

# Sid4p is required to localize components of the septation initiation pathway to the spindle pole body in fission yeast

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**A mutation in the *Schizosaccharomyces pombe* *sid4*<sup>+</sup> (septation initiation defective) gene was isolated in a screen for mutants defective in cytokinesis. We have cloned *sid4*<sup>+</sup> and have found that *sid4*<sup>+</sup> encodes a previously unknown 76.4-kDa protein that localizes to the spindle pole body (SPB) throughout the cell cycle. Sid4p is required for SPB localization of key regulators of septation initiation, including the GTPase Spg1p, the protein kinase Cdc7p, and the GTPase-activating protein Byr4p. An N-terminally truncated Sid4p mutant does not localize to SPBs and when over-produced acts as a dominant-negative mutant by titrating endogenous Sid4p and Spg1p from the SPB. Conversely, the Sid4p N-terminal 153 amino acids are sufficient for SPB localization. Biochemical studies demonstrate that Sid4p interacts with itself, and yeast two-hybrid analysis shows that its self-interaction domain lies within the C-terminal half of the protein. Our data indicate that Sid4p SPB localization is a prerequisite for the execution of the Spg1p signaling cascade.**

**T**o ensure proper segregation of genetic material and organelles to daughter cells during cell division, the onset of cytokinesis must be coordinated with the completion of mitosis. The regulation of cytokinesis has been extensively studied in *Schizosaccharomyces pombe* because it is amenable to both genetic and biochemical studies. Furthermore, *Sz. pombe* divides by using a medial actomyosin contractile ring, a process similar to cell division in animal cells (1–3).

Through genetic and biochemical studies in *Sz. pombe*, key components of a GTPase signaling pathway involved in regulating and initiating cytokinesis have been identified (reviewed in refs. 4–6). Mutations in many of these components give rise to the *sid* (septation initiation defective) phenotype. In *sid* mutants, the medial actomyosin ring forms, but it fails to constrict, and septum formation does not occur. However, nuclear division and growth continue, producing elongated multinucleate cells. The *sid* mutants display genetic interactions (7, 8) that are consistent with the Sid proteins acting in the same biochemical pathway. Several of the *sid* pathway components have now been analyzed at the molecular level, permitting positioning of components within a signaling cascade. The *spg1*<sup>+</sup> gene encodes a small Ras-superfamily GTPase, whose activation is thought to trigger the *sid* pathway (8–10). The *sid1*<sup>+</sup>, *sid2*<sup>+</sup>, and *cdc7*<sup>+</sup> genes encode protein kinases that act downstream of Spg1p activation to initiate septation (refs. 8, 10, and 11; D. Guertin and D. McCollum, personal communication). The two-component GTPase-activating protein (GAP) for Spg1p, composed of Cdc16p and Byr4p (12), works antagonistically to prevent septation.

Several proteins regulating the *sid* pathway have been shown to localize to the spindle pole body (SPB) during at least some portion of the cell cycle (10, 11, 13). Spg1p is present at the SPB throughout the cell cycle. It is in an inactive GDP-bound form on both SPBs during interphase, an active GTP-bound form on both SPBs during early mitosis, and an active GTP-bound form on one SPB in anaphase B (11). Both Cdc16p and Byr4p are present on the SPB during interphase (13), presumably inacti-

vating Spg1p. During early mitosis, Cdc16p levels decrease at the SPBs, which permits the activation of Spg1p at both SPBs (11, 13). In late anaphase, Cdc16p and Byr4p levels increase at one SPB, resulting in asymmetric inactivation of Spg1p at one SPB (11, 13). Cdc7p kinase activity, which is not cell cycle regulated, is apparently spatially regulated by Spg1p such that its SPB localization is coincident with the activated GTP-bound form of Spg1p (11). Unlike Cdc7p, Sid2p is present at the SPB throughout the cell cycle, but its kinase activity is cell cycle regulated and peaks during medial ring constriction and septation (10). Sid2p activity depends on several components of the *sid* pathway, including *cdc7*<sup>+</sup> and *spg1*<sup>+</sup> (10). Unlike Cdc7p, Sid2p localizes to the actomyosin ring at late anaphase (10). Thus, it appears that Spg1p recruits Cdc7p to the SPB, which sets in motion the events leading to cell division.

The Spg1p signaling pathway is related to a pathway required for cyclin destruction and exit from mitosis in *Saccharomyces cerevisiae*. Spg1p, Cdc7p, and Sid2p are homologous with *Sc. cerevisiae* Tem1p (9, 14, 15), Cdc15p (16), and Dbf2p (8), respectively. In the absence of these protein functions, *Sc. cerevisiae* cells become arrested in telophase as large-budded cells with segregated chromosomes, an elongated spindle, and high Cdc28/Clb2 protein kinase activity (reviewed in ref. 17). In addition, *Sz. pombe* Cdc16p and Byr4p are homologues of *Sc. cerevisiae* Bub2p and Byr4p/Bfa1p, respectively (18–21). The existence of similar pathways in *Sz. pombe* and *Sc. cerevisiae* indicates that this signal transduction pathway is conserved, although its target(s) may vary between species.

