The Meiosis-Specific Sid2p-related Protein Slk1p Regulates Forespore Membrane Assembly in Fission Yeast

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Cytokinesis in all organisms involves the creation of membranous barriers that demarcate individual daughter cells. In fission yeast, a signaling module termed the septation initiation network (SIN) plays an essential role in the assembly of new membranes and cell wall during cytokinesis. In this study, we have characterized Slk1p, a protein-kinase related to the SIN component Sid2p. Slk1p is expressed specifically during meiosis and localizes to the spindle pole bodies (SPBs) during meiosis I and II in a SIN-dependent manner. Slk1p also localizes to the forespore membrane during sporulation. Cells lacking Slk1p display defects associated with sporulation, leading frequently to the formation of asci with smaller and/or fewer spores. The ability of *slk1Δ* cells to sporulate, albeit inefficiently, is fully abolished upon compromise of function of Sid2p, suggesting that Slk1p and Sid2p play overlapping roles in sporulation. Interestingly, increased expression of the syntaxin Psy1p rescues the sporulation defect of *sid2-250 slk1Δ*. Thus, it is likely that Slk1p and Sid2p play a role in forespore membrane assembly by facilitating recruitment of components of the secretory apparatus, such as Psy1p, to allow membrane expansion. These studies thereby provide a novel link between the SIN and vesicle trafficking during cytokinesis.

INTRODUCTION

Cytokinesis in all organisms requires the formation of new membranes between segregated chromosomes, leading eventually to the formation of independent daughter cells (Guertin *et al.*, 2002; Wu *et al.*, 2003; Glotzer, 2001, 2005). In animal cells, assembly of new membranes occurs in coordination with constriction of the actin and myosin containing contractile ring and proceeds in a centripetal direction (Balasubramanian *et al.*, 2004; Gromley *et al.*, 2005; Matheson *et al.*, 2005; Baluska *et al.*, 2006). In plant cells, new membrane growth occurs in a centrifugal manner, and this centrifugal expansion is mediated by targeted vesicle delivery to the spindle midzone (Jurgens, 2005; Baluska *et al.*, 2006). Fungi appear to use two distinct mechanisms depending on the mode of cell cycle regulation. During vegetative growth, as in the case of animal cells, new membrane assembly occurs concomitant with constriction of an actomyosin ring (Balasubramanian *et al.*, 2004; Wu and Pollard, 2005; Wu *et al.*, 2006). In contrast, during meiosis and sporulation, new membrane assembly is initiated around the spindle pole bodies, in an apparently actomyosin ring-independent manner (Shimoda, 2004; Neiman, 2005). Although different cell types use different modes of cytokinesis, elements important for membrane targeting and vesicle fusion are highly con-

served. How these conserved molecules participating in membrane biogenesis respond to different signals remains largely unanswered.

In recent years, the fission yeast *Schizosaccharomyces pombe* has become particularly attractive for the study of cytokinesis, due to the ease with which genetic and cell biological analysis are applied in this organism (Gould and Simanis, 1997; McCollum and Gould, 2001; Wu and Pollard, 2005; Wu *et al.*, 2006). During vegetative growth fission yeast cells divide through the use of an actomyosin-based contractile ring (Marks *et al.*, 1986; Balasubramanian *et al.*, 1992, 1994, 1998; Fankhauser *et al.*, 1995; McCollum *et al.*, 1995; Chang *et al.*, 1997; Eng *et al.*, 1998; Le Goff *et al.*, 2000; Naqvi *et al.*, 2000; Wong *et al.*, 2000; Wu *et al.*, 2003). This ring undergoes constriction after completion of anaphase. New membranes and cell wall material are deposited concomitant with constriction of the actomyosin ring (Rajagopalan *et al.*, 2003; Wu *et al.*, 2003). Actomyosin ring maintenance, new membrane, and cell wall assembly in fission yeast require the function of the septation initiation network (SIN; McCollum and Gould, 2001; Simanis, 2003). The SIN is a signaling cascade comprising a small GTPase (Spg1p) and three protein kinases (Cdc7p, Sid1p, and Sid2p; Fankhauser and Simanis, 1994; Schmidt *et al.*, 1997; Sohrmann *et al.*, 1998; Sparks *et al.*, 1999; Guertin *et al.*, 2000; Hou *et al.*, 2000; Salimova *et al.*, 2000). These and other members of the SIN (Cdc11p, Cdc14p, Sid4p, and Mob1p) localize to the spindle pole bodies and are thereby thought to coordinate cytokinesis with completion of chromosome segregation (Chang and Gould, 2000; Hou *et al.*, 2000; Salimova *et al.*, 2000; Krapp *et al.*, 2001; Tomlin *et al.*, 2002; Morrell *et al.*, 2004). The protein kinase Sid2p and its binding partner Mob1p, which are considered

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as the downstream effectors of the SIN, localize to the division site, in addition to the spindle pole bodies (SPBs; Hou *et al.*, 2000, 2004). The Sid2p kinase has thus been hypothesized to phosphorylate molecules important for actomyosin ring maintenance, membrane and cell wall assembly, although the identities of substrates and the mechanism by which the SIN physically regulates cytokinesis remains unknown. Recent studies have shown that the SIN components also localize to the SPBs during meiosis and play an important role in forespore membrane assembly (Krapp *et al.*, 2006). The defect in forespore membrane assembly has been correlated with improper localization of the t-SNARE (syntaxin) Psy1p in some mutants (Krapp *et al.*, 2006). As in the case of mitotic cells, the precise mechanism linking SIN and forespore membrane assembly is not fully understood.

In this study we characterize Slk1p, a protein kinase related to Sid2p. We show that Slk1p localizes to the SPB and the forming spore membranes during meiosis. Slk1p and Sid2p function downstream of the SIN and appear to regulate forespore membrane assembly, perhaps by regulating membrane growth by facilitating recruitment of proteins such as the syntaxin Psy1p to the forming spore membranes.

MATERIALS AND METHODS

Yeast strains, Media, and Culture Conditions

Schizosaccharomyces pombe strains used in this study are listed in Table 1. Yeast strains were constructed by either random spore method or by tetrad analysis. Yeast cells were grown on YES medium or minimal media with appropriate supplements (Moreno *et al.*, 1991). Solid yeast extract-peptone-dextrose (YPD) medium was used for mating and sporulation. Growth temperatures were 24°C (permissive) for temperature-sensitive strains, and 30°C for all other strains. Homothallic yeast strains were induced to enter meiosis and sporulation on YPD for 24 h at 24°C (for *cdc 11-123* and *sid4-A1* under permissive

conditions) and then for 12 h at 24°C and then were shifted to 35°C for 10 h (*cdc 11-123* under restrictive conditions) and for 12 h at 24°C and then were shifted to 28°C for 14 h (for *sid4-A1* under restrictive conditions and for all other strains) and processed for staining or imaging experiments as the case may be. Thiamine was used at a final concentration of 25 µM to repress transcription from the *nmt1* and *nmt41* promoter (Basi *et al.*, 1993). Procedures for DNA-mediated transformation of *S. pombe* by the lithium acetate method have been described previously (Okazaki *et al.*, 1990).

The kan^R:*rad21-sid4* strain (MBY3507) and the kan^R:*rad21-sid4-gfp pcp1-mCherry* strain (MBY4933) were generated as follows: the oligonucleotides 5'-GAAGATCTAGACGAGTAGCTATAGCGGACA-3' (MOH2329) and 5'-CCTTAATTAAAAGAGCTTAATATGATTAGGAG-3' (MOH2330) were used to amplify the promoter of the *rad21* gene using wild-type genomic DNA as a template by PCR. A 1.5-kb DNA fragment carrying the *rad21* promoter was cloned into the BglII/PacI sites to replace *nmt1* promoter in the plasmid of pFA6a-kanMX6-P3nmt1-mGFP (pCDL956), thereby generating the plasmid pCDL1076. A 3-kb DNA fragment containing *rad21* promoter and kan^R gene was generated by PCR using the plasmid pCDL1076 as the template with the forward primer: 5'-CATGTGACTTACACTCTGGGTAACAAT-ATTAGTTTAAAGTCAGCTACTCACAACTAAACCTTTTAATCATGTTT-TAGAGAATTTCGAGCTCGTTTAAAC-3' (MOH2407) and the reverse primer: 5'-GATGGATGACTATCAAAATGAGAATGACCTAACCATCGGTAATCT-GTACTCAAATATCACAAAAGCCTCATCCATAAGAAGCTTAATATG-ATTAGGAG-3' (MOH2408). This 3-kb DNA fragment was introduced into the yeast strains MBY1238 (wild-type) and MBY4972 (*Sid4-GFP*, *Pcp1p-mCherry*). Desired transformants were selected on solid medium containing Geneticin (0.01 mg/ml, Sigma).

