

The role of the Sid1p kinase and Cdc14p in regulating the onset of cytokinesis in fission yeast

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Coordination of mitosis and cytokinesis is crucial for ensuring proper chromosome segregation and genomic stability. In *Schizosaccharomyces pombe*, the *sid* genes (*cdc7*, *cdc11*, *cdc14*, *spg1*, *sid1*, *sid2* and *sid4*) define a signaling pathway that regulates septation and cytokinesis. Here we describe the characterization of a novel protein kinase, Sid1p. Sid1p localizes asymmetrically to one spindle pole body (SPB) in anaphase. Sid1p localization is maintained during medial ring constriction and septum synthesis and disappears prior to cell separation. Additionally, we found that Cdc14p is in a complex with Sid1p. Epistasis analysis places Sid1p–Cdc14p downstream of Spg1p–Cdc7p but upstream of Sid2p. Finally, we show that cyclin proteolysis during mitosis is unaffected by inactivating the *sid* pathway; in fact, loss of Cdc2–cyclin activity promotes Sid1p–Cdc14p association with the SPB, possibly providing a mechanism that couples cytokinesis with mitotic exit.

Keywords: *cdc7/cdc14*/cytokinesis/*Schizosaccharomyces pombe*/*sid1*

Introduction

Cytokinesis occurs immediately following mitosis and results in irreversible cell cleavage. Mitosis and cytokinesis are tightly regulated both temporally and spatially so that cleavage occurs between separated chromosomes and not before mitotic spindle assembly and chromosome segregation. This ensures that each daughter cell receives only one copy of the genome. Cellular signals that regulate mitotic events have been studied extensively. Cellular mechanisms that coordinate mitosis and cytokinesis are less well defined.

The fission yeast *Schizosaccharomyces pombe* has proven to be an excellent model organism for studying cytokinesis (for reviews, see Gould and Simanis, 1997; Le Goff *et al.*, 1999). Like animal cells, *S. pombe* divides through use of an actin–myosin-based contractile ring. During interphase, F-actin patches localize to the growing ends of cells. In early mitosis, the medial ring, which is composed of F-actin, myosin II and numerous other proteins, forms at the cell center. F-actin patches sub-

sequently re-localize to the medial ring. Once anaphase is complete, the spindle disassembles, the medial ring constricts and synthesis of a septum is initiated. Following septum formation, primary septal material is degraded and daughter cells physically separate.

At least eight genes are involved in triggering cytokinesis in *S. pombe*. They include the *sid* genes (septation initiation-deficient), *cdc7*, *cdc11*, *cdc14*, *spg1*, *sid1*, *sid2* and *sid4* (Nurse *et al.*, 1976; Schmidt *et al.*, 1997; Balasubramanian *et al.*, 1998), as well as *plp1* kinase (Ohkura *et al.*, 1995). Under restrictive conditions, cells bearing temperature-sensitive alleles of the *sid* genes show normal medial ring assembly and redistribution of actin patches, but fail to initiate ring constriction and septum deposition. These cells proceed through additional nuclear division cycles without septation, becoming long and multinucleate before eventually lysing (Fankhauser *et al.*, 1995; Balasubramanian *et al.*, 1998). *Plp1* seems to play a role in cytokinesis since it is required for septum formation and its overexpression in interphase results in septum formation (Ohkura *et al.*, 1995).

Genetic and biochemical studies suggest that the *sid* gene products function in a novel signaling cascade regulating cytokinesis, yet their order of function is unclear (Marks *et al.*, 1992; Balasubramanian *et al.*, 1998; Sohrmann *et al.*, 1998). Based on localization studies, at least part of this cascade appears to function by transducing septum-promoting signals from spindle pole bodies (SPBs) to the division site. *Spg1p*, a small GTPase of the ras superfamily, localizes to SPBs throughout interphase in an active GDP-bound form. Upon entry into mitosis, *Spg1p* is converted to an active GTP-bound state until anaphase, when conversion of *Spg1p*–GTP to *Spg1p*–GDP occurs at one SPB (Sohrmann *et al.*, 1998). *Bry4p* and *Cdc16p* function together as the GTPase-activating protein (GAP) for *Spg1p* (Furge *et al.*, 1998); however, the GDP/GTP exchange factor (GEF) has not yet been identified. Moreover, *Byr4p*/*Cdc16p* co-localizes with *Spg1p*–GDP but not *Spg1p*–GTP (Cerutti and Simanis, 1999). The key function of *Spg1p*–GTP appears to be in localizing *Cdc7p* kinase to the SPB during mitosis since *Cdc7p* kinase activity does not seem to be regulated (Sohrmann *et al.*, 1998). *Sid2p* kinase is localized to the single SPB in interphase, both SPBs during mitosis and transiently to the cleavage site during medial ring constriction and septation. *Sid2p* kinase activity peaks during late anaphase and its localization and kinase activity require the other *sid* genes, suggesting that it functions late in the cascade (Sparks *et al.*, 1999). *Cdc14p* has been cloned but does not display significant sequence similarity to any known proteins (Fankhauser and Simanis, 1993). Overproduction of *Cdc14p* results in a G₂–M block; however, *Cdc14p* localization and where it functions in the *sid* pathway have not been described.

In this study, we investigated the role of *S. pombe* Sid1p and Cdc14p in the *sid* pathway. We show that Sid1p is a PAK/GC family protein kinase that functions in a complex with Cdc14p, downstream of Spg1p–Cdc7p and upstream of Sid2p. We also show that Sid1p localization may be regulated by Cdc2–cyclin activity, suggesting a mechanism that may couple cytokinesis with completion of mitosis.

Results

Cloning and disruption of *sid1*

The *sid1* gene was cloned by complementation of a *sid1-239* temperature-sensitive mutant. *sid1* corresponded to an open reading frame with GenBank accession No. CAB11493. *sid1* encodes a 471 amino acid protein kinase of the PAK/GC kinase family (for a review, see Sells and Chernoff, 1997). A sequence comparison of the catalytic domain of Sid1p and catalytic domains from related proteins is shown in Figure 1A. Sid1p is most related to GC kinases that have an N-terminal kinase domain and a C-terminal half with little homology to known proteins. A null allele of *sid1* was constructed using a one-step gene replacement strategy in a diploid strain, and *sid1* was found to be essential for viability (see Materials and methods). *sid1* null cells become long and multinucleate, identical to the phenotype of cells with *sid1* temperature-sensitive alleles at restrictive temperature (data not shown).

Sid1p is a protein kinase

To determine if Sid1p displayed kinase activity *in vitro*, we constructed a strain that expressed a 13Myc epitope-tagged version of Sid1p from the chromosomal *sid1* locus. 13Myc-Sid1p immune complexes were prepared from cell lysates and kinase assays were performed using myelin basic protein (MBP) as a substrate. Immune complexes prepared from 13Myc-*sid1* cell lysates displayed high kinase activity. To confirm that kinase activity is specific to Sid1p, a hemagglutinin (HA)-tagged kinase-dead version of Sid1p (HA-Sid1p-K38R) was constructed and expressed from the pREP41 vector under the control of the thiamine-repressible *nmt1* promoter (see Materials and methods). Kinase activity was associated with HA-Sid1p immune complexes (Figure 1B) and was absent in preparations from cells expressing kinase-dead HA-Sid1-K38R or the empty plasmid vector (Figure 1B). We next tested the ability of the HA-Sid1-K38R mutant to rescue the *sid1-125* temperature-sensitive defect. As shown in Figure 1C, only the wild-type version of Sid1p rescued the *sid1-125* phenotype.