In a previous screen for mutants defective in cytokinesis, we identified a mutation affecting the *Sz. pombe* *sid* pathway, *sid4-SAI* (8). This temperature-sensitive (ts) mutation is synthetically lethal with *spg1* and *sid2* mutations, and Spg1p-induced septum formation requires Sid4p function (8). Here, we further characterize the *sid4*<sup>+</sup> gene and its product and demonstrate that Sid4p localizes to the SPB. Sid4p localization is independent of other components required for septum formation, but all other characterized components of the Spg1p GTPase signaling pathway require Sid4p for their localization to the SPB.

## Materials and Methods

**Strains, Media, Genetic Methods, and Plasmid Manipulations.** Strains were grown in yeast extract (YE) or minimal medium with

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Abbreviations: GFP, green fluorescent protein; HA, hemagglutinin; SPB, spindle pole body; ts, temperature-sensitive; DAPI, 4',6-diamidino-2-phenylindole.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF153475).

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appropriate supplements (22). Crosses were performed on glutamate medium, and double-mutant strains were constructed by tetrad analysis (22). Unless otherwise indicated, yeast transformations were performed by electroporation (23). Plasmid manipulations and bacterial transformations used standard procedures (24). Strains and plasmids used in this study are listed in Tables 1 and 2 (supplementary material at [www.pnas.org](http://www.pnas.org)).

**Cloning and Sequencing of *sid4*<sup>+</sup>.** *sid4-SAI* mutant cells (KGY1234) were transformed with a pUR19-based *Sz. pombe* genomic library (25), and one plasmid, pKG986, permitted growth at restrictive temperature after isolation and retransformation in KGY1234. For integration mapping, the 4.0-kb genomic fragment was subcloned into the *Sz. pombe ura4*-based integrating vector pJK210 (26) to generate pKG1068. pKG1068 was linearized at the unique *Nde*I site in *sid4*<sup>+</sup> and transformed into KGY1234. *Sid4*<sup>+</sup> *Ura*<sup>+</sup> transformants were picked and outcrossed to either *sid4*<sup>+</sup> or *sid4-SAI* strains to verify cosegregation of the *sid4*<sup>+</sup> phenotype with the *ura4*<sup>+</sup> marker. Approximately 2.7 kb of the 4.0-kb insert was sequenced; it corresponded to a sequenced region of the *Sz. pombe* genome on cosmid c244. The *sid4*<sup>+</sup> ORF (GenBank accession no. AF153475) corresponds to g3184072 of the Sanger Center (SPBC244.01c). To sequence the *sid4-SAI* allele, the *sid4* locus was amplified by PCR from genomic DNA prepared from KGY1234. The PCR products were sequenced directly, and a single nucleotide change was detected, at position 2116 (T to A).

**Deletion of *sid4*<sup>+</sup>.** pSK *sid4*<sup>+</sup> (pKG1098) was cut with *Nde*I/*Stu*I to release a fragment containing 1,256 nucleotides of *sid4*; this fragment was replaced with the *ura4*<sup>+</sup> gene, creating pKG1269. This procedure left sequences encoding the first 153 and last 48 amino acids of Sid4p. A fragment containing *ura4*<sup>+</sup>, as well as 670 bp of 5' and 1 kb of 3' *sid4*<sup>+</sup> flanking sequences, was isolated from pKG1269 and transformed into a diploid strain (KGY137). Transformants were selected on medium lacking uracil, and stable integrants were identified. Replacement of one copy of *sid4*<sup>+</sup> with the *ura4*<sup>+</sup> cassette was confirmed by Southern hybridization analysis in strain KGY1358. Tetrads segregated 2:2 for viability and all viable colonies were *Ura*<sup>-</sup>, indicating that deletion of *sid4* was lethal.

**Overexpression Analysis.** To study the overexpression phenotype of *sid4*<sup>+</sup> constructs, strains were grown to mid-logarithmic phase in the presence of 2  $\mu$ M thiamin and then grown in medium lacking thiamin to induce the thiamin-repressible *nmt1* promoter at temperatures and times indicated.

**Epitope Tagging of *sid4*<sup>+</sup>.** The *sid4*<sup>+</sup> chromosomal locus was tagged at its 3' end with sequences encoding either three copies of the hemagglutinin epitope (3xHA) or nine copies of the myc epitope (9xmyc) as described previously (27).

**Immunoprecipitations and Immunoblots.** Denatured and native protein lysates were prepared from *Sz. pombe* as detailed previously (28, 29). Immunoprecipitations with anti-HA or anti-myc antibodies were performed as described (30). Proteins were resolved by SDS/PAGE on a 6–20% gradient gel and analyzed by immunoblotting as described (30).

**Glycerol Gradient Analysis.** Native cell lysates were layered onto a 10–30% glycerol gradient and centrifuged at 40,000 rpm for 18 h in an SW-50 Ti rotor (Beckman). Gradient fractions were collected from the bottom and mixed with 5 $\times$  sample buffer, and fractions were analyzed by SDS/PAGE and immunoblotting. Size standards were sedimented in a parallel gradient and analyzed by Coomassie blue staining.

**Yeast Two-Hybrid Analysis.** YPB2 cells were cotransformed with bait (pBI770) and prey (pBI771) plasmids that either did or did not contain *sid4*<sup>+</sup> fragments by the LiOAc/single-stranded DNA/PEG procedure (31) and plated on synthetic dextrose medium lacking leucine and tryptophan (32). *Leu*<sup>+</sup> *Trp*<sup>+</sup> transformants were scored for positive interactions by plating on synthetic dextrose medium lacking leucine, tryptophan, and histidine and containing 5 mM 3-amino-1,2,4-triazole (3AT; Sigma) at 25°C.