The strain *slk1K191R::ura4⁺* (MBY4436) was generated as follows: the oligonucleotides 5'-GGGGTACCAACATAGATAGGACCTACTGG-3' (MOH2773) and 5'-CCCCGGGCTGATTAGTTTCGAGCGGAATACG-3' (MOH2772) were used to amplify by PCR the *slk1* coding sequences using wild-type genomic DNA as a template. The PCR product was cloned into pJK210, yielding the plasmid pJK210-*slk1* (pCDL1254). Then, the plasmid pJK210-*slk1* (pCDL1254) was then mutagenized by the PCR based method using two oligonucleotides: 5'-CACACGAATTGTTAGCAATGAGAATGATGAAAAA-ATCAACATTAC-3' (MOH2792) and 5'-GTAATGTTGATTTTTCATCAT-TCTCATTGCTAACAAATTCGTGTG-3' (MOH2793); the underlined nucleotides denote the artificially introduced nucleotides that serve to replace lysine 191 with arginine, yielding the plasmid pJK210-*slk1K191R* (pCDL1255). The plasmid pCDL1255 was linearized with Sall restriction enzyme (~260 nucle-

Table 1. Yeast strains used in this study

Strains	Genotype	Reference or Source
MBY192	<i>h⁻ ura4-D18 leu1-32</i>	Our stock
MBY1238	<i>h⁹⁰ ade6-M210 leu1-32 ura4-D18</i>	Our stock
MBY1850	<i>h⁹⁰ slk1::ura4⁺ ade6-M210 leu1-32 ura4-D18</i>	Bimbo <i>et al.</i> (2005)
MBY3507	<i>h⁹⁰ kan^R:rad21-sid4 ade6-M210 leu1-32 ura4-D18</i>	This study
MBY3655	<i>h⁹⁰ slk1-gfp::ura4⁺ ade6-M210 leu1-32 ura4-D18</i>	This study
MBY3663	<i>h⁹⁰ spo3-gfp::leu2 leu1-32</i>	Nakamura <i>et al.</i> (2001)
MBY3664	<i>h⁹⁰ leu1-32::gfp-psy1</i>	Nakase <i>et al.</i> (2008)
MBY3698	<i>h⁹⁰ slk1::ura4⁺ spo3-gfp::leu2</i>	This study
MBY3699	<i>h⁹⁰ slk1::ura4⁺ leu1-32::gfp-psy1</i>	This study
MBY3975	<i>h⁹⁰ meu14-gfp::ura4⁺ leu1-32 ura4-D18</i>	Okuzaki <i>et al.</i> (2003)
MBY4355	<i>h⁹⁰ sid2-250 slk1::ura4⁺ ade6-M210 leu1-32 ura4-D18</i>	This study
MBY4363	<i>h⁹⁰ sid2-250 ade6-M210 leu1-32 ura4-D18</i>	This study
MBY4364	<i>h⁹⁰ slk1-gfp::ura4⁺ pcp1-mCherry::ura4⁺ ade6-M210 leu1-32 ura4-D18</i>	This study
MBY4377	<i>h⁹⁰ leu1-32::gfp-psy1 sid2-250 slk1::ura4⁺</i>	This study
MBY4415	<i>h⁹⁰ slk1K191R-gfp::ura4⁺ ade6-M210 leu1-32 ura4-D18</i>	This study
MBY4436	<i>h⁹⁰ slk1K191R::ura4⁺ ade6-M210 leu1-32 ura4-D18</i>	This study
MBY4492	<i>h⁹⁰ slk1::ura4⁺ meu14-gfp::ura4⁺ ade6-M210 leu1-32 ura4-D18</i>	This study
MBY4558	<i>h⁹⁰ sid2-250 slk1Δ/pREP1</i>	This study
MBY4559	<i>h⁹⁰ sid2-250 slk1Δ/pREP41</i>	This study
MBY4562	<i>h⁹⁰ sid2-250 slk1Δ/pREP1-psy1</i>	This study
MBY4563	<i>h⁹⁰ sid2-250 slk1Δ/pREP41-psy1</i>	This study
MBY4594	<i>h⁹⁰ meu14-gfp::ura4⁺ slk1::ura4⁺ sid2-250</i>	This study
MBY4599	<i>h⁹⁰ sid2-250 slk1Δ/pREP41-slk1</i>	This study
MBY4882	<i>h⁹⁰ cdc11-123 slk1-gfp::ura4⁺ pcp1-mCherry::ura4⁺ ade6 leu1-32 ura4-D18</i>	This study
MBY4883	<i>h⁹⁰ sid4-A1 slk1-gfp::ura4⁺ pcp1-mCherry::ura4⁺ ade6 leu1-32 ura4-D18</i>	This study
MBY4884	<i>h⁹⁰ kan^R:rad21-sid4 slk1-gfp::ura4⁺ pcp1-mCherry::ura4⁺ ade6 leu1-32 ura4-D18</i>	This study
MBY4933	<i>h⁹⁰ kan^R:rad21-sid4-gfp pcp1-mCherry::ura4⁺ ade6 leu1-32 ura4-D18</i>	This study
MBY4972	<i>h⁹⁰ sid4-gfp::ura4⁺ pcp1-mCherry::ura4⁺ ade6 leu1-32 ura4-D18</i>	This study
MBY5118	<i>h⁹⁰ slk1K191R-gfp::ura4⁺ pcp1-mCherry::ura4⁺ ade6 leu1-32 ura4-D18</i>	This study
MBY5210	<i>h⁹⁰ slk1-gfp::ura4⁺/pREP1-mCherry-atb2</i>	This study

otides from the initiation codon) and integrated into the strain MBY1238 to generate the strain MBY4436.

Gene Tagging

To fuse *Slk1p* with green fluorescence protein (GFP), the following oligonucleotides were used to amplified C-terminal coding sequences of *slk1* using wild-type genomic DNA as a template by PCR: 5'-CGGGGTACCTTACCTAGCAGAAATGGTCG-3' (MOH2114) and 5'-TCCCCCGGGGAGCAA-AAATTCATACAGGTC-3' (MOH2115). The PCR product was cloned into pJK210-GFP, yielding pJK210-*slk1*-C1.1-GFP (pCDL1008). The plasmid pCDL1008 was linearized with *NdeI* restriction enzyme (~1247 nucleotides from the initiation codon) and integrated into the strain MBY1238 to generate the strain MBY3655.

The strain *slk1K191R-gfp* (MBY4415) was generated as follows: the *slk1* genomic DNA was amplified by PCR using wild-type genomic DNA as a template and with the oligonucleotides: 5'-GGGGTACCACAAACATAGAT-AGGACCTACTGG-3' (MOH2773) and 5'-CCCCCGGGGAGCAAAATTCATACAGGTC-3' (MOH2115). The PCR fragment was cloned into pJK210-GFP, yielding the plasmid pJK210-*slk1-gfp* (pCDL1252). The plasmid pCDL1252 was mutagenized by the PCR-based method using the following oligonucleotides: 5'-CACACGAATTGTTAGCAATGAGAATGATGAAAAAATCAACATTAC-3' (MOH2792) and 5'-GTAATGTTGATTTTTCATCATTCCTCATTGCTAACAAATTCGTGTG-3' (MOH2793), where the underlined nucleotides denote the artificially introduced nucleotides that serve to replace the codon for lysine 191 with the codon for arginine, yielding the plasmid pJK210-*slk1K191R-gfp* (pCDL1253). The plasmid pCDL1253 was linearized with *Sall* restriction enzyme (~260 nucleotides from the initiation codon) and integrated into the strain MBY1238 to generate the *slk1K191R-gfp* strain (MBY4415).