Localization of Sid1p

Cells expressing green fluorescent protein (GFP)–Sid1p from the chromosomal locus were stained with antibodies against tubulin to assess clearly the cell cycle-specific intracellular distribution of Sid1p. In cells with an interphase array of microtubules (Figure 2A, cells 1 and 2), diffuse Sid1p signal was observed. In contrast, cells in anaphase (Figure 2A, cells 3–5) or telophase (Figure 2A, cell 6) exhibited strong GFP signal at one SPB. Because weak GFP signals are often difficult to see following immunofluorescence processing, we also examined cells

just prior to processing. As before, interphase cells displayed diffuse GFP–Sid1p signal (data not shown), while anaphase or telophase cells showed strong GFP–Sid1p signal at one SPB (Figure 2B, iii and iv). Interestingly, GFP–Sid1p could be seen faintly at both SPBs in metaphase or early anaphase (Figure 2B, i and ii). To determine the temporal relationship between Sid1p appearance at the SPB and initiation of septation, we stained GFP–Sid1p-expressing cells with calcofluor, which preferentially stains the septum. Sid1p arrived at the SPB prior to initiation of septation since cells could be observed that had not yet initiated septation but had GFP–Sid1p signal at one SPB (Figure 2C, cell 1). Sid1p was always observed at one SPB in septating cells that had not completed septum formation (Figure 2C, cells 2 and 3). Once cells had completed septum formation but prior to cell separation, Sid1p disappeared from the SPB (Figure 2C, cell 4). Cdc7p, after localizing initially to both SPBs during mitosis, also localizes to only one SPB in late anaphase (Sohrmann *et al.*, 1998). We tested whether Sid1p localized to the same SPB as Cdc7p. Cells expressing both Cdc7-HA (Figure 2D, i) and GFP–Sid1p (Figure 2D, ii) gave signals that overlapped (Figure 2D, iii), suggesting that Cdc7p and Sid1p localize to the same SPB during septum formation.

We next tested whether factors that affect Cdc7p localization also affect Sid1p localization. Inactivation of *cdc16* results in localization of Cdc7p to both SPBs in late anaphase, an effect that is also observed upon overexpression of Spg1p (Sohrmann *et al.*, 1998). Furthermore, overexpression of Spg1p results in inappropriate recruitment of Cdc7p to the SPB in interphase cells. Like Cdc7p, Sid1p localized to both SPBs in *cdc16-116* cells (Figure 3A) and in cells overexpressing Spg1p (Figure 3B; see arrows), as well as to the SPB in interphase cells overexpressing Spg1p (Figure 3B). After shift to restrictive temperature, *cdc16-116* cells display two phenotypes termed type I and type II cells (Minet *et al.*, 1979; Cerutti and Simanis, 1999). Type I cells have two nuclei and make multiple septa. Type II cells have a single nucleus and a septa. It has been proposed that the type II cells immediately septate again after division because they inherit the SPB that contains active Spg1p (Cerutti and Simanis, 1999). In support of this hypothesis, Sid1p was present at the SPB in type II cells (Figure 3A; see arrow). These observations suggest that Sid1p may function downstream of Spg1p. Consistent with this model, Spg1p overexpression causes wild-type cells to form multiple septa (Schmidt *et al.*, 1997) (Figure 3C); however, septa are not formed when Spg1p is overexpressed in a *sid1-125* mutant (Figure 3D).

Dependencies of localization of Sid1p, Cdc7p and Cdc14p

To determine where Sid1p functions with respect to the other *sid* gene products, we examined whether GFP–Sid1p localized to the SPB in other *sid* mutants. *cdc7-24*, *cdc11-123*, *cdc14-118*, *sid2-250*, *spg1-106* and *sid4-A1* cells expressing GFP–Sid1p were shifted to restrictive temperature and localization of GFP–Sid1p was assessed. Sid1p localized to the SPB in 12% (32/227) of wild-type cells and 22% (69/240) of *sid2-250* mutants; however, examination of >200 cells showed that Sid1p was unable

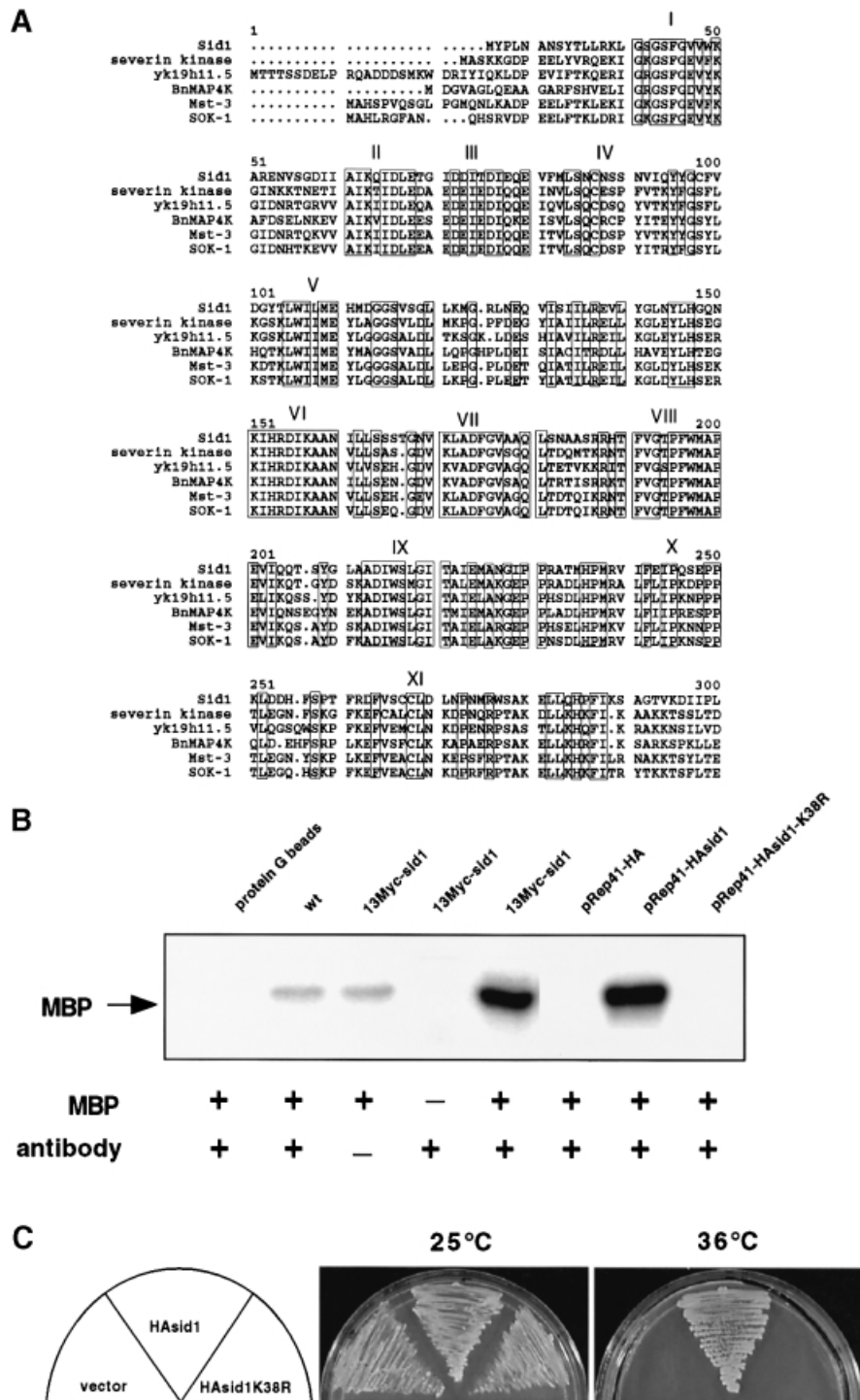


Fig. 1. Kinase activity is essential. (A) Alignment of the catalytic domain of Sid1p with the catalytic domains of the most closely related PAK/GC family members as determined by the BLAST and MULTALIN programs. Relatives include severin kinase from *Dictyostelium discoideum* (AAC24522), *Caenorhabditis elegans* contig yk19h11.5 (AAC9038), BnMAPK4 from *Brassica napus* (CAA08757), human Ste20-like kinase Mst-3 (4505261) and human oxidant stress-activated Ste20-like kinase SOK-1 (5454174). Roman numerals indicate the 11 subdomains characteristic of Ser/Thr protein kinases (Hanks *et al.*, 1988). Boxes enclose conserved residues. (B) Immune complex kinase assays were performed using Myc or HA antibodies as appropriate (see Materials and methods) on lysates prepared from wild-type cells, or cells expressing 13Myc-Sid1p, HA-Sid1p or HA-Sid1-K38R. Assays were carried out with (+) or without (-) antibody or the artificial substrate MBP. A control assay was also carried out in the absence of cell lysate (protein G beads). (C) *sid1-125* cells (YDM435) transformed with vector alone or with plasmids expressing either HA-Sid1p or HA-Sid1-K38R were plated at 25 or 36°C.