**In Vitro Transcription and Translation.** pBluescript SK<sup>+</sup> (Stratagene) containing *sid4-myc* (pKG1935) or *sid4 $\Delta$ N-HA* (pKG1936) served as templates for *in vitro* transcription/translation reactions in the TNT coupled reticulocyte lysate system (Promega).

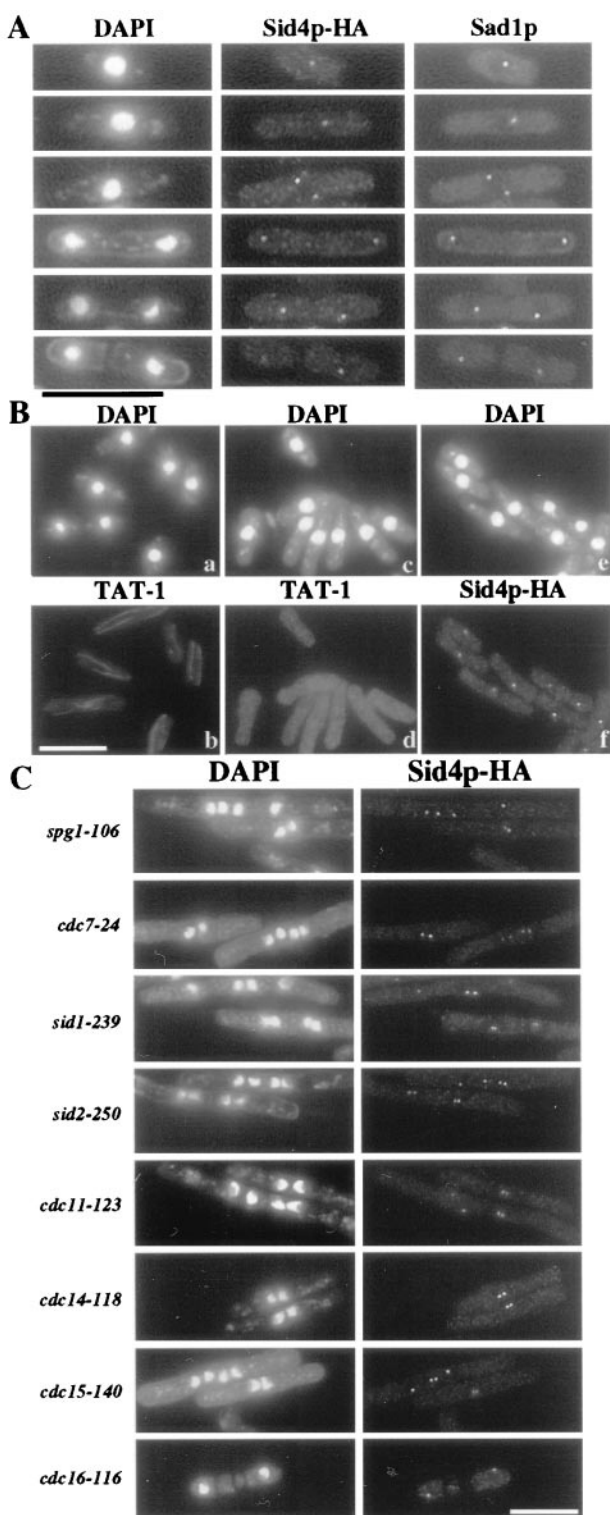
**Microscopy.** All fluorescence microscopy was performed on a Zeiss Axioskop, and images were captured with a cooled charge-coupled device camera (Optronics ZVS47DEC). For staining the cell wall or for immunofluorescence, cells were fixed in formaldehyde or methanol, respectively, and processed as described previously (14). 4',6-Diamidino-2-phenylindole (DAPI) was used to visualize DNA, Calcofluor was used to visualize cell wall material, TAT-1 antibody was used to visualize microtubules (33), polyclonal rabbit anti-Sad1p antibody was used to visualize SPBs (34), and Byr4p localization was determined by using affinity-purified polyclonal anti-Byr4p antibody (13, 18). To visualize Sid4p-HA, Cdc7p-HA, or Spg1p-HA, the 12CA5 monoclonal antibody (anti-HA) was used at 6  $\mu$ g/ml. After incubation with primary antibody, cells were washed with PBAL as described previously (14). Secondary antibodies, Alexa<sup>488</sup> goat anti-rabbit IgG or Alexa<sup>594</sup> goat anti-mouse IgG (Molecular Probes), were used at 5  $\mu$ g/ml.