Plasmid Constructs

The plasmids, pREP1-*psy1* and pREP41-*psy1* were constructed by cloning *psy1* cDNA into the vectors pREP1 (pCDL32) and pREP41 (pCDL38), respectively. The *psy1* cDNA was amplified by PCR using wild-type genomic DNA as a template and with the two primer pairs: 5'-CCGGTCCGACATGAATAAAGCAAACGATTATACAC-3' (MOH2883) and 5'-CCGGGATCCCTCAATGTC-TATTGCCAAGAACAGG-3' (MOH2884).

Microscopy

To detect GFP-fusion proteins, yeast cells were cultured on YPD plates to induce meiosis. Meiotic cells were fixed using glutaraldehyde and paraformaldehyde as described by Hagan and Hyams (1988). The cells were processed and stained with anti-GFP primary antibody (ab1218, Abcam, Cambridge, MA). Alexa Fluor-488-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR) was used as a secondary antibody. Nuclear DNA was stained with DAPI (4', 6-diamidino-2-phenylindole, Sigma, St. Louis, MO). Microscopic images were captured using Olympus IX71 fluorescence microscope (Melville, NY) equipped with a Photometrics Cool SNAP ES camera (Tucson, AZ). For confocal microscopy, cells were observed under Zeiss meta inverted LSC (LSM510; Thornwood, NY), and images were taken and analyzed with LSM 5 browser software. For time-lapse microscopy, cells were first induced to enter meiosis and spotted on a glass slide containing 2% agar pad with appropriate medium at room temperature (22–24°C).

Spore Viability Test

sid2-250 slk1Δ cells carrying the plasmids pREP1, pREP41, pREP41-*slk1*, pREP1-*psy1*, and pREP41-*psy1* were cultured in minimal medium lacking leucine and containing 25 μM thiamine to midlog phase. The cells were washed and cultured in minimal medium (lacking nitrogen, leucine, and thiamine) to OD₅₉₅ = 1.0. Approximately 5 μl of the concentrated cells was spotted and grown on minimal medium (lacking leucine) plates for sporulation at 24°C. Five days later, the sporulated cells were collected and digested with NEE-154 glucosylase (PerkinElmer Life Sciences, Boston, MA) overnight at 24°C. The digested sporulation mixture was treated with 30% ethanol to kill nonsporulated vegetative cells and washed three times with sterile water. Thereafter, around 2000 spores were spread on YES plates to score for viability.

Iodine Staining Assay

Cells were first grown in YES medium to midlog growth phase. Cells were washed three times and concentrated to OD₅₉₅ = 1.0. Approximately 5 μl of 10-fold serial dilutions of these wild-type and mutant cells was spotted and grown onto YPD plates, and the plates were incubated for 5 d at 24°C. Plates were then exposed to iodine vapor (I₂) to stain spore walls.

Electron Microscopy

Cells were incubated on YPD plates at 28°C for 18 h, except for the *sid2-250 slk1Δ* strain (MBY4355), which was incubated on YPD plates at 24°C for 30 h. Cells were collected and fixed with 3% glutaraldehyde in potassium phosphate buffer (pH 7.0). After washing several times with PBS, cells were postfixed in 2% OsO₄ for 2 h at room temperature. Then they were soaked in

a 0.5% aqueous solution of uranyl acetate for 2 h and mounted on a copper grid to form a thin layer. The cells were dehydrated by passing through graded ethanol and acetone and then were embedded in Spurr's resin. Sections were stained with uranyl acetate and viewed/photographed using a JEOL 200CX electron microscope (Peabody, MA) at 100 kV.

RESULTS

Slk1p, a Protein Kinase Related to the SIN-Component *Sid2p*, Localizes to the SPBs and Spore Periphery during Meiosis

The *Sid2p* kinase is one of several proteins that function as part of the SIN signaling pathway, which is required for actomyosin ring maintenance, constriction, and septation (Sparks *et al.*, 1999). Sequencing of the fission yeast genome revealed the presence of a novel *Sid2p*-related open reading frame that we refer to as *Slk1p* and the gene encoding this kinase as *slk1* (*sid2*-like kinase 1, encoded by SPCC417.06c). *slk1* has been referred to as *ppk35* as well as *mug27* based on functional genomic studies (Mata *et al.*, 2002; Bimbo *et al.*, 2005; Martin-Castellanos *et al.*, 2005). *Slk1p* consists of 624 amino acids with a putative 304 amino acid Ser-Thr kinase domain (Figure 1A). Homology searches using the Blast algorithm indicated that *Slk1p* is most related to fission yeast *Sid2p* and the budding yeast proteins *Dbf2p* and *Dbf20p* (Toyn and Johnston, 1994; Balasubramanian *et al.*, 1998; Komarnitsky *et al.*, 1998). Figure 1B shows an alignment of the kinase domains of these four proteins. The invariant lysine residue, known to be important for ATP binding, was found at position 191 of *Slk1p*.

To gain insight into the cellular role of *Slk1p* the subcellular localization of this protein was analyzed. To this end, a strain was generated in which the GFP was fused in-frame to the C-terminus of *Slk1p*. *Pcp1p*-mCherry (a marker for the spindle pole body: SPB) was introduced into this strain to visualize the SPB and monitor cell cycle progression (Flory *et al.*, 2002). Consistent with the absence of *slk1* mRNA in vegetative cells (Bimbo *et al.*, 2005), *Slk1p*-GFP was not visualized in vegetative cells (Figure 2Ai). We then observed *Slk1p*-GFP localization in cells undergoing cell fusion, meiosis, and sporulation. *Slk1p*-GFP was not detected during cell fusion and during horsetail movement of chromosomes (Figure 2Aii). Interestingly, *Slk1p*-GFP was visible in cells completing the first meiotic division as two discrete foci that colocalized with the *Pcp1p*-mCherry signal (Figure 2Aiii). The colocalization of *Slk1p* and *Pcp1p* suggested that *Slk1p* localized in the vicinity of the SPB in meiotic cells. During meiosis II, *Slk1p*-GFP was detected at all four SPBs (Figure 2Aiv). As sporulation commenced and progressed, *Slk1p*-GFP gradually disappeared from the four SPBs. Concomitantly, *Slk1p*-GFP signal appeared to spread from each of the four SPBs, forming a cup-like structure that moved toward the medial plane between each pair of segregating chromosomes (Figure 2A, v and vi). Time-lapse imaging further confirmed the apparent movement of *Slk1p*-GFP from the SPBs to the forming spore periphery (Figure 2B). *Slk1p*-GFP signal was not detected in mature spores (Figure 2Avii). Visualization of microtubules, using mCherry-tagged α -tubulin, further revealed that *Slk1p* localized to the meiotic SPBs after separation of the SPBs in meiosis I (Figure 2Ci). *Slk1p* persisted at the SPBs throughout meiosis II and gradually disappeared from the SPBs upon passage through anaphase B and upon full elongation of the meiosis II spindle (Figure 2C, ii–iv). It was difficult to ascertain if the *Slk1p* signal was lost from each pair of SPBs (connected by the meiosis II spindle) simultaneously. Collectively, these studies established that *Slk1p* specifically associated with the

Figure 1. The protein kinase Slk1p is related to the SIN component Sid2p. (A) Schematic representation of Slk1p. The conserved kinase domain of 304 amino acids (162–465) is in the middle of Slk1p. The arrow indicates the lysine residue within the putative ATP binding site at position 191. (B) Alignment of the predicted amino acid sequence of the kinase domains of Slk1p with *S. pombe* Sid2p and *S. cerevisiae* Dbf2p and Dbf20p. Identical amino acids are highlighted in black and similar amino acids are shaded in gray. Slk1p shares 51, 50, and 50% amino acid sequence identity with Sid2p, Dbf2p, and Dbf20p, respectively. The arrow indicates the conserved lysine residue in the consensus ATP-binding site.