to localize in *cdc7*, *cdc11*, *cdc14*, *spg1* or *sid4* mutant cells (Figure 4; Table I; data not shown). This is consistent with Sid1p functioning upstream of Sid2p but downstream of the other *sid* proteins. Similar experiments examining

Cdc7p localization in other mutant backgrounds showed that Cdc7p cannot localize to the SPB(s) in *cdc11* (Figure 4; Table I) and *spg1* (Sohrmann *et al.*, 1998) mutant cells (>200 cells examined). In contrast, Cdc7

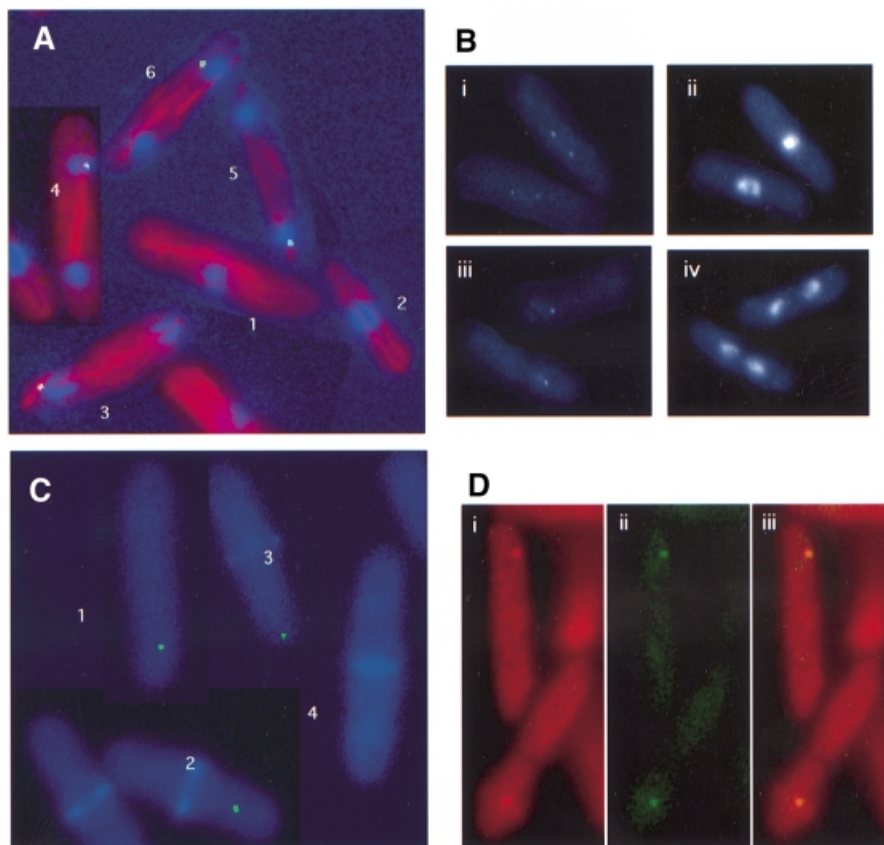


Fig. 2. Localization of GFP-Sid1p. (A) Cells expressing GFP-Sid1p (green) were processed for immunofluorescence and stained for tubulin (red) and DNA (blue). Numbers indicate cells at stages of the cell cycle described in the text. (B) GFP-Sid1p cells in early (i and ii) and late (iii and iv) anaphase were fixed and stained for DNA. Panels i and iii show GFP-Sid1p fluorescence, and the corresponding DNA fluorescence is displayed in panels ii and iv. (C) Cells were fixed and stained for septum material (blue) with calcofluor. (D) Cells expressing Cdc7p-HA and GFP-Sid1p were fixed and processed for immunofluorescence using HA antibodies. Cdc7-HA fluorescence (i) and GFP-Sid1p fluorescence (ii) were merged (iii) to show co-localization of the two proteins.

localized to the SPBs in 27% (47/124) of *cdc14* mutant cells and in 27% (102/277) of *sid1* mutant cells, which is similar to that seen in *sid2* mutants at permissive and restrictive temperature (28 and 53%, respectively; Sparks *et al.*, 1999; Figure 4; Table I). These results indicate that Cdc14p, Sid1p and Sid2p probably function downstream of Cdc7p, with Cdc14p either functioning upstream of or in conjunction with Sid1p.

Cdc14p localizes to one spindle pole body during septum formation

Previous studies have shown synthetic lethal interactions between *sid1-239* and *cdc14-118* (Balasubramanian *et al.*, 1998). Furthermore, our epistasis studies suggest that Cdc14p functions just upstream of or at the same point as Sid1p. Therefore, we further characterized the relationship between Sid1p and Cdc14p. We first examined the intracellular distribution of Cdc14p in a strain expressing an integrated copy of GFP-Cdc14p from the Cdc14 promoter. In wild-type cells, GFP-Cdc14p had an identical localization pattern to that of Sid1p. Cdc14p localized to a single spot on the nuclear periphery of cells at the end of anaphase or telophase (Figure 4). GFP-Cdc14p presumably localized to the same SPB as Sid1p and Cdc7p, but we were unable to confirm this since the signal did not survive immunofluorescence processing. To circumvent this problem, a strain was constructed that expressed a

GFP-tagged version of both Sid1p and Cdc14p. Similarly to cells expressing GFP-Sid1p or GFP-Cdc14p alone, all late anaphase cells (30/30) expressing both GFP-Sid1p and GFP-Cdc14p showed GFP signal at just one SPB, suggesting that Sid1p and Cdc14p co-localize. Examination of Cdc14p localization in different mutant backgrounds revealed that Cdc14p localized normally to the SPB in *sid2* mutant cells (30/139) compared with wild type (22/199), and was unable to localize in *sid1*, *sid4*, *spg1*, *cdc7* and *cdc11* mutant cells (Figure 4; Table I; data not shown). Thus it appears that Sid1p and Cdc14p may function together since they depend on each other for localization.

Cdc14p and Sid1p show physical interaction

Since localization experiments suggested that Sid1p and Cdc14p may function together, we tested whether the two proteins formed a complex. To facilitate these studies, we constructed strains expressing epitope-tagged versions of both Sid1p and Cdc14p (see Materials and methods). Examination of lysates prepared from cells expressing 13Myc-Sid1p and GFP-Cdc14p showed that 13Myc-Sid1p could be co-immunoprecipitated with GFP-Cdc14p (Figure 5A). When immunoprecipitations were immunoblotted with anti-GFP antibodies, we did not detect GFP-Cdc14p in either 13Myc-Sid1p immune complexes or control GFP-Cdc14p immune complexes

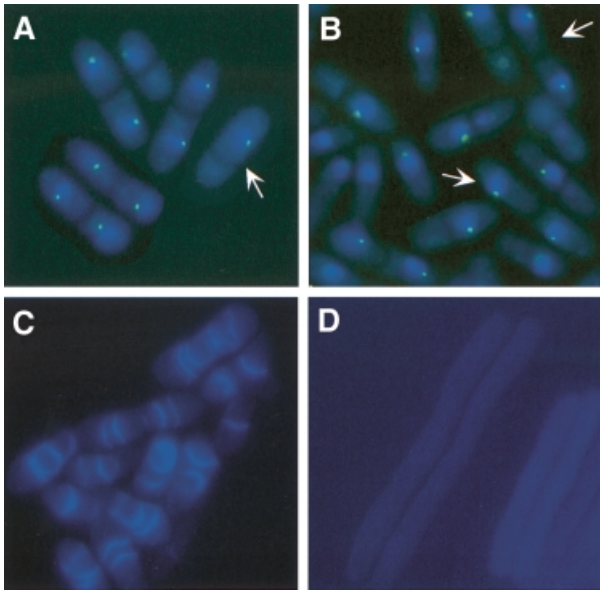


Fig. 3. *spg1* overexpression or *cdc16* inactivation cause Sid1p to localize to the SPB. (A) *GFP-sid1 cdc16-116* cells (YDM560) were grown at 25°C, shifted to 36°C for 2 h and then fixed. Cells were stained for DNA (blue), and images of the DNA staining and the GFP-Sid1p signal (green) were captured. (B) *spg1* expression was induced for 16 h in *nmt1-spg1 GFP-sid1* cells (YDM564) and images of DNA (blue) and GFP-Sid1p (green) were captured. (C) *nmt1-spg1* (YDM551) or (D) *sid1-125 nmt1-spg1* cells (YDM566) were grown to mid-log phase at 25°C in medium containing thiamine. The culture was then shifted to medium lacking thiamine, grown at 25°C for an additional 23 h and subsequently placed at 36°C for 5 h. Cells were then fixed and stained with calcofluor.