## Results

***sid4*<sup>+</sup> Encodes an Essential Protein of  $\approx$ 76.4 kDa.** A recessive ts mutant, *sid4-SAI*, was isolated in a screen for mutants defective in cytokinesis (8). At restrictive temperature, *sid4-SAI* cells did not form septa but continued growth and underwent nuclear division, such that cells were elongated and contained multiple nuclei (ref. 8; Fig. 2). We cloned *sid4*<sup>+</sup> by complementation of *sid4-SAI* and confirmed by integration mapping that the rescuing plasmid encoded *sid4*<sup>+</sup> and not a high-copy suppressor. The *sid4*<sup>+</sup> genomic clone contains a single ORF interrupted by one intron and encodes a protein with a predicted molecular mass of 76.4 kDa. Database searches using the Basic Local Alignment Sequence Tool (BLAST; ref. 35) indicated that Sid4p has no obvious similarities to other proteins currently in the database. Sequence analysis of the *sid4-SAI* mutant allele revealed a single mutation in nucleotide 2116 (T to A), resulting in a single amino acid change (L629P). By replacing one copy of *sid4*<sup>+</sup> with *ura4*<sup>+</sup> in a diploid background and performing tetrad analysis, we found that *sid4*<sup>+</sup> encodes an essential protein (data not shown).

**Sid4p Localizes to SPBs Independent of Microtubules and Other Sid Proteins.** To allow detection of Sid4p, we constructed *sid4-HA* and *sid4-myc* strains (see *Materials and Methods*). Both of these strains were morphologically indistinguishable from wild type at all temperatures, indicating that the epitope tags did not alter the function of Sid4p. Anti-HA antibody detected a protein of  $\approx$ 80 kDa in the *sid4-HA* strain (Fig. 4 and data not shown), and anti-myc antibody detected a protein of  $\approx$ 100 kDa in the *sid4-myc* strain (Fig. 4 and data not shown), as predicted.

The intracellular localization of Sid4p-HA was examined by immunofluorescence using antibodies to the HA and myc epitopes. In wild-type cells, no discrete staining was observed (data not shown); however, in *sid4-HA* and *sid4-myc* cells, dots were detected at the periphery of the nuclei throughout the cell cycle (Fig. 1A; data not shown). These dots coincided with those



**Fig. 1.** (A) Sid4p-HA localizes to the SPBs throughout the cell cycle. *sid4-HA* cells (KGY1576) were fixed and stained with DAPI, anti-HA, and anti-Sad1p. (B) Sid4p-HA SPB localization does not require intact microtubules. Before (a and b) or after (c–f) cold shock, KGY1576 cells were fixed and stained with DAPI and either TAT-1 or anti-HA. (C) Sid4p SPB localization is independent of the known proteins required for cytokinesis. Endogenous *sid4-HA* was visualized in *spg1-106* (KGY1593), *cdc7-24* (KGY1594), *sid1-239* (KGY1591), *sid2-250* (KGY1592), *cdc11-123* (KGY1595), *cdc14-118* (KGY1596), *cdc15-140* (KGY1597), or *cdc16-116* (KGY1598). Cells were grown to mid-logarithmic phase at 25°C, shifted to 36°C for 4 h, and fixed and stained with DAPI and anti-HA. (Bar = 10  $\mu$ m).

visualized by staining for Sad1p (Fig. 1A), a known constituent of the SPB (34). We conclude that Sid4p, like Spg1p, localizes to SPBs irrespective of cell cycle stage.

To determine whether Sid4p localization is dependent upon microtubules, *sid4-HA* cells were placed on ice for 25 min to disrupt the microtubule array (34). After cold shock, Sid4p-HA was still detected at the SPBs (Fig. 1B). Similarly, Sid4p-HA localized to the SPBs when a strain carrying the  $\beta$ -tubulin mutation, *nda3-KM311* (36, 37), was incubated at its restrictive temperature to disrupt microtubule arrays (data not shown).

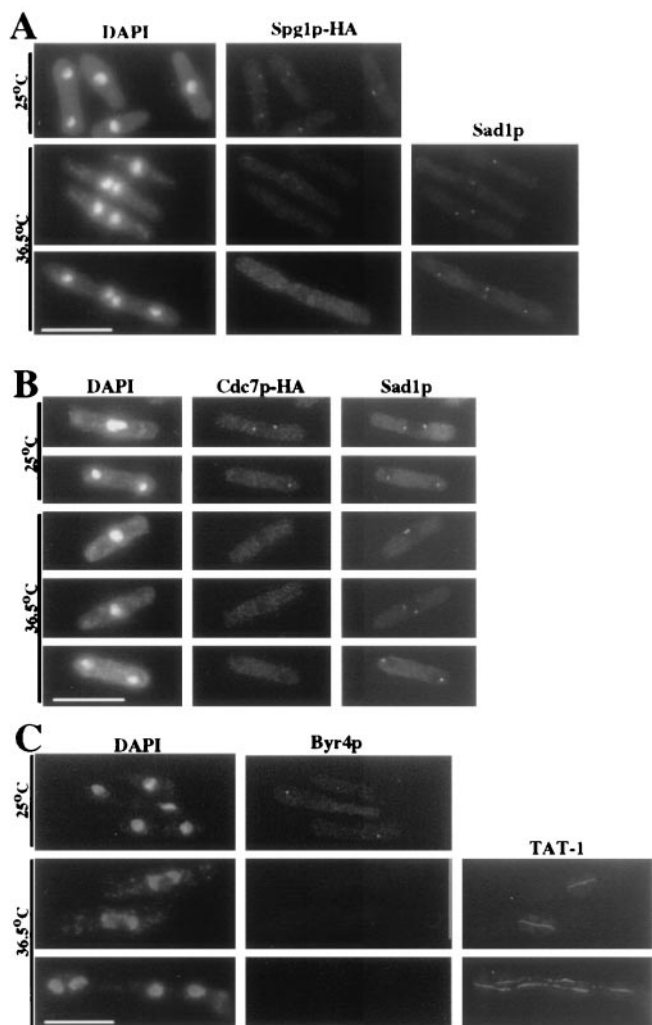
Localization of the Cdc7p and Sid2p protein kinases to the SPB depends upon Spg1p (10, 11). To determine whether Sid4p localization also depends on Spg1p or other known proteins required for septum formation, Sid4p-HA localization was examined in various *ts* septation mutants. Sid4p-HA localized to SPBs in all of the *ts* mutants tested (Fig. 1C), as well as in a *byr4*<sup>+</sup> shut-off strain (18) (data not shown).