SPB during meiosis I and II and also associated with the developing spore periphery.

Slk1p is highly related to Sid2p, the most downstream member of the SIN. We therefore tested if Slk1p localization, like that of Sid2p, depended on the SIN. To this end, we first created a strain (referred to as P_{rad21} -sid4-gfp) in which expression of the SIN scaffold protein Sid4p (as a GFP fusion) was under the control of the promoter of the *rad21* gene, which is strongly repressed during meiosis (Kitajima *et al.*, 2004). On transfer of this strain to conditions that promoted meiosis and sporulation, the level of Sid4p was dramatically reduced in two of the four SPBs present in meiosis II cells (Figure 3A). Thereby, we ascertained that Sid4p levels could be significantly reduced in two of the four SPBs in meiosis II cells, by meiotic shut-off of Sid4p expression using the *rad21* promoter. The stronger signals in two of the four SPBs might result from carry over of Sid4p-GFP from the vegetative cells. In contrast, Sid4p-GFP levels were roughly of comparable intensity in all four SPBs in wild-type cells undergoing meiosis II (Figure 3A).

We then checked if Slk1p localization was affected in cells expressing reduced levels of Sid4p. To this end, we first created a P_{rad21} -sid4 strain, in which the untagged Sid4p

coding sequences were placed downstream of the promoter sequence of the *rad21* gene. The P_{rad21} -sid4 strain was able to proceed through meiosis I and II, like wild-type cells. Interestingly, however, sporulation was abnormal in P_{rad21} -sid4 cells; the majority of tetranucleate cells were only capable of assembling two spores with a nucleus each, whereas the other two nuclei were not encapsulated within spore membranes and spore walls (Figure 3, B and C). We then evaluated the localization of Slk1p in P_{rad21} -sid4 cells. As a control, Slk1p localization was monitored in wild-type cells. Interestingly, the level of Slk1p at the SPB was significantly reduced in P_{rad21} -sid4 cells (Figure 3D; cf. the wild-type and P_{rad21} -sid4 panels). Reduction of Slk1p-GFP signal at the SPB was particularly evident in cells undergoing meiosis II (Figure 3, D and E; SPBs are marked with Pcp1p-mCherry). The weak Slk1p-GFP signal was only detected in two of the four SPBs during sporulation of P_{rad21} -sid4 cells. Consistently, Slk1p was detected in spore membranes in the vicinity of two SPBs (that contained Slk1p; shown in Figure 3D). Quantitation of observed localization patterns revealed that in wild-type cells with four visible SPBs, Slk1p was detected either on all four SPBs (~48% cells) or on spore membranes (~50% cells). In <2% cells with four SPBs, Slk1p was found

Figure 2. Slk1p localizes to the SPBs and the spore periphery during meiosis. (A) Visualization of Slk1p-GFP and the SPB marker Pcp1p-mCherry. (i) No detectable signal of Slk1p-GFP during vegetative growth. (ii) No detectable signal of Slk1p-GFP during horsetail stage of meiosis. (iii) Slk1p-GFP colocalizes with Pcp1p-mCherry on the two SPBs during meiosis I. (iv) Slk1p-GFP colocalizes with Pcp1p-mCherry on the four SPBs during early meiosis II. (v) Slk1p-GFP moves gradually from SPBs to the FSM during late meiosis II. (vi) Slk1p-GFP is distributed on the FSM at the end of meiosis II. (vii) Slk1p-GFP signal is not observed in mature spores. Slk1p-GFP/Pcp1p-mCherry-expressing cells (MBY4364) were cultured on YPD plates to induce meiosis. Scale bar, 10 μ m. (B) Time-lapse imaging of redistribution of Slk1p-GFP from the SPB to the spore periphery. Arrows show that Slk1p-GFP localizes to the SPBs. Scale bar, 5 μ m. (C) Observation of live cells (MBY5210) expressing mCherry-Atb2p and Slk1p-GFP, during the first meiotic division (i), metaphase II (ii), anaphase II (iii), and sporulation (iv). The tubulin marker mCherry-Atb2p was expressed under the control of *nmt1* promoter from the plasmid (pREP1-mCherry-*atb2*) on MM lacking leucine plates with 5 μ M thiamine. Scale bar, 10 μ m.

to exhibit a diffuse staining pattern. Interestingly, Slk1p was never detected on all four SPBs in *P_{rad21}-sid4* cells with four SPBs. In contrast, Slk1p was found to be present largely in a diffuse cytosolic pattern (~35%) or on two SPBs or on spore membranes in two spores (~55%; Figure 3E). Slk1p localization was also significantly altered in temperature-sensitive mutations affecting Sid4p, as well as another SIN component, Cdc11p (Figure 3, F and G). Collectively, these studies

established that Slk1p localization to the SPBs, and spore membranes during meiosis depends on the SIN scaffold proteins Cdc11p and Sid4p.

During mitosis, the localization of all other SIN components is independent of Sid2p function (Fankhauser and Simanis, 1994; Schmidt *et al.*, 1997; Sohrmann *et al.*, 1998; Sparks *et al.*, 1999; Chang and Gould, 2000; Guertin *et al.*, 2000; Hou *et al.*, 2000; Salimova *et al.*, 2000; Krapp *et al.*, 2001;

Figure 3. Slk1p-GFP localization to the SPB is dependent on the SIN scaffold proteins Sid4p and Cdc11p. (A) $P_{rad21}\text{-}sid4\text{-}gfp$ is a strain (MBY4933) in which Sid4p expression is under the control of the promoter of the *rad21* gene. Top panel, Sid4p-GFP levels were roughly of comparable intensity in all four SPBs in wild-type cells (MBY4972) undergoing meiosis II. Bottom panels, the level of Sid4p was dramatically reduced in two of the four SPBs present in meiosis II cells. Scale bar, 10 μ m. (B) Meiotic sporulation phenotype resulting from reduction of Sid4p function. Shown are images of $P_{rad21}\text{-}sid4$ cells containing two-spored asci. DAPI staining of these cells (demarcated with dotted lines) reveals the presence of four nuclei. $P_{rad21}\text{-}sid4$ cells (MBY3507) were induced to undergo meiosis, fixed, and stained with DAPI. Arrows show asci with two spores. The dotted lines trace the outline of the indicated two-spored asci with four nuclei. Scale bar, 10 μ m. (C) Quantification of the sporulation phenotypes in $P_{rad21}\text{-}sid4$ cells. Wild-type (MBY1238) and $P_{rad21}\text{-}sid4$ cells (MBY3507) were induced to undergo meiosis and sporulation on YPD plates for 2 d at 28°C. At least 500 tetranucleate cells were counted for each sample. (D) $P_{rad21}\text{-}sid4$ expressing Slk1p-GFP and Pcp1p-mCherry was induced to undergo meiosis on YPD plates. Top panel, Slk1p-GFP was detected in all four SPBs in wild-type cells (MBY4364) undergoing meiosis II. Bottom panel, Slk1p-GFP was detected in two or less SPBs in $P_{rad21}\text{-}sid4$ cells (MBY4884). The dotted lines

Tomlin *et al.*, 2002; Morrell *et al.*, 2004). We therefore tested the localization of all SIN components in *slk1Δ* meiotic cells. The localization of all known components of the SIN was unaffected in *slk1Δ* cells (Figure S1A). In addition, Slk1p-GFP localization to the SPB was unaffected in cells lacking two key components of the sporulation machinery, Spo3p (coiled-coil protein expressed in forespore membrane) and Spo15p (coiled-coil protein required for SPB modification during meiosis II; Ikemoto *et al.*, 2000; Nakamura *et al.*, 2001; Figure S1, B and C). Collectively, these experiments indicated that Slk1p localization, like that of Sid2p, depended on the SIN scaffold proteins Sid4p and Cdc11p and that Slk1p might function downstream of the SIN and upstream of the sporulation machinery during meiosis.