(data not shown). We presumed that this was because Western blotting using anti-GFP antibody was not as sensitive as when anti-Myc antibody was used against the 13Myc tag. To test this, we switched the tags and prepared lysates from cells expressing 13Myc-Cdc14p and GFP-Sid1p. Samples were analyzed as above and we found that 13Myc-Cdc14p co-immunoprecipitated with GFP-Sid1p (Figure 5B). Consistent with the observation that Cdc14p and Sid1p form a complex, we found that Cdc14p has Sid1-dependent associated kinase activity (Figure 5C).

Sid1p cell cycle regulation

We next tested if Sid1p and Cdc14p protein levels or their ability to form a complex fluctuated throughout the cell cycle. *cdc25-22 13Myc-sid1 GFP-cdc14* cells were synchronized by shifting them to 36°C to block them in G₂, then they were released by returning them to permissive temperature (25°C). Samples were collected every 15 min and lysates were prepared and analyzed directly for Sid1p protein (Figure 6A) or subjected to co-immunoprecipitation experiments (Figure 6B). Western analysis of lysates revealed that Sid1p (Figure 6A) and Cdc14p (data not shown) protein levels remain unchanged throughout the cell cycle. Moreover, Western analysis of GFP-Cdc14p immune complexes with Myc antibody revealed that 13Myc-Sid1p remained in a complex with GFP-Cdc14 throughout the cell cycle (Figure 6B). These results suggest that neither the levels of Sid1p nor its association with Cdc14p are regulated in a cell cycle-dependent manner.

Cdc7p kinase remains active throughout the cell cycle (Sohrman *et al.*, 1998) while Sid2p kinase activity peaks at the end of anaphase (Sparks *et al.*, 1999). Therefore, we wondered if Sid1p kinase activity was cell cycle regulated. *cdc25-22 13Myc-sid1* cells were released from a G₂ block as described above, and samples were collected at 20 min intervals through two rounds of the cell cycle. Kinase assays were then performed on 13Myc-Sid1p immune complexes. Sid1p kinase activity peaked at anaphase/ telophase (Figure 6C), indicating that Sid1p becomes more active at around the same time that it localizes to the SPB (Figure 2). This suggests that Sid1p kinase activity is cell cycle regulated.

Sid1p recruitment to the SPB may be triggered by cyclin destruction

Our localization studies suggest that the Sid1p-Cdc14p complex functions immediately downstream of Cdc7p and requires Cdc7p to localize to the SPB. However, the mere presence of Cdc7p at the SPB is not sufficient to recruit Sid1p-Cdc14p since Cdc7p appears at the SPB(s) at the beginning of mitosis and Sid1p-Cdc14p does not localize to the SPB until anaphase. We reasoned that some other signal, perhaps spindle elongation or anaphase proteolysis events, might contribute to Sid1p-Cdc14p recruitment. To address this issue, we looked at Sid1p and Cdc7p localization in the tubulin mutant *nda3-km311*, which arrests in a pre-metaphase state, unable to form a mitotic spindle. After 4 h at restrictive temperature, 70% of *nda3-km311* cells become arrested in mitosis as judged by the presence of condensed chromatin and absence of septa (Table II). GFP-Cdc7p signal was observed at the SPB(s) in 69% of cells, consistent with GFP-Cdc7p localizing to the SPB(s) in cells blocked in mitosis (Table II; Figure 7B). In contrast, GFP-Sid1p signal was not observed at SPBs in these cells although faint nuclear signal was observed (Figure 7A). This suggests that some aspect of completion of mitosis is required in order for Sid1p to localize. Mitotic progression in *nda3-km311* mutants is prevented by the spindle assembly checkpoint. We therefore addressed whether GFP-Sid1p localized in septating *nda3-km311* cells under conditions in which the spindle assembly checkpoint was rendered inactive by introduction of a *mad2* mutation in the *nda3-km311 GFP-sid1* strain. Unlike *nda3-km311* mutants alone, double mutant cells enter mitosis but are unable to inhibit anaphase proteolysis events. This triggers mitotic exit and cytokinesis, often resulting in cleavage of the undivided nucleus (He *et al.*, 1997). We found that after 4 h at restrictive temperature, only 6% of these cells were in mitosis as judged by condensed chromatin, 46% had septated without undergoing chromosome separation, and GFP-Sid1p could be observed in 50% of the aberrantly septating cells (Table II; Figure 7C, see arrowheads). GFP-Sid1p was not observed in every aberrantly septated cell since many cells had presumably finished septation and Sid1p normally disappears from the SPB once the septum has formed. We conclude that Sid1p localization to the SPB requires anaphase-induced proteolysis events but not chromosome segregation and spindle elongation.

Multiple proteins, such as Cut2p (Funabiki *et al.*, 1996) and the B-type cyclin Cdc13p (Yamano *et al.*, 1996), are ubiquitinated and degraded in anaphase; therefore, we