**Spg1p, Cdc7p, and Byr4p SPB Localization Depend on Sid4p.** We next asked whether the localization of Cdc7p, Spg1p, or Byr4p depends on Sid4p. Because Cdc7p localizes to SPBs only during mitosis, cells were stained with anti-Sad1p to identify cells in the early and late stages of anaphase. At permissive temperature, Spg1p-HA, Cdc7p-HA, and Byr4p localized to SPBs in the *sid4-SAI* mutant (Fig. 2), but at restrictive temperature, these proteins were not detected at SPBs (Fig. 2), indicating that Sid4p is essential for their localization to the SPB.

**Effects of Sid4p Overproduction.** Overproduction of proteins involved in cytokinesis can lead either to inhibition of septum formation as seen with Byr4p (18) or to uncontrolled septation as seen with Spg1p (8, 9) or Plo1p (38). To determine whether overproduction of Sid4p affects cytokinesis, we examined cells overproducing Sid4p from the *nmt1* promoter (see *Materials and Methods*). Sid4p overproduction rescued the temperature sensitivity of the *sid4-SAI* mutant (data not shown) and did not generate a detectable phenotype in wild-type cells. The overproduction of Sid4p in these strains was confirmed by immunoblot analysis (data not shown).

In attempts to map the functional domains of Sid4p, we found that a construct expressing the N-terminal 153 amino acids (pKG1386) or the N-terminal 153 amino acids fused to three copies of the HA epitope (pKG1534) did not rescue the *sid4* *ts* or deletion mutants. In contrast, a construct expressing moderate levels of Sid4p lacking the N-terminal 153 amino acids (Sid4 $\Delta$ Np, pKG1225) rescued the *sid4* *ts* mutant although not the *sid4* deletion mutant. Overproduction of either pKG1386 or pKG1534 in a wild-type background did not affect cell growth or morphology (data not shown). That N153Sid4p-HA was overproduced was confirmed by immunoblot analysis (data not shown). Interestingly, overproduction of Sid4 $\Delta$ Np inhibited septum formation and produced elongated cells containing multiple nuclei. This was seen best when the *nmt1-sid4 $\Delta$ N* construct was integrated in single copy (KGY1347) (Fig. 3A). The dominant-negative phenotype of Sid4 $\Delta$ Np suggested that it may titrate out a factor required for septum formation.

To test this possibility, the localization of endogenous Sid4p-HA was examined in cells overproducing untagged Sid4 $\Delta$ Np under the control of the *nmt1* promoter (KGY1742). When Sid4 $\Delta$ Np was produced at moderate levels, Sid4p-HA SPB staining was strong (Fig. 3B). However, when Sid4 $\Delta$ Np was overproduced, Sid4p-HA SPB staining was reduced and most often undetectable (Fig. 3B). In contrast, overproduction of Byr4p (18), which also inhibits septation, did not reduce the amount of Sid4p detectable at the SPBs (data not shown). If Sid4 $\Delta$ Np titrates Sid4p-HA from the SPB, then compensatory overproduction of Sid4p might rescue the dominant-negative phenotype of Sid4 $\Delta$ Np overproduction. Indeed, when *sid4*<sup>+</sup> was

