SpIpk1 function. We propose that the unique N-terminal domain of SpIpk1 allows the spatially restricted production of IP₆ for roles in mRNA export, endocytosis, polarized cell growth, and cell separation.

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