Slk1p Is Required for Proper Pore Formation

Previous studies have shown that Slk1p was dispensable for vegetative growth (Bimbo *et al.*, 2005). However, because Slk1p expression was significantly up-regulated during meiosis (Martin-Castellanos *et al.*, 2005) and Slk1p was detected at the SPBs and spore periphery, we investigated possible function(s) of this protein in meiosis. Homothallic (h^{90}) wild-type cells placed under nitrogen-limiting conditions underwent meiosis and sporulation, leading to the formation of four-spored asci (Figure 4A). In contrast, several abnormalities related to sporulation and/or meiosis were observed when *slk1Δ* h^{90} cells were placed under nitrogen-limiting conditions. First, *slk1Δ* ascospores were smaller than ascospores formed upon meiosis in wild-type cells (Figure 4, A and B). Second, ~89% of wild-type cells formed four mature ascospores in an ascus (Figure 4C). However, the frequency of four-spored asci in *slk1Δ* cells was only ~30%. Instead, a significant fraction of *slk1Δ* asci contained one, two, or three spores. Furthermore, the percentage of asci without spores in the *slk1Δ* mutant was ~58%, which is dramatically higher than that in wild-type cells (Figure 4, A and C). The small size of spores in *slk1Δ* asci suggested that these spores might not retain viability. Because the many of the spores were too small to be individually manipulated, we assessed the proportion of asci that contained at least one viable spore. To this end, asci from wild-type and *slk1Δ* cells were separated by micromanipulation and incubated for 3 d on growth medium. Although 98% of wild-type asci contained viable spores, only 35% of *slk1Δ* asci contained at least one viable spore (Figure 4D). Because cells expressing *slk1K191R*, in which ATP binding site of Slk1p was altered, also generated phenotypes similar to that displayed by *slk1Δ* cells (Figure 4, A, C, and D), it is likely that the kinase activity of Slk1p is important for its function in ascospore formation. Interestingly, Slk1p-K191R was able to localize to the SPBs, suggesting that the localization of Slk1p to the SPBs was indepen-

dent of the function of the putative ATP-binding site and potentially of the kinase activity of this protein (Figure 4, E and F). However, even though *slk1K191R* cells assemble aberrant and smaller spores, we have been unable to detect Slk1p-K191R on membranous structures, suggesting that the function of the putative ATP-binding site (and potentially the kinase activity) is important in relocation of Slk1p from the SPBs to the forming spore membranes.

We used electron microscopy (EM) to gain further insight into the phenotype resulting from loss of Slk1p function. All asci generated from wild-type cells contained four nuclei, each of which was surrounded by spore membranes and spore walls (Figure 4G). In contrast, nuclei were not packaged into spore membranes and spore walls frequently in cells lacking Slk1p. Thus loss of Slk1p led the formation of asci containing unpackaged nuclei as well as “anucleate shells” of spore walls and membranes (Figure 4H, marked with a white arrow). Collectively, these experiments established that Slk1p was important for proper sporulation and for the coordination of nuclear division with formation of spore membrane and spore wall.

*Spore Membrane Assembly Is Aberrant in *slk1Δ* Cells*

The analysis of *slk1Δ* cells by EM revealed the presence of anucleate spores and nuclei devoid of surrounding spore membranes and walls. To quantify the extent of these phenotypes, we simultaneously labeled the spore membranes and nuclei with GFP-Psy1p and DAPI, respectively. The GFP-Psy1p used in this experiment was expressed under control of the native chromosomal promoter as a single copy (Nakase *et al.*, 2008). Psy1p is related to t-SNARE syntaxins and localizes to spore membranes as well as in mature spores. Interestingly, we found that in 47.9% of *slk1Δ* asci, nuclei were not encapsulated within spore membranes, whereas in the other 52.1% asci, like in wild-type asci, nuclei were encapsulated within the spore membranes (Figure 5, A and B).

In fission yeast, spore formation proceeds through a series of temporally regulated events. First, the outer plaque of the SPB is modified to a crescent-shaped structure known as the meiotic outer plaque (Ikemoto *et al.*, 2000; Shimoda, 2004). This maturation event requires the SPB associated protein Spo15p (Ikemoto *et al.*, 2000; Shimoda, 2004). After this event, membranous vesicles are targeted to the meiotic outer plaque. The coiled-coil-containing protein Spo3p and the syntaxin-related protein Psy1p participate in this process (Nakamura *et al.*, 2001). Subsequently, membrane outgrowth is initiated at each of the modified SPBs and proceeds toward the midspindle region. Meu14p assembles into a ring structure at the leading edge of the forespore membrane and allows for orderly membrane expansion (Okuzaki *et al.*, 2003). After completion of spore membrane assembly, the spore wall is assembled by the combined action of enzymes, such as Ags1p/Mok1p and Bgs2p (Hochstenbach *et al.*, 1998; Liu *et al.*, 2000; Martin *et al.*, 2000; Vos *et al.*, 2007), which are involved in biosynthesis of α - and β -glucan, respectively.

To understand the molecular basis of the spore formation defect in *slk1Δ* cells, we investigated the localization of several proteins that participate in the individual steps. The localization of Spo15p (and the assembly of the Spo15p crescents) was unaffected in *slk1Δ* cells (Figure S2A and Table S3). In addition, we found that the spores were stained by iodine vapor (Bimbo *et al.*, 2005) and Bgs2p localized normally in the forming spores (Figure S2B), suggesting that elements of the spore wall biosynthetic machinery were active in *slk1Δ* cells.

Figure 3 (cont). in the Pcp1p-mCherry panel trace the outline of the cell. Scale bar, 10 μ m. (E) Quantification of Slk1p-GFP during meiosis II in wild-type (MBY4364) and *P_{rad21}-sid4* (MBY4884) cells. Cells were induced to undergo meiosis on YPD plates at 28°C for 16 h. At least 200 cells with four clearly discernable SPBs, marked by Pcp1p-mCherry, were counted for each sample. (F) Quantification of Slk1p-GFP during meiosis II in *sid4-A1* (MBY4883) cells. Cells were induced to undergo meiosis on YPD plates at 28°C for 14 h. At least 100 cells with four clearly discernable SPBs, marked by Pcp1p-mCherry, were counted for each sample. (G) Quantification of Slk1p-GFP during meiosis II in *cdc 11-123* cells (MBY4882). Cells were induced to undergo meiosis on YPD plates at 35°C for 10 h. At least 100 cells with four clearly discernable SPBs, marked by Pcp1p-mCherry, were counted for each sample.

Figure 4. Slk1p is required for proper sporulation. (A) Bright field images of asci. Wild-type (MBY1238), *slk1Δ* (MBY1850), and *slk1K191R* (MBY 4436) cells were sporulated on YPD plates for 2 d at 28°C and were observed by differential interference contrast microscopy. Scale bar, 10 μm. (B) Quantification of spore diameter in wild-type cells (MBY1238) or *slk1Δ* (MBY1850). Cells were induced to undergo meiosis and sporulation on YPD plates for 2 d at 28°C. Values are the mean ± SD. At least 200 spores were counted for each sample. (C) Quantification of spore number per ascus. At least 500 tetranucleate cells were counted for each sample. (D) Germination efficiency of wild-type, *slk1Δ* and *slk1K191R* spores. Asci formed on YPD plates for 2 d were randomly chosen and grown on YES solid medium after micromanipulation at 30°C. Colonies formed after 3 d of incubation at 30°C were counted. At least 200 asci were selected from each sample. (E) Slk1pK191R-GFP can localize to SPB during meiosis. The dotted lines indicate the outline of the asci. Pcp1p-mCherry was used as a marker of the SPB. Scale bar, 10 μm. (F) Quantification of localization patterns of Slk1p-GFP and Slk1p K191R-GFP during meiosis II. Cells expressing Slk1p-GFP (MBY 4364) and Slk1pK191R-GFP (5118) were induced to undergo meiosis on YPD plates at 28°C for 16 h. At least 200 cells with four clearly discernable SPBs, marked by Pcp1p-mCherry, were counted for each sample. (G) Thin-section EM images of the ascospores (white arrowhead) of wild-type cells. Scale bar, 1 μm. (H) Electron microscopy of ascospores of *slk1Δ* cells (MBY1850) to show anucleate spores and naked nuclei. Black arrows show small but normally encapsulated ascospores. Black arrowheads show naked nuclei. The white arrow points to an anucleate spore. Nuclei are marked by N. Scale bar, 1 μm.