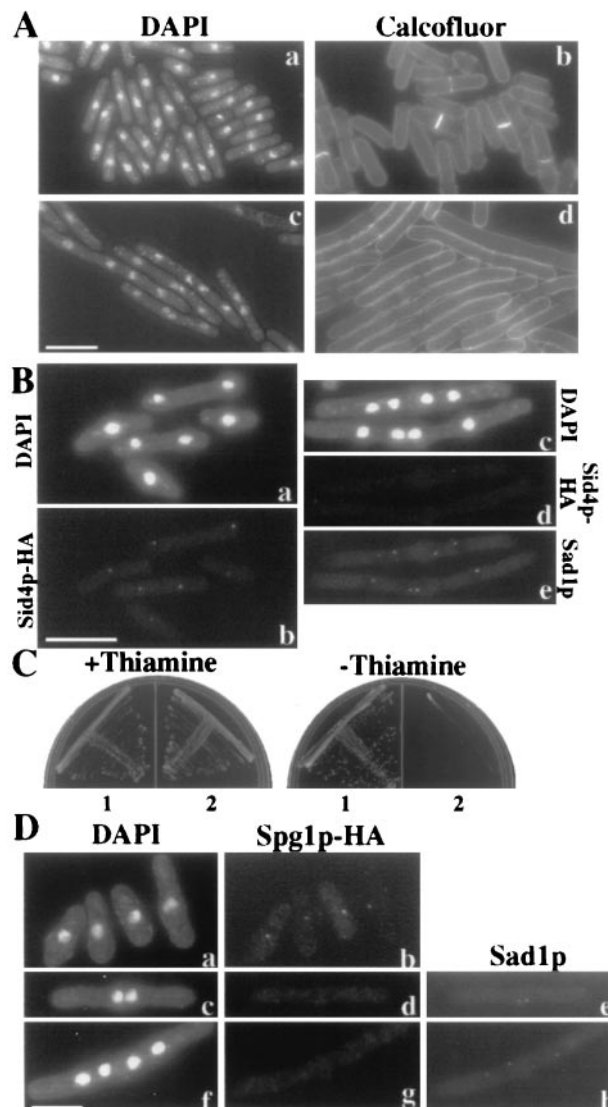


**Fig. 2.** Spg1p, Cdc7p, and Byr4p SPB localization requires Sid4p function. (A and B) *spg1-HA sid4-SA1* (KGY1725; A) or *cdc7-HA sid4-SA1* (KGY1600; B) cells were grown to mid-logarithmic phase at 25°C and then shifted to 36.5°C for 4 h (A) or 2 h (B). Cells were fixed and stained with DAPI, anti-HA, and anti-Sad1p. (C) *sid4-SA1* (KGY1234) cells were grown to mid-logarithmic phase at 25°C and then shifted to 36.5°C for 4 h. Cells were fixed and stained with DAPI, anti-Byr4p, and TAT-1. (Bar = 10  $\mu$ m.)

expressed from a multicopy plasmid along with Sid4 $\Delta$ Np, cell growth was restored (Fig. 3C).

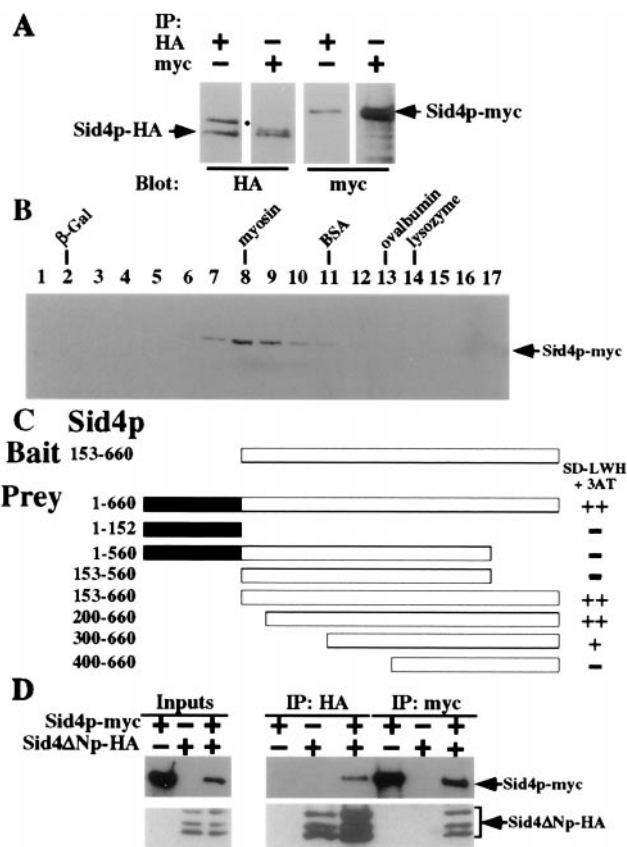
Although the data of Fig. 2A showed that Spg1p localization to the SPB depends on Sid4p, it was not clear whether this depended upon Sid4p localizing to the SPB. To address this question, we removed Sid4p from the SPB by overproducing Sid4 $\Delta$ Np. Under these conditions, Spg1p-HA staining was not detected at the SPBs (Fig. 3D). Sid4 $\Delta$ Np overproduction also blocked Sid2p localization to the SPB (data not shown). We conclude that the function of Sid4p in recruiting other proteins to the SPB depends on its own localization to the SPB.

**Dimerization of Sid4p.** Sid4 $\Delta$ Np rescues the *sid4* ts mutant even though it lacks a domain essential for function. This observation suggested that *sid4* $\Delta$ N and *sid4-SA1* exhibit intragenic complementation that might depend on the formation of dimers or larger multimers; moreover, the domain allowing this interaction must be present within Sid4 $\Delta$ Np. To examine this possibility, a heterozygous Sid4p-HA/Sid4p-myc diploid strain was constructed. When either anti-HA or anti-myc was used, both