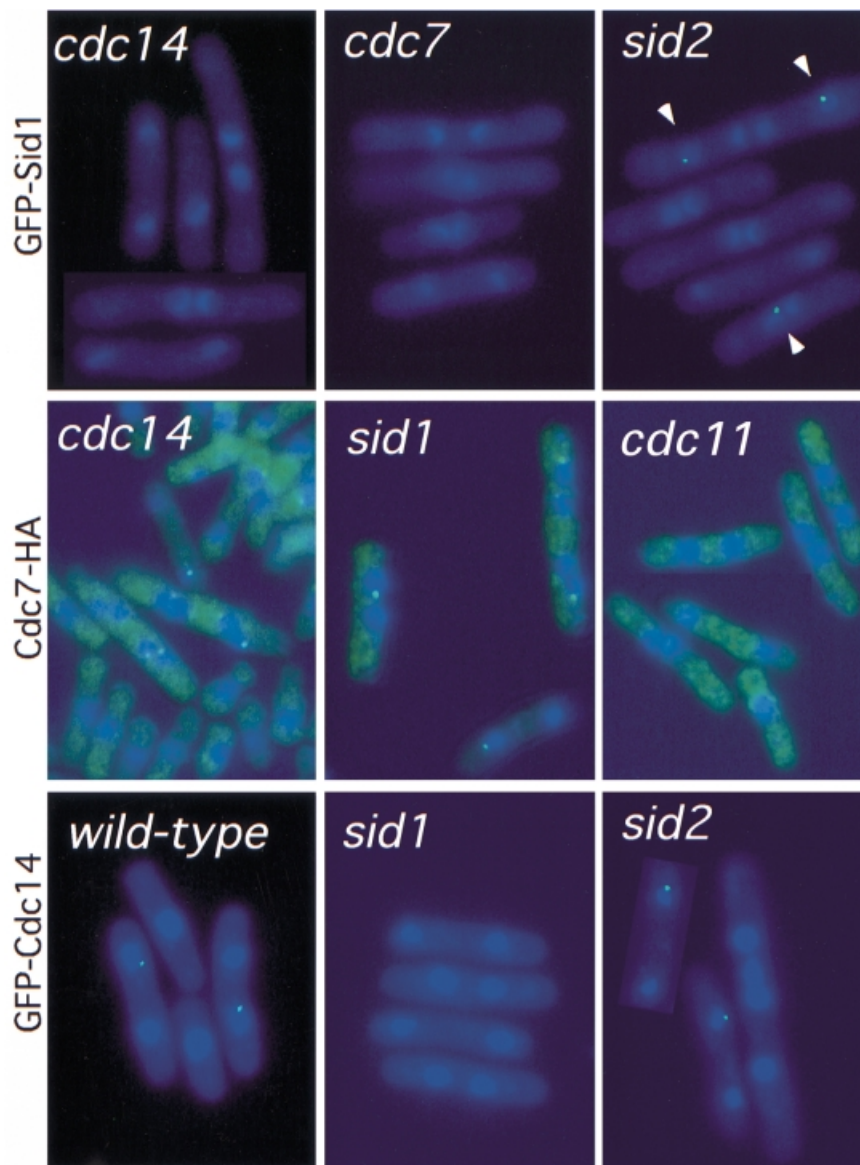


Fig. 4. Dependencies for localization of Sid1p, Cdc14p and Cdc7p. The localizations of GFP-Sid1p, Cdc7-HA and GFP-Cdc14p were examined in wild-type and different *sid* mutant cells after incubation at 36°C for 2 h. All samples were fixed and stained for DNA (blue), GFP-Sid1p and GFP-Cdc14p signal, and Cdc7-HA staining is shown in green. Cdc7-HA cells have a bright green haze as a result of background staining from the secondary antibody.

Table I. Dependencies for localization of Cdc7p, Sid1p and Cdc14p

	Cdc7p localization	Sid1p localization	Cdc14p localization
<i>sid2-250</i>	+	+	+
<i>sid1-125</i>	+	–	–
<i>cdc14-118</i>	+	–	–
<i>cdc7-24</i>	–	–	–
<i>cdc11-123</i>	–	–	–
<i>spg1-106</i>	n.d.	–	–
<i>sid4-A1</i>	n.d.	–	–

+ and – denote whether or not the protein was able to localize properly in the given mutant background. n.d. indicates that this particular strain was not assayed.

attempted to determine which proteins need to be destroyed for Sid1p to localize to the SPB. Previous experiments suggested that inactivation of Cdc2p kinase is

essential for cytokinesis (He *et al.*, 1997). He *et al.* showed that overexpression of *mad2* resulted in a metaphase block. If *mad2* was overexpressed in a temperature-sensitive *cdc2* or *cdc13* mutant strain at permissive temperature (25°C), cells arrested in metaphase. When arrested cells were shifted to restrictive temperature (36°C), they exited mitosis without executing anaphase and underwent cytokinesis. These observations suggest that loss of Cdc2p kinase activity, presumably brought about by cyclin degradation, is essential for triggering cytokinesis. We asked if GFP-Sid1p could be targeted to the SPB by inactivating *cdc2* in Mad2p-overproducing cells. *mad2* was overexpressed in *cdc2-33* cells that also expressed Cdc7p-HA and GFP-Sid1p. Cells were stained with anti-tubulin or anti-HA monoclonal antibodies. It was reported previously that *mad2* overexpression in *cdc2-33* cells at permissive temperature resulted in 50% of cells becoming blocked in metaphase (He *et al.*, 1997). In our strains,

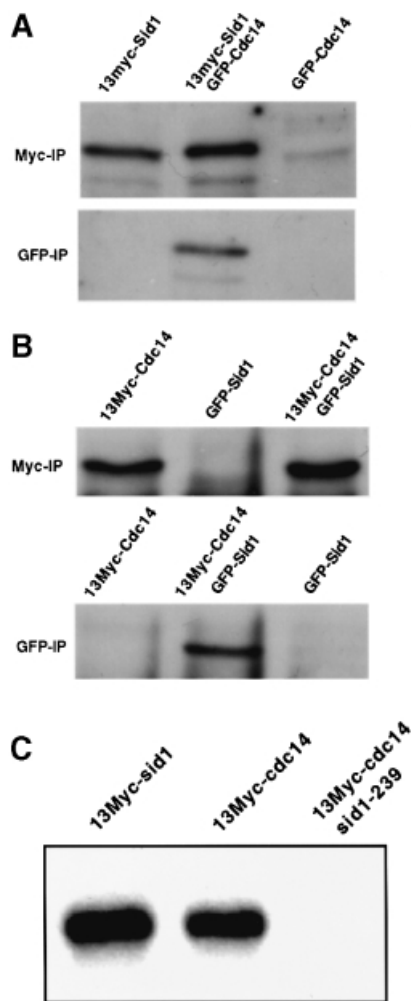


Fig. 5. Sid1p and Cdc14p associate *in vivo*. (A) Lysates were prepared from cells expressing 13Myc-Sid1p (YDM617), GFP-Cdc14p (YDM627) or both (YDM629). (B) Lysates were prepared from cells expressing 13Myc-Cdc14p (YDM617), GFP-Sid1p (YDM558) or both (YDM626). Each lysate was split in two, and immunoprecipitations were performed using anti-Myc or anti-GFP antibodies. Immune complexes were analyzed by Western blotting using anti-Myc antibodies. (C) Wild-type cells expressing 13Myc-Sid1p (YDM587) or 13Myc-Cdc14p (YDM617), or *sid1-239* (YDM637) cells expressing 13Myc-Cdc14p were started at 25°C, shifted to 36°C for 3 h, and kinase assays were performed on 13Myc-Cdc14 or 13Myc-Sid1 immune complexes from these cells.

~20% of cells became blocked with metaphase spindles. However, this percentage is still substantially elevated over the 2% that are observed in asynchronous wild-type cells (Hagan and Hyams, 1988). Twenty-one percent of cells displayed a metaphase Cdc7p staining pattern, consistent with Cdc7p being present at the SPBs of metaphase-arrested cells (Figure 7D, see arrow). Sid1p was never observed at the SPBs in these cells (Figure 7D, see arrow). All cells that displayed Sid1p SPB signal (87/87) were in late anaphase and presumably not yet arrested. After a shift to restrictive temperature for 50 min to inactivate Cdc2p, cells could now be observed that were septating without undergoing anaphase (Figure 7E and F, see arrows). At this point, 96% (68/71) of cells displaying Sid1p signal at the SPB were septating without having undergone anaphase (Figure 7E and F, see arrows). Thus,

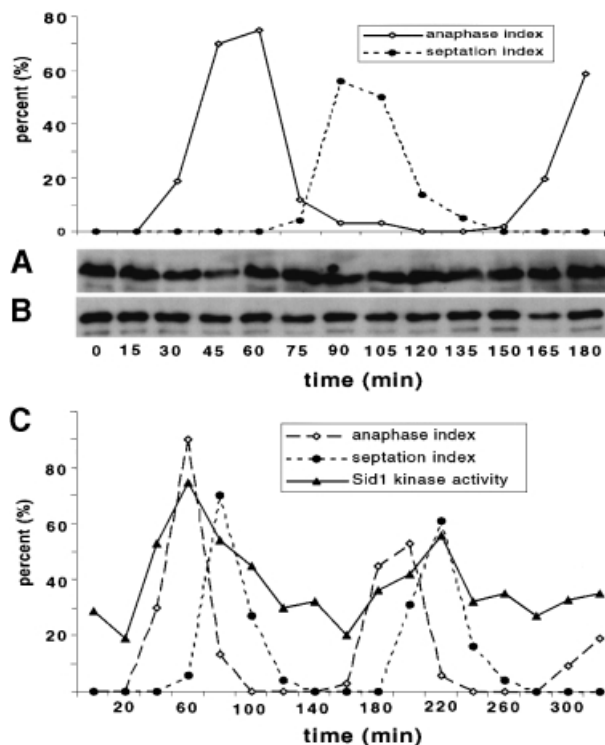


Fig. 6. Sid1p kinase activity, but not protein level or ability to co-immunoprecipitate with Cdc14p, is cell cycle regulated. (A and B) *cdc25-22 13myc-sid1 GFP-cdc14* cells were released in synchrony from a Cdc25-dependent G₂ block. Cells were collected at 15 min intervals and scored for the percentage of total cells in anaphase or septation. (A) Western analysis was performed directly on lysates with Myc antibody to detect Sid1p. (B) GFP-Cdc14 immune complexes were prepared from lysates using GFP antibody and subjected to Western analysis with Myc antibody. (C) *cdc25-22 13Myc-sid1* cells were released from a G₂, samples were collected at 20 min intervals and kinase assays were performed on 13Myc-Sid1p immune complexes. Kinase activity is displayed in arbitrary units.

it appears that loss of Cdc2p activity in metaphase is sufficient to allow Sid1p to localize to the SPB and for initiation of cytokinesis.