We then investigated the behavior of Meu14p, which forms the leading edge of the forespore membrane. In wild-type cells Meu14p assembled into ring-structures near the SPBs in metaphase/early anaphase of meiosis II. As forespore membrane assembly proceeded, Meu14p rings migrated away from the SPBs and toward the midspindle. Finally, the Meu14p rings underwent constriction, after which Meu14p was detected in a diffuse pattern in the nuclei/spores (Figure 5C). Meu14p was able to assemble

rings in *slk1Δ* cells, and these rings also exhibited normal behavior, in that they migrated away from the SPBs. Interestingly, although Meu14p rings were able to constrict, constriction appeared to bisect the nucleoplasmic Meu14 signals, leading to the formation of more than four Meu14p-containing structures (Figure 5C, marked with arrows).

We then investigated the localization and behavior of syntaxin-related protein Psy1p in wild-type and *slk1Δ* cells. To further understand the development of this phenotype,

Figure 5. Dynamics of Meu14p and Psy1p in wild-type and *slk1Δ* asci. (A) Localization of GFP-Psy1p in wild-type and *slk1Δ* asci. Wild-type cells (MBY3664) and *slk1Δ* cells (MBY3699) expressing GFP-Psy1p were induced to undergo meiosis, fixed, and stained with DAPI. Green panel reveals GFP-Psy1p localization, whereas the red panel reveals the nuclear architecture. Several examples of failure of encapsulation of nuclei within spore membranes in *slk1Δ* are shown. (B) Quantification of normal and abnormal encapsulation of nuclei within spores of wild-type (MBY3664) and *slk1Δ* cells (MBY3699). (C) Time-lapse imaging of Meu14p-GFP in wild-type (MBY3975) and *slk1Δ* cells (MBY4492). In both cases, Meu14p assembles into rings. Although constriction of Meu14p rings leads to four Meu14p-containing structures in wild-type cells (Arrows), more than four (and up to eight) Meu14p-containing structures are detected upon constriction of Meu14p rings in *slk1Δ* cells (arrows). Scale bar, 10 μ m. (D) Time-lapse fluorescence microscopy of GFP-Psy1p in wild-type and *slk1Δ* cells. Top panel, wild-type cells (MBY3664). The rest of the panels show imaging of *slk1Δ* cells (MBY3699). In

all cases, Psy1p localizes initially and assembles cup-shaped structures. In some cases (I; 13/40 cells imaged) Psy1p localization and sporulation proceeded normally, but generated smaller spherically shaped spores. In other cases (II; 27/40 cells imaged) Psy1p and sporulation were abnormal and led to the defective spore formation. Scale bar, 10 μ m. (E) Quantification of the time taken for the assembly of the spore membranes after meiosis II in wild-type (MBY3664; n = 15) and *slk1Δ* cells (MBY3699; n = 21).

we compared behavior Psy1p-GFP by time-lapse microscopy in asci generated from wild-type and *slk1Δ* cells. GFP-Psy1p in wild-type cells initially appeared as a crescent shape near the SPBs at meiosis II, expanded as four cup-like structures in the asci, later became four round structures at the spore periphery, and persisted after spore maturation (Figure 5D). Similarly, GFP-Psy1p in *slk1Δ* cells appeared as two pairs of bright arcs at metaphase II and formed two pairs of cup-shaped membranes facing each other. However, in some *slk1Δ* cells, the cup-shaped GFP-Psy1p reorganized into round but smaller than normal structures, which led to smaller spores (Figure 5, D, I). In other *slk1Δ* cells, GFP-Psy1p assembled into cup-shaped structures, whose expansion led to the formation of abnormally shaped and unequally sized structures (Figure 5D, II). Time-lapse imaging studies also revealed that the time taken for the assembly of the spore membrane was longer in *slk1Δ* cells compared with that in wild-type cells (Figure 5E). Staining of wild-type and *slk1Δ* cells expressing GFP-Psy1p, with antibodies against GFP and tubulin revealed that in metaphase II and anaphase II localization of Psy1p was indistinguishable. However, GFP-Psy1p assumed abnormal localizations during progression through spore membrane assembly (data not shown). Collectively, time-lapse studies suggested that the inability of *slk1Δ* cells to form proper ascospores might result from a premature constriction of Meu14p-containing, leading edge rings and/or the insufficient delivery of Psy1p and associated factors to the forming spore membranes.

Sid2p and Slk1p Perform Overlapping Roles in Spore Formation

We have shown that Slk1p regulates the fidelity of the process of sporulation, but is not essential for it. Because Sid2p, the protein most related to Slk1p, and the entire SIN are expressed during meiosis and sporulation (Mata *et al.*, 2002; Krapp *et al.*, 2006), we considered the possibility that Sid2p and Slk1p might perform overlapping functions in the regulation of sporulation. To test if this was the case, we constructed a double mutant of the genotype *sid2-250 slk1Δ*. We have previously shown that the *sid2-250* allele is only partially functional at 24°C and is nonfunctional at 36°C (Mishra *et al.*, 2005). The homothallic *sid2-250* strain underwent meiosis and sporulation normally at 24°C. Interestingly, homothallic *sid2-250 slk1Δ* cells were completely unable to sporulate, under conditions in which *slk1Δ* was partially compromised for sporulation and the *sid2-250* was not compromised for sporulation (Figure 6A). Consistent with the inability of *sid2-250 slk1Δ* to sporulate, these double mutants did not stain with iodine vapor (Figure 6B). These studies established that the pair of highly related protein kinases Slk1p and Sid2p are indispensable for sporulation. Time-lapse imaging of Meu14p-GFP and Psy1p-GFP in the *sid2-250 slk1Δ* double mutant revealed similar behavior of these proteins in *slk1Δ* cells and *sid2-250 slk1Δ* cells (Figure 6, C and D). Electron microscopic analysis of *sid2-250 slk1Δ* cells revealed a more penetrant sporulation defect characterized by the presence of small spores that lacked nuclei (Figure 6E).

A strong reduction in the efficiency of sporulation was also observed in double mutants lacking Slk1p and two other components of the SIN; *mob1-R4* and *spg1-106* (Figure S3 and data not shown). The other SIN components did not combine to generate synthetic effects with *slk1Δ* at 24°C, upon iodine vapor staining. However, the observations that *cdc11-136*, *sid4-SA1*, *spg1-B8*, and *sid1-C14* are defective in sporulation (Krapp *et al.*, 2006) might indicate that Slk1p

might synergize with a significant proportion of the SIN to regulate sporulation.