**Fig. 3.** (A) Overproduction of Sid4 $\Delta$ Np inhibits cytokinesis. Wild-type cells carrying a single integrated copy of *sid4* $\Delta$ N under control of the thiamin-repressible *mnt1* promoter (KGY1347) were grown to mid-logarithmic phase at 32°C in medium containing thiamin (a and b). To induce overexpression of *sid4* $\Delta$ N, cells were then grown in medium lacking thiamin for 22 h at 32°C (c and d). Cells were fixed and stained with DAPI or with Calcofluor. (B) Sid4 $\Delta$ Np overproduction prevents Sid4p SPB localization. *sid4-HA* cells carrying a single integrated copy of *mnt1-sid4* $\Delta$ N (KGY1742) were grown to mid-logarithmic phase at 32°C under repressing conditions (a and b) or were shifted to inducing conditions for 20 h at 32°C (c–e). Cells were stained with DAPI, anti-HA, and anti-Sad1p. (C) Full-length Sid4p rescues Sid4 $\Delta$ Np overproduction phenotype. KGY1347 was transformed with plasmid pUR19 *sid4*<sup>+</sup> carrying full-length Sid4p (sector 1) or control plasmid pUR19 (sector 2) and grown on minimal medium with or without thiamin to repress or induce production of Sid4 $\Delta$ Np. (D) Sid4 $\Delta$ Np overproduction prevents Spg1p SPB localization. *spg1-HA* cells carrying a single integrated copy of *mnt1-sid4* $\Delta$ N (KGY1741) were grown to mid-logarithmic phase at 32°C under repressing (a and b) or inducing conditions (c–h). Cells were stained with DAPI, anti-HA, and anti-Sad1p to detect SPBs. (Bar = 10  $\mu$ m.)

Sid4p-HA and Sid4p-myc were co-immunoprecipitated from native cell lysates (Fig. 4A). To examine the apparent size of the Sid4p complex, lysates containing Sid4p-myc were resolved by sedimentation on a glycerol gradient and analyzed by immunoblot analysis (Fig. 4B). Sid4p-myc was detected in fractions 7 through 11 and peaked in fractions 8 and 9, similar to myosin

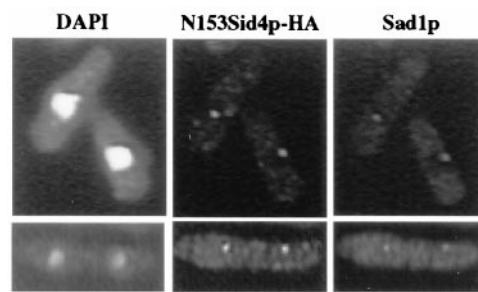


**Fig. 4.** Sid4p interacts with itself. (A) Cell lysates were prepared under native conditions from a *sid4-HA/sid4-myc* heterozygous diploid strain (KGY2732), immunoprecipitated with either anti-HA or anti-myc, and analyzed by immunoblotting with either anti-HA or anti-myc. The positions of Sid4p-HA and Sid4p-myc are indicated. The position of a nonspecific HA-crossreactive protein is indicated (●). (B) Sid4p-myc sediments with an apparent mass of ≈200 kDa. Cell lysates were prepared under native conditions from a *sid4-myc* strain (KGY1340) and analyzed by sedimentation in a glycerol gradient. Fraction numbers are indicated. Peak fractions for molecular mass markers sedimented in a parallel gradient are indicated (lysozyme, 14 kDa; ovalbumin, 45 kDa; BSA, 66 kDa; myosin, 220 kDa; β-galactosidase (β-Gal), 464 kDa). (C) Sid4p dimerization domain. Sid4ΔNp in pB1770 (Bait) and Sid4p fragments in pB1771 (Prey) were cotransformed into strain YPB2 (KGY1400) and interactions were analyzed as described in the text. ++, Strong interaction; +, moderate interaction; -, no interaction. The amino acid residues encoded by each fragment are indicated. (D) Sid4p-myc binds directly to Sid4ΔNp-HA. Sid4p-myc and Sid4ΔNp-HA were translated *in vitro* either separately or together and immunoprecipitated with either anti-HA or anti-myc, and immunoprecipitates were analyzed by immunoblot analysis with either anti-HA or anti-myc. Ten percent of the input into the immunoprecipitation reactions was also resolved by SDS/PAGE and immunoblotted. The positions of Sid4p-myc and Sid4ΔNp-HA (and its internal initiation products) are indicated.

(220 kDa); these data suggest that Sid4p-myc (≈100 kDa) may form a homodimer.

The hypothesis that a Sid4p dimerization domain resided within Sid4ΔNp was supported by the ability of Sid4ΔNp-HA to be co-immunoprecipitated with myc antibodies when expressed in a *sid4-myc* strain (data not shown). To further define the interaction domain, the yeast two-hybrid system was used (32, 39). As expected, Sid4ΔNp did not interact with empty prey plasmid (data not shown) but was able to interact with itself (Fig. 4C). N- and C-terminal truncations of Sid4ΔNp demonstrated that the entire C-terminal half of the protein was important for Sid4p-Sid4p interaction in this assay (Fig. 4C).

To rule out the possibility that an unidentified yeast protein



**Fig. 5.** Sid4p contains an SPB localization domain. Wild-type cells expressing the N-terminal 153 amino acids of Sid4p tagged with the HA epitope under control of the *nmt1* promoter (pKG1534) were grown to mid-logarithmic phase under inducing conditions at 32°C. Cells were fixed and stained with DAPI, anti-HA to detect N153Sid4p-HA, and anti-Sad1p. (Bar = 10 μm.)

was bridging the interaction of Sid4p with itself in the assays just described, Sid4p-myc and Sid4ΔNp-HA were translated either separately or together *in vitro*. Immunoprecipitation and immunoblot analysis indicated that, when cotranslated, they were able to associate with one another (Fig. 4D). These results strongly suggest that Sid4p can interact directly with itself.