If loss of Cdc2p activity triggers Sid1p localization to the SPB, one would expect Sid1p localization to follow Cdc2p kinase inactivation. To test this directly, H1 kinase activity and GFP-Sid1p localization were monitored as cells progressed through mitosis. As expected, Sid1p localization peaked as cells completed anaphase, coincident with loss of Cdc2 kinase activity, but prior to septation (Figure 8A). APC/C-dependent degradation of Cdc13p upon anaphase onset results in decreased Cdc2 kinase activity. It has been shown previously using indirect immunofluorescence that Cdc13p accumulates in the nucleus until anaphase onset, when it becomes degraded (Alfa *et al.*, 1990). Consistent with loss of Cdc2p kinase activity triggering Sid1p localization, cells in early mitosis that have not degraded Cdc13p do not display GFP-Sid1p at the SPB (Figure 8B, cells 1 and 2), but do show Cdc7p SPB signal (Figure 8C). GFP-Sid1p does not become localized to the SPB until cells degrade Cdc13p (Figure 8B, cells 3 and 4). Interestingly, post-mitotic cells that finished septation and no longer show GFP-Sid1p SPB signal have begun to accumulate cyclin (Figure 8B, cell 5). These experiments are consistent with the notion

Table II. Cdc7p-GFP and GFP-Sid1p localization in an *nda3* block

Strain	% Condensed chromatin	% Pole Staining	% Septa
<i>cdc7-GFP nda3-km311</i>	70	69	1
<i>GFP-sid1 nda3-km311</i>	71	0	0
<i>GFP-sid1 nda3-km311 mad2Δ</i>	6	23	46

For all data points, at least 200 cells were scored.

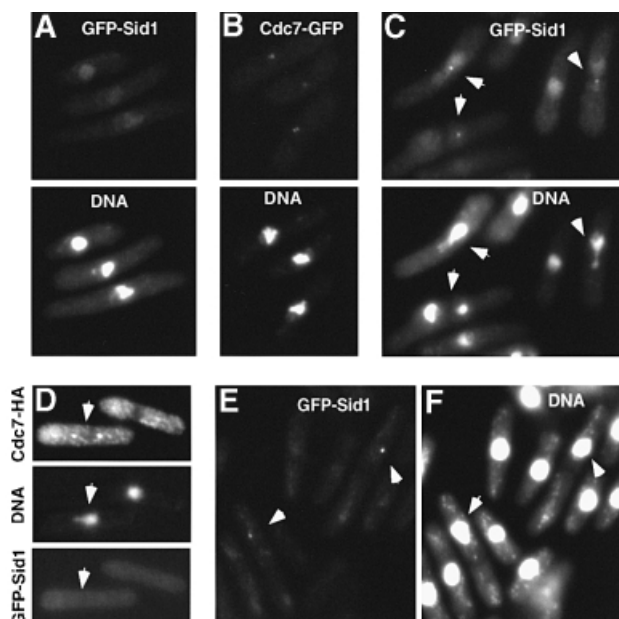


Fig. 7. Cdc2p inactivation triggers localization of Sid1p to the SPB. The *nda3-km311 GFP-sid1* (A), *nda3-km311 GFP-cdc7* (YDM667) (B) and *nda3-km311 GFP-sid1 mad2Δ* (YDM555) (C) strains were grown at 30°C and then shifted to 19°C for 4 h, fixed, stained for DNA and the GFP signals were visualized. (D-F) *cdc2-33 GFP-sid1 cdc7-HA* cells (YDM651) containing a *nmt1-mad2* plasmid were grown at 25°C in the presence of thiamine, then thiamine was removed to induce *mad2* expression and cells were allowed to continue growth at 25°C for 20 h. At this time, a portion of the culture was fixed and stained for DNA, Cdc7-HA and GFP-Sid1p. The rest of the culture was shifted to 36°C for 50 min, fixed and stained for DNA (F) and the GFP-Sid1p signal was visualized (E). Arrowheads indicate cells described in the text.

that Sid1p does not localize to the SPB until anaphase proteolysis of cyclin (and inactivation of Cdc2p kinase) occurs.

The *sid* mutants are not defective in Cdc13p cyclin proteolysis

Our results suggest that the *sid* pathway in *S.pombe*, while clearly functioning in cytokinesis, may be regulated by cyclin proteolysis. The observation that *sid* mutants continue to undergo multiple nuclear division cycles also suggests that the *sid* pathway does not play a major role in regulating cyclin proteolysis in *S.pombe*; however, *sid* mutants have not been examined previously for fluctuations in cyclin levels. We therefore investigated whether the *S.pombe sid* genes were required for cyclin proteolysis. Synchronous populations of *cdc7-24* (Figure 9A) and wild-type (Figure 9D) cells were analyzed for Cdc13p levels, DNA content and number of nuclei (Figure 9A). This analysis showed that in *cdc7-24* cells, like wild-type

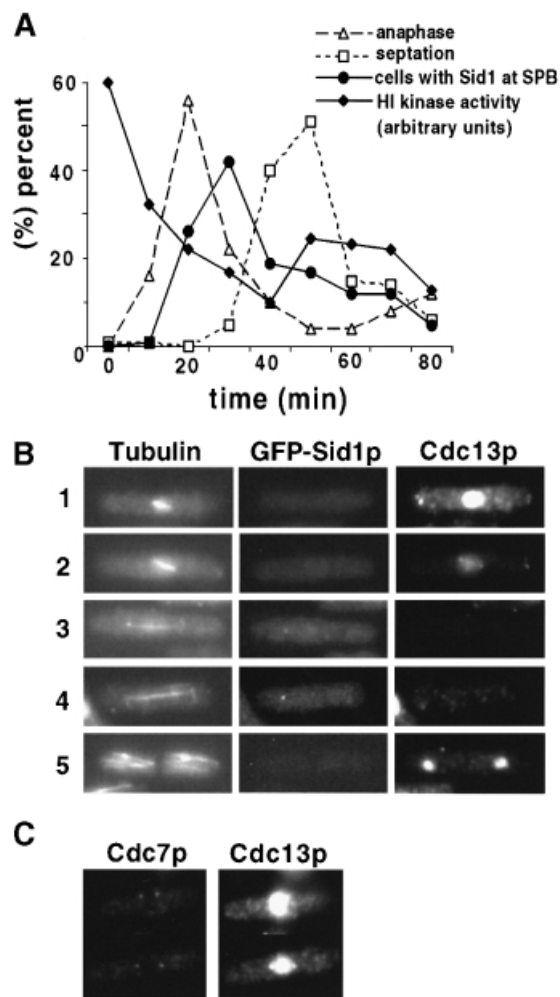


Fig. 8. Sid1p SPB localization coincides with Cdk inactivation and cyclin destruction. (A) *nda3-km311 GFP-sid1* cells were blocked in early mitosis by incubation at 19°C for 6 h, released from the block by shifting to 30°C and samples were collected every 10 min and scored for percentage anaphase, septation, GFP-Sid1p localization and H1 kinase activity. (B) *GFP-sid1* cells were fixed and stained for tubulin and cyclin (Cdc13p). Numbers indicate the cell cycle stages described in the text. (C) *cdc7-HA* cells were fixed and stained for Cdc7-HA and cyclin.