Overexpression of psy1 Rescues Sporulation Defect of the sid2-250 slk1Δ Mutant

In *S. pombe*, previous studies have shown that the general protein secretion apparatus plays an important role in fore-spore membrane (FSM) assembly (Nakamura *et al.*, 2005). Because the sporulation phenotype of *sid2-250 slk1Δ* appeared to result from insufficient membrane delivery, we tested if overproduction of known components of the secretory pathway that function in sporulation were able to rescue *sid2-250 slk1Δ* cells. In particular, SpSec9p, related to SNAP-25, and its interacting partner, Psy1p, related to syntaxins, are essential for FSM assembly (Nakamura *et al.*, 2001, 2005). We therefore tested if increased dosage of Psy1p and Sec9p were able to suppress the sporulation defect of *sid2-250 slk1Δ* mutants. The *sid2-250 slk1Δ* strain (rather than the *slk1Δ* single mutant) was chosen because of the increased severity of the FSM formation defect in this double mutant. The homothallic *sid2-250 slk1Δ* double mutant (MBY4355) was transformed with the plasmids pREP41-*psy1*, pREP1-*psy1*, pREP41-*slk1*, pREP41-*sec9*, pREP1-*sec9*, pREP41, and pREP1. As expected, cells carrying the empty vector were incapable of sporulation (Figure 7, A and B). Under the conditions used, high-dosage expression of Sec9p did not lead to rescue of the sporulation defect of *sid2-250 slk1Δ* (data not shown). Interestingly, increased dosage of Psy1p led to a significant suppression of the sporulation defect of the *sid2-250 slk1Δ* double mutant (Figure 7, A and B). Quantification showed that there were ~37% asci in pREP1-*psy1* transformants (MBY4563) and 20% asci in pREP41-*psy1* transformants (MBY4562) containing spores, although the spores in the asci were smaller than wild-type spores, and the spore numbers in each ascus varied (Figure 7, A and B). Although spores from *sid2-250 slk1Δ* rescued by pREP41-*slk1* retained nearly 94% viability, ~35–50% of spores overexpressing Psy1p were viable (Figure 7C). These studies established that the syntaxin-related Psy1p might function as a key element downstream of Slk1p and Sid2p in the regulation of FSM assembly.

Genetic Interactions between spo3 and slk1 Mutants

We have shown that over expression of Psy1p rescues the sporulation defect of *slk1Δ sid2-250* mutants. Previous studies have shown over production of Psy1p rescued the sporulation defect of cells defective in the coiled-coil protein Spo3p, which plays an important role in FSM assembly (Nakamura *et al.*, 2001, 2005). We therefore attempted to study the behavior of Spo3p-GFP in *slk1Δ* cells. Surprisingly, we found that sporulation was completely abolished in *slk1Δ* cells expressing Spo3p-GFP (Figure 8A). This might result from the fact that Spo3p-GFP might be partially compromised for function. Like Psy1p-GFP, Spo3p-GFP localized normally to the forming membranes initially in both *slk1Δ* and *sid2-250 slk1Δ* cells (Figure S4 and data not shown). EM analysis confirmed that sporulation was drastically affected in the *slk1Δ spo3-gfp* strain, and unpackaged nuclei and “anucleate shells” were routinely detected in asci generated from this strain (Figure 8B). Thus it is likely that Spo3p functions in parallel with Slk1p or that loss of Slk1p function might result in reduction of Spo3p function and further reduction of function of Spo3p-GFP.

Figure 6. Overlapping role for Slk1p and Sid2p in sporulation. (A) Synthetic genetic interaction between *sid2-250* and *slk1Δ* mutants. Left panel, proper spores are formed in *sid2-250* cells (MBY4363) at 24°C. Middle Panel, aberrant spores are formed in *slk1Δ* cells (MBY1850). Right panel, no spores are formed in *sid2-250 slk1Δ* cells (MBY4355). Scale bar, 10 μ m. (B) Iodine vapor staining of *slk1Δ*, *sid2-250*, and *sid2-250 slk1Δ* cells. A heterothallic wild-type strain (h^-) and a homothallic wild-type strain (h^{90}) were used as negative and positive control, respectively. (C) Time-lapse imaging of Meu14p-GFP in *sid2-250 slk1Δ* cells (MBY4594). Initially Meu14p assembles into rings in these cells. However, constriction of Meu14p rings leads to more than four (and up to eight) Meu14p-containing structures (arrows). Scale bar, 10 μ m. (D) Time-lapse fluorescence microscopy of GFP-Psy1p in *sid2-250 slk1Δ* cells (MBY4377). Psy1p localizes initially and assembles cup-shaped structures. However, spore membrane expansion was abnormal leading to defective sporulation. Scale bar, 10 μ m. (E) EM images of the ascospores of *sid2-250 slk1Δ* cells (MBY4355) show anucleate spore wall and naked nuclei. The black arrowheads show naked nuclei. White arrows show misshapen anucleate spore wall. Nuclei are marked by N. Cells were cultured on YPD plates for 2 d at 24°C to induce meiosis. Scale bar, 1 μ m.

DISCUSSION

The Protein Kinases Slk1p and Sid2p Localize to the SPB during Meiosis

The SIN is a signaling cascade that is essential for cytokinesis in vegetative *S. pombe* cells (Balasubramanian *et al.*, 1998; McColm and Gould, 2001; Simanis, 2003). The SIN comprises a GTPase (Spg1p) and three protein kinases (Cdc7p, Sid1p, and

Sid2p), all of which localize to the SPBs, whereas the Sid2p kinase and its binding partner Mob1p also localize to the division site (Sparks *et al.*, 1999; Hou *et al.*, 2000; Simanis, 2003; Hou *et al.*, 2004; Krapp *et al.*, 2004). Recent studies using DNA microarrays have shown that the SIN is also expressed during meiosis (Mata *et al.*, 2002). Components of the SIN localize to the SPBs during meiosis (Krapp *et al.*, 2006). However, none of the SIN components have been shown to localize to the divi-

Figure 7. Increased expression of *psy1* suppresses the sporulation defect of *sid2-250 slk1Δ* double mutant cells. (A) Bright field microscopy of meiotic *sid2-250 slk1Δ* cells carrying the indicated plasmids. Expression of *psy1* or *slk1* was induced on minimal medium at 24°C for 5 d. Scale bar, 10 μ m. (B) Sporulation efficiency of the transformants. Transformants were scored for the percentage of asci that contained present spores. (C) Quantification of spore germination of the transformants carrying the indicated plasmids. Vegetative cells were cultured to midlog phase in liquid minimal medium supplemented with 25 μ M thiamine, concentrated to OD₅₉₅ = 1.0 and then spotted on MM complete without leucine plates to allow sporulation. The sporulated cells were collected and digested with NEE-154 glucosylase, and 2000 spores were plated on a YES plate and incubated for 5 d at 24°C.

sion site (i.e., newly forming membranes) during sporulation. Previous studies have, however, shown that SIN components are important for proper sporulation and for forespore membrane assembly (Krapp *et al.*, 2006).

In this study we have characterized Slk1p, a protein highly related to Sid2p, the most downstream component of the SIN. Slk1p has been identified as the product of the *mug27* gene (meiotic up-regulated gene-27) from a microarray based analysis of genes expressed during meiosis, and its expression has not been detected in mitotically growing vegetative cells (Martin-Castellanos *et al.*, 2005). Slk1p has been independently characterized by two other research groups recently and these authors arrive largely at similar conclusions (Ohtaka *et al.*, 2008; Perez-Hidalgo *et al.*, 2008).

Although the regions of high similarity between Slk1p and Sid2p lie in the protein kinase domain (~51% identity), the nonkinase domains are also related, in that the amino acids upstream of the kinase domain exhibit 18% identity, whereas those downstream of the kinase domain exhibit 28% identity. Slk1p, like Sid2p, contains activatory phosphorylation sites within the kinase domain (serine 356) and the nonkinase domain (threonine 537). Phosphorylation of these sites is thought to facilitate binding of the activatory subunit Mob1p (Hou *et al.*, 2004). Interestingly, we have found a two-hybrid interaction (data not shown) between Slk1p and Mob1p as well as synthetic genetic interactions between cells defective in *slk1* and *mob1*, suggesting that Mob1p might serve as an activating subunit of Slk1p.