**The N Terminus of Sid4p Is Sufficient for SPB Localization.** Because overproduction of Sid4ΔNp prevented assembly of other pathway components at the SPB, we inferred that the SPB localization signal within Sid4p resided in the N-terminal fragment that had been removed from Sid4ΔNp. To test this possibility directly, six copies of the HA epitope were appended to N153Sid4p and the localization of this fusion protein was determined. Anti-HA detected dots in cells expressing N153Sid4p-HA, and these dots colocalized with Sad1p (Fig. 5). Similarly, when green fluorescent protein (GFP) was appended to amino acids N153Sid4p at the C terminus and expressed from a plasmid, GFP was detected at SPBs in live cells (data not shown). Together these data indicate that the N-terminal 153 amino acids of Sid4p contain a SPB localization domain.

## Discussion

Through genetic and biochemical studies in *Sz. pombe*, a signal transduction pathway involving Spg1p, Cdc7p, Cdc11p, Cdc14p, Cdc16p, Byr4p, Sid1p, Sid2p, and Sid4p proteins has been shown to regulate the onset of cytokinesis (4–6). Several components of this signaling cascade localize to the SPB. These include the GTPase Spg1p, the two-component GTPase-activating protein for Spg1p, and two protein kinases, Cdc7p and Sid2p, which act downstream of activated Spg1p (10, 11, 13). Like Spg1p and Sid2p, Sid4p-HA was detected at the SPB throughout the cell cycle. Using several tagged versions of Sid4p, we did not detect Sid4p at the medial ring (see above; additional data not shown). We and Sparks *et al.* (10) have found that Sid4p function is critical for the ability of at least four other proteins (Spg1p, Cdc7p, Bry4p, and Sid2p) to localize to the SPB, suggesting that Sid4p either acts upstream of Spg1p, Cdc7p, and Sid2p or perhaps serves as a scaffold for this pathway.

Like other proteins in this pathway, Sid4p is essential for viability, but unlike most other components thus far described, it does not have an obvious homologue in *Sc. cerevisiae*. The COILS program (version 2.2, window 28; ref. 40) indicates the probable presence of two coiled-coil domains within the C-terminal half of Sid4p that may be involved in interactions of Sid4p with itself or with another protein(s). The *sid4-SA1* mutant allele contains a single amino acid change from leucine to proline within the second putative coiled-coil domain. As a proline would interrupt the α-helical structure of the coiled-coil,

the ts phenotype of *sid4-SAI* might be explained by the disruption of a protein-protein interaction mediated by this domain.

Although overproduction of Sid4 $\Delta$ Np is lethal to cells, moderate levels of Sid4 $\Delta$ Np rescue the *sid4* ts mutant but not the *sid4* null mutant. This observation suggests that Sid4 $\Delta$ Np lacks a domain essential for function. As tagged versions of the N-terminal 153 amino acids of Sid4p can localize to the SPB, it appears that the missing domain in Sid4 $\Delta$ Np is the SPB localization domain. However, localization of the N-terminal Sid4p fragment to the SPB requires its overproduction (data not shown), suggesting that dimerization of Sid4p might improve the efficiency of localization. In future studies, it will be of interest to define the amino acids essential for SPB localization and to identify other SPB protein(s) with which these amino acids interact.

Although the Spg1p pathway is conserved between *Sz. pombe* and *Sc. cerevisiae*, the *Sz. pombe* guanine nucleotide exchange factor (GEF) for Spg1p has not yet been identified. It remains possible that Sid4p participates in the GEF activity for Spg1p, but this seems unlikely for several reasons. First, Sid4p has no sequence similarity to *Sc. cerevisiae* Lte1p, which is predicted to provide GEF activity for Tem1p (15, 41). Second, if Sid4p were the GEF for Spg1p, mutation of *sid4*<sup>+</sup> might rescue mutations of *cdc16*<sup>+</sup>; this is not the case (data not shown). Similarly, overproduction of Sid4p might result in an increase in activated, GTP-bound Spg1p, which would in turn produce a phenotype similar to Spg1p-induced uncontrolled septum formation. However, overproduction of full-length Sid4p has no effect on cell growth or morphology.

Our data suggest that Sid4p might act as a scaffold at the SPB upon which other components of the Spg1p signaling pathway assemble. However, we have been unable to detect a direct

physical interaction between Sid4p and Spg1p, Cdc7p, or Sid2p by either yeast two-hybrid or co-immunoprecipitation analyses (data not shown). Indeed, in yeast cell lysates, we can detect only interactions of Sid4p with itself. There are at least three possible explanations for these results. First, the form of Sid4p interacting with other pathway components might be embedded within the SPB, a structure well known for its resistance to solubilization. Perhaps pertinent to this hypothesis, Sid4p contains two predicted coiled-coil domains, as do several essential structural SPB components, including Cut12p (42), Spc110p (43, 44), Spc72p (45), Spc42p (46), and Mps2p (47). Second, there may exist an unidentified component of the pathway, such as the presumed GEF for Spg1p that links Sid4p to Spg1p. Third, Sid4p might not interact directly with any pathway component but instead be involved in organizing the SPB such that pathway components can bind to it. Further studies of the interaction between Sid4p and SPB components, as well as between Sid4p and the signaling pathway components, should not only enhance our understanding of the regulation of cytokinesis in *Sz. pombe* but also provide insight into the conserved features between the septation initiation pathway of *Sz. pombe* and the mitotic exit pathway of *Sc. cerevisiae*.

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