cells, Cdc13p proteolysis proceeded normally as cells entered anaphase. The loss of Cdc13p in *cdc7-24* cells could also be observed by immunofluorescence. Cdc13p was observed in the nucleus of interphase cells as judged by the presence of interphase microtubule arrays (Figure 9B, see arrows). However, in cells that displayed anaphase spindles, Cdc13p had disappeared from the nucleus (Figure 9B, see arrowheads). Similar results were obtained when all of the other *sid* mutants were examined (data not shown). To determine whether Cdc2p kinase activity dropped as cells entered anaphase, Cdc2p kinase activity was also analyzed (Figure 9C). It was only feasible to synchronize sufficient cells for analysis of Cdc2p kinase activity through the first mitosis; however, we analyzed the number of nuclei and DNA content through another mitosis to demonstrate that cells were undergoing a first cell cycle block. This analysis showed that like Cdc13p levels, Cdc2p kinase activity dropped as cells entered anaphase. Thus, it seems clear that in *S.pombe* the *sid* pathway is not essential for cyclin destruction and mitotic

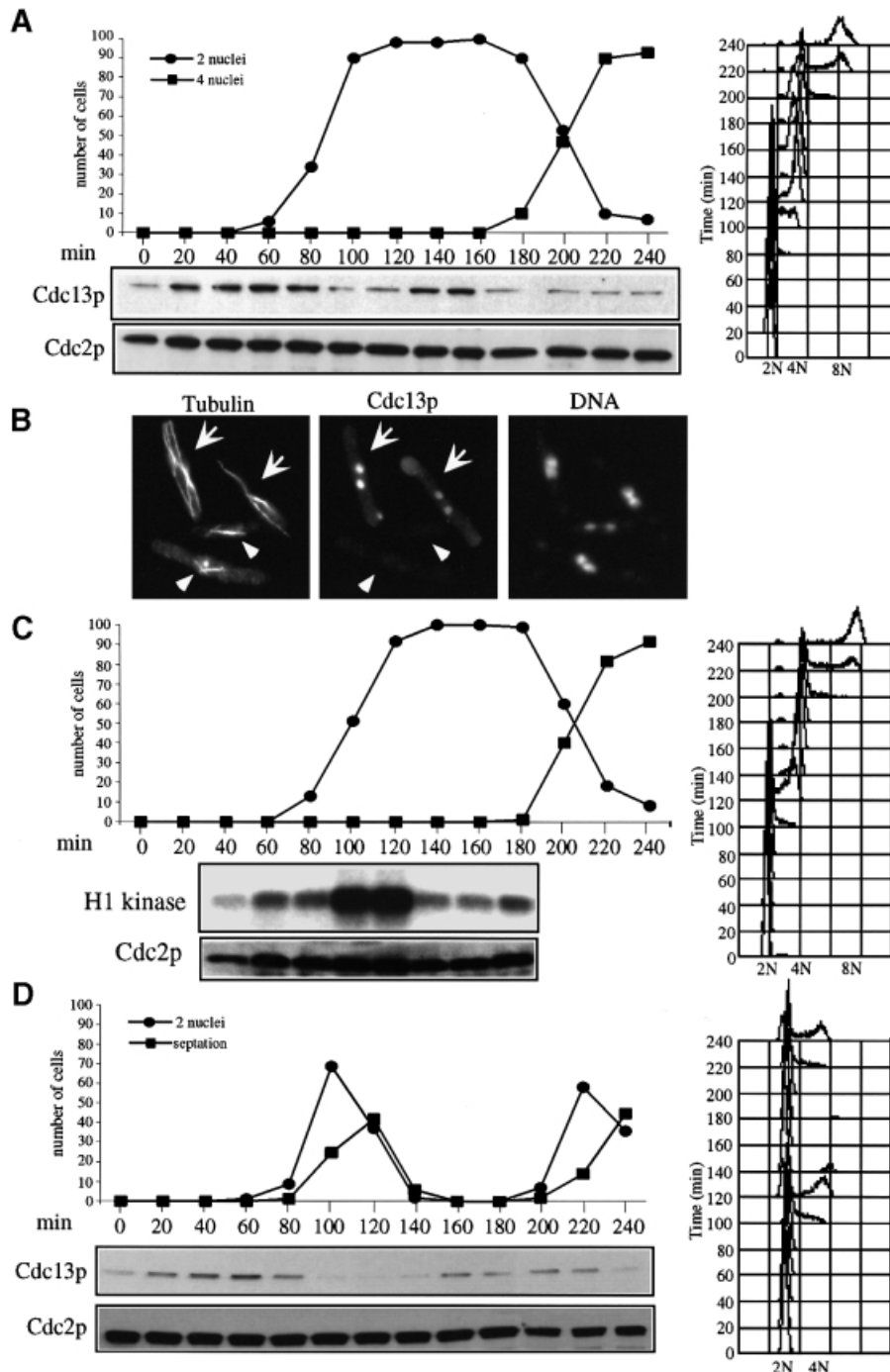


Fig. 9. The *sid* genes are not required for Cdc13p–cyclin destruction in mitosis. (A) *cdc7-24* (YDM168) cells, grown at 25°C, were synchronized in early G₂ phase by centrifugal elutriation and then shifted to 36°C. Every 20 min, samples were removed and examined for number of nuclei, Cdc13p and Cdc2p levels by Western blotting, and DNA content by FACS. (B) Asynchronous *cdc7-24* cells were grown at 25°C, shifted to 36°C for 2 h, fixed and stained for tubulin, Cdc13p and DNA. Arrows indicate interphase cells, and arrowheads indicate post-metaphase cells. (C) *cdc7-24* cells were grown as in (A) and H1 kinase assays were performed on samples through the first mitosis. (D) Wild-type cells were analyzed as in (A).

exit; however, it remains possible that the *sid* pathway could play a minor role in cyclin destruction.

Discussion

A signaling pathway to initiate septation and cytokinesis

Several studies suggest that the *sid* gene products function in a signal transduction pathway (Marks *et al.*, 1992;

Schmidt *et al.*, 1997; Balasubramanian *et al.*, 1998; Sohrmann *et al.*, 1998; Sparks *et al.*, 1999). Overexpression of Cdc7p protein kinase rescues the cytokinesis defects of *cdc11* and *spg1* mutants (Fankhauser and Simanis, 1994; Schmidt *et al.*, 1997), whereas overexpression of Spg1p rescues *cdc11* mutants (Schmidt *et al.*, 1997), suggesting that Cdc7p functions downstream of Spg1p, which, in turn, functions downstream of *cdc11*. Interestingly, Spg1p overexpression can induce septum

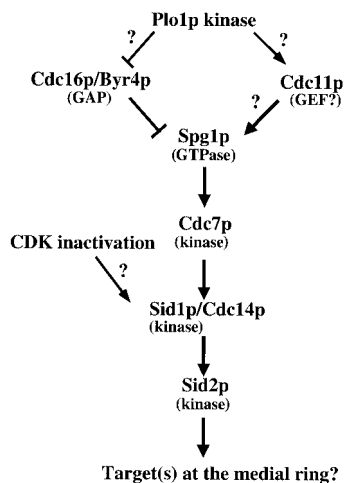


Fig. 10. A model for regulation of septation initiation in *S.pombe* (described in the text).