Slk1p, like other components of the SIN, localizes to the SPB during meiosis I and II. Interestingly, unlike other components of the SIN, Slk1p is also detected at the forming forespore membrane. It is presently unclear if Sid2p, which is most related to Slk1p, does not localize to the forespore membranes or that the Sid2p signal at the FSM is too weak to be clearly detected. Previous studies have identified a pathway, composed of proteins such as Spo15p and Spo3p, which participates in the maturation of the SPB during meiosis II as well as in the process of FSM assembly (Ikemoto *et al.*, 2000; Nakamura *et al.*, 2001). We have shown that the localization of Slk1p to the SPBs is independent of the function of Spo3p and Spo15p. Although Slk1p localizes normally in the improperly formed FSMs in *spo3Δ* cells, Slk1p is not detected at the FSM in *spo15Δ* cells. Presently it

is unclear if this reflects a requirement for Spo15p in the localization of Slk1p to the FSM or if the absence of Slk1p at the FSM is purely a consequence of the lack of FSM in *spo15Δ* cells. Interestingly, we have found that reduction of the level of the SIN scaffold protein, Sid4p (upon meiotic shut-off of *sid4* expression in *P_{rad21}-sid4* cells) or impairment of function of the SIN scaffold Cdc11p leads to a dramatic reduction in the levels of Slk1p at the SPBs, suggesting that Slk1p, like Sid2p, might function downstream of the SIN. This conclusion is also consistent with the finding that localization of all SIN components tested is unaffected in *slk1Δ* cells. The sequence similarity between Slk1p and Sid2p, taken together with the localization dependencies between the SIN and Slk1p, suggest that Slk1p might be a novel downstream component of the SIN during meiosis and sporulation.

Slk1p and Sid2p Are Essential for Sporulation and Regulate FSM Assembly

Previous studies have shown that Slk1p is not essential for viability although minor defects in chromosome segregation during meiosis have been reported (Bimbo *et al.*, 2005; Martin-Castellanos *et al.*, 2005). We have found a variety of defects pertaining to sporulation in *slk1Δ* cells. These defects include the formation of asci that contain less than four spores, the formation of asci with no spores, as well as the formation of those with four small-sized spores. We have found that a mutant allele of *slk1* (*slk1K191R*), that specifically affects the putative ATP binding site in Slk1p leads to defective sporulation, suggesting that the ATP binding site (and by extension the protein kinase activity of Slk1p) is essential for its role in sporulation. Because Slk1pK191R is capable of localizing to the SPB but not the forming spore membranes of the improper spores, it is likely that the protein kinase activity might control functions of Slk1p directly pertaining to later events of spore membrane or wall assembly.

Consistent with the high sequence similarity between Slk1p and Sid2p, we have found that the simultaneous loss of both of these proteins leads to a complete inability in sporulation. Stronger sporulation defects were also observed when *slk1Δ* was combined with *mob1-R4* and *spg1-106*, mutations affecting two key components of the SIN

Figure 8. Genetic interactions between *spo3-gfp* and *slk1* mutants. (A) Bright-field images of asci from strains of indicated genotypes. *spo3-gfp* (MBY3663), *slk1Δ* (MBY1850) and *slk1Δ spo3-gfp* (MBY 3698) cells were sporulated on YPD plates at 28°C for 2 d and were observed by phase contrast microscopy. Scale bar, 10 μ m. (B) EM images of the ascospores of *slk1Δ spo3-gfp* cells (MBY3698) show anucleate spore wall and naked nuclei. The black arrowheads show naked nuclei. White arrows show misshapen anucleate spore wall. Nuclei are marked by N. Scale bar, 1 μ m. (C) Schematic representation of data described in the study.

(Figure S3 and data not shown). The genetic interaction between *slk1* and upstream SIN mutants might indicate that Slk1p functions in parallel with the SIN to regulate sporulation. However, it is more likely that the compromise of upstream SIN mutants might result in reduced function of the downstream component of the SIN, Sid2p. This compromise of *sid2* function might in turn result in the strong additive effect between upstream SIN mutants and *slk1*, as in the case of *slk1* and *sid2-250*.

What is the molecular function of Slk1p and Sid2p in sporulation? Although under some conditions, cells lacking *slk1* display minor chromosome segregation defects (Mata *et al.*, 2002; Bimbo *et al.*, 2005; Martin-Castellanos *et al.*, 2005), the vast majority of *slk1Δ* and *sid2-250 slk1Δ* cells undergo meiosis I and II without obvious defects (data not shown). These observations suggest that the inability of *sid2-250 slk1Δ* mutants to sporulate is unrelated to defects in progres-

sion through meiosis I and II. The fact that Slk1p localizes to the forespore membrane during sporulation, instead, suggests a more direct role for Slk1p (and possibly Sid2p) in spore assembly.

Sporulation in fission yeast involves a series of highly coordinated events, starting with the maturation of the meiosis II SPB to the eventual assembly of spore membranes and spore walls (Shimoda, 2004). The coiled-coil domain containing protein Spo15p plays a key role in the initiation of forespore membrane assembly, by allowing the maturation of meiosis II SPB, leading to the formation of a Spo15p crescent-structure (Ikemoto *et al.*, 2000). Cells defective in *slk1Δ* as well as those doubly defective in *slk1Δ* and *sid2-250* are able to assemble Spo15p crescents, suggesting that Slk1p and Sid2p do not play an important role in the maturation of the meiosis II SPB. After maturation of the SPB, secretory vesicles are directed toward the mature SPBs. Spo3p, a

coiled-coil domain containing protein, and the syntaxin-related protein Psy1p are important for assembly of new membranes (Nakamura *et al.*, 2001). Assembly of membranes is initiated near the meiosis II SPB and a leading edge composed of a ring of Meu14p and associated factors allows growth of membranes, initiating at the SPB and moving toward the midspindle (Nakamura *et al.*, 2001; Okuzaki *et al.*, 2003; Shimoda, 2004). We have found that Meu14p assembles leading edges in *slk1Δ* and *sid2-250 slk1Δ* mutants. However, unlike in wild-type cells these Meu14p leading edge-rings constrict and bisect the nucleoplasm. Analysis of the syntaxin Psy1p provided further clues into a potential molecular function of Slk1p and Sid2p in sporulation. Psy1p (and its interacting partner Spo3p; data not shown) assembles normally near the SPB in *slk1Δ* and *sid2-250 slk1Δ* double mutants. However, during forespore membrane extension, the Psy1p and Spo3p are not maintained properly, leading to the formation of smaller spores lacking nuclei. These observations suggest at least two possibilities. First, it is possible that Slk1p and Sid2p delay Meu14p (leading edge) ring constriction until the chromosomes have fully segregated to the daughter nuclei. Second, Slk1p and Sid2p might play a partial role in the recruitment of components important for secretory vesicle targeting, failure of which might lead to constriction of the Meu14p ring at inappropriate locations leading to defective spatial coordination of spore membrane assembly with nuclear position. We favor this second scenario because over expression of Psy1p leads to reversal of the sporulation defect of *sid2-250 slk1Δ* mutants, as well as to increased spore viability. The decreased speed of spore formation in *slk1Δ* cells (this study and Perez-Hidalgo *et al.*, 2008) as well as the formation of smaller sized spores in *slk1Δ* cells are also consistent with a role for Slk1p in recruiting components important for spore membrane biogenesis.

Although ascertaining the precise mechanism of action of Slk1p and Sid2p during meiosis requires the identification of substrates of these kinases, genetic analyses suggest a few possibilities (Figure 8C). In particular genetic interactions have been uncovered between *slk1* and *spo3* and *slk1* and *psy1*. Given the strong interactions between these three proteins, it is possible that Slk1p might phosphorylate either Spo3p or Psy1p, or both. *Saccharomyces cerevisiae* Sso1p, a protein highly related to fission yeast Psy1p has been shown to be recruited to membranes upon dephosphorylation (Marash and Gerst, 2001). It is possible that activation of Slk1p and Sid2p might lead to phosphorylation of Spo3p or indirectly to the dephosphorylation of Psy1p, leading to membrane recruitment and membrane expansion. Future studies should test these possibilities.

Although the current study has focused on the links between the SIN and vesicle targeting in meiosis and sporulation, it will be interesting to assess if the SIN might play a similar role in vesicle targeting during normal cytokinesis. In this context, it is interesting to note that the mammalian protein centriolin, which is related to the fission yeast SIN component Cdc11p, plays an important role in recruitment of the exocyst and SNAREs during terminal stages of cytokinesis (Gromley *et al.*, 2005). It is therefore likely that the SIN might emerge as a key regulator of the assembly of new membranes during cytokinesis.

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