formation during interphase in a Cdc7p-, Cdc14p-, Sid4p-, Sid1p- and Sid2p-dependent manner, consistent with Spg1p functioning early in the cascade (Schmidt *et al.*, 1997; Balasubramanian *et al.*, 1998; Sparks *et al.*, 1999). Cdc16p and Byr4p have been identified as a two-component GAP for Spg1 (Furge *et al.*, 1998). Because *cdc11* mutants can be rescued by loss-of-function mutations in *cdc16* (Marks *et al.*, 1992), it has been proposed that Cdc11p may act as the GEF for Spg1p, although proof awaits the molecular cloning and characterization of *cdc11* (Schmidt *et al.*, 1997). Since localization of Cdc7p to the SPB has been shown to depend on Spg1p-GTP, the fact that Cdc7p does not localize in *cdc11* mutants coincides with the proposed function of Cdc11p as an activator of Spg1p. Sid2p kinase localizes to the SPB throughout the cell cycle and transiently to the cleavage furrow in late anaphase. Sid2p kinase activity and localization require the function of the other *sid* gene products, suggesting that it functions late in the pathway (Sparks *et al.*, 1999). In the present study, we found that Sid1p–Cdc14p localizes to one SPB after Cdc7p, just before Sid2p localizes to the cleavage furrow. Our localization studies suggest that Sid1p–Cdc14p functions at an intermediate step in the pathway, downstream of Cdc7p and upstream of Sid2p. We propose a model in which Spg1p, regulated by a two-component GAP comprised of Cdc16p/Byr4p and potential activator Cdc11p, functions upstream of Cdc7p kinase, while Sid1p kinase–Cdc14p functions downstream of Cdc7p and upstream of Sid2p kinase (Figure 10). While this model is consistent with genetic and localization studies presented here and elsewhere, further biochemical studies will be necessary to determine if the model is correct.

The role of Sid4p and Plo1p kinase in the *sid* pathway is unclear. Plo1p may function early in the pathway since its overexpression can induce septum formation in interphase, suggesting that Plo1p drives both actin ring formation and activation of the *sid* pathway (Ohkura *et al.*, 1995). Plo1p overexpression also results in recruitment of Cdc7p to the SPB, presumably by activating Spg1p (Mulvihill *et al.*, 1999). One prediction based on these data is that Plo1p might function to activate the GEF or inactivate the GAP for Spg1p.

The target of Sid1p phosphorylation *in vivo* is unknown; however, since Sid2p seems to function immediately downstream of Sid1p–Cdc14p, one model is that Sid2p or an associated protein could be substrates for Sid1p. Consistent with this idea, Sid1p peak kinase activity and Sid1p–Cdc14p SPB localization coincide with activation of Sid2p kinase activity and localization to the cleavage site. At present, it is unclear whether Sid1p acts directly on Sid2p or through another molecule(s). The function of Cdc14p is also unclear. Determination of how the *sid* proteins regulate each other will require studies using purified components *in vitro*.

Recruitment of Sid1p–Cdc14p to the SPB may be regulated by Cdc2–cyclin proteolysis

Studies involving expression of non-degradable forms of cyclin in *S.pombe* (Yamano *et al.*, 1996), *Saccharomyces cerevisiae* (Surana *et al.*, 1993) and mammalian cells (Wheatley *et al.*, 1997) suggest that APC/C-dependent cyclin proteolysis and subsequent loss of Cdk activity is required to initiate cytokinesis. Surprisingly, inactivation of some APC/C subunits in *S.pombe*, which are required for cyclin ubiquitylation in mitosis, does not block cytokinesis (Hirano *et al.*, 1988; Samejima and Yanagida, 1994; Berry *et al.*, 1999). It is possible that Cdc2p activity is inhibited in these APC mutants through tyrosine phosphorylation or the action of the inhibitor Rum1p (Moreno and Nurse, 1994), thus allowing cells to undergo cytokinesis. Activation of the *sid* pathway by inactivation of *cdc16* can cause interphase cells to septate, but only if Cdc2p activity is low (Cerutti and Simanis, 1999). Similarly, Spg1p overproduction will not cause *nda3* cells, which are blocked in early mitosis with high Cdc2p activity, to septate (Schmidt *et al.*, 1997). Finally, overexpression of the spindle checkpoint protein Mad2p induces metaphase arrest by hyperactivating the spindle checkpoint (He *et al.*, 1997). These cells maintain high Cdc2p kinase activity and do not septate. If the mitotic kinase (*cdc2*) or the associated mitotic cyclin (*cdc13*) is inactivated in cells blocked in metaphase by *mad2* overexpression, cells form a division septum without undergoing anaphase. These data are consistent with the idea that high Cdc2p activity acts to inhibit initiation of septation. In this study, we show that activation of the spindle checkpoint by two different methods blocks Sid1p localization to the SPB, whereas Cdc7p can clearly be seen on spindle poles under these conditions. When we inactivated Cdc2p or the spindle checkpoint, septum formation was induced in the absence of anaphase and Sid1p localized to the SPB. It is unclear whether loss of Cdc2p activity directly regulates Sid1p–Cdc14p localization or functions through some intermediary; however, it is clear from our studies that cyclin degradation and Cdc2p inactivation occur prior to Sid1p–Cdc14p SPB localization. Both Sid1p and Cdc14p contain potential Cdc2p phosphorylation sites, but their functional significance is not known. In any case, although the data presented here are correlative, they suggest that Cdc2p inactivation, perhaps triggered by cyclin proteolysis, regulates Sid1p–Cdc14p localization to the SPB, which could provide a key control mechanism to link completion of mitosis with cytokinesis in *S.pombe*.

Table III. Strains used

Strain	Genotype	Source
YDM435	<i>sid1-125 ade6-210 ura4-D18 leu1-32 h⁺</i>	laboratory stock
YDM75	<i>sid1-239 ade6-210 ura4-D18 leu1-32 h⁺</i>	laboratory stock
YDM105	<i>leu1-32 ura4-D18 ade6-210 h⁻</i>	laboratory stock
YDM108	<i>leu1-32 ura4-D18 ade6-216 h⁺</i>	laboratory stock
YDM579	<i>sid1::ura4⁺/sid1⁺ ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-210/ade6-216 h⁺/h⁻</i>	this study
YDM558	<i>GFP-sid1::ura4⁺ ura4-D18 leu1-32 ade6-210 h⁻</i>	this study
YDM587	<i>13Myc-sid1::ura4⁺ ura4-D18 leu1-32 ade6-210 h⁻</i>	this study
YDM627	<i>GFP-cdc14::leu1⁺ ura4-D18 leu1-32 ade6-210 h⁻</i>	this study
YDM617	<i>13Myc-cdc14::leu1⁺ ura4-D18 leu1-32 ade6-210 h⁻</i>	this study
YDM553	<i>GFP-sid1::ura4⁺ cdc7-HA::ura4⁺ ura4-D18 leu1-32 ade6-210 h⁻</i>	this study
YDM560	<i>GFP-sid1::ura4⁺ cdc16-116 ura4-D18 leu1-32 h⁻</i>	this study
YDM564	<i>GFP-sid1::ura4⁺ nmt1-spg1::leu1⁺ ura4-D18 leu1-32 ade6-210 h⁺</i>	this study
YDM551	<i>nmt1-spg1::leu1⁺ ura4-D18 leu1-32 ade6-210 h⁺</i>	K.Gould
YDM566	<i>sid1-125 nmt1-spg1::leu1⁺ ura4-D18 leu1-32 ade6-210 h⁻</i>	this study
YDM561	<i>GFP-sid1::ura4⁺ sid4-A1 ura4-D18 leu1-32 ade6-210h⁻</i>	this study
YDM562	<i>GFP-sid1::ura4⁺ cdc11-123 ura4-D18 leu1-32 ade6-210h⁻</i>	this study
YDM568	<i>GFP-sid1::ura4⁺ cdc14-118 ura4-D18 leu1-32 ade6-210h⁻</i>	this study
YDM569	<i>GFP-sid1::ura4⁺ cdc7-24 ura4-D18 leu1-32 ade6-210h⁻</i>	this study
YDM567	<i>GFP-sid1::ura4⁺ sid2-250 ura4-D18 leu1-32 ade6-210h⁻</i>	this study
YDM563	<i>GFP-sid1::ura4⁺ spg1-106 ura4-D18 leu1-32 ade6-210h⁻</i>	this study
YDM572	<i>cdc7-HA::ura4⁺ cdc11-123 ura4-D18 leu1-32 ade6-210h⁻</i>	this study
YDM571	<i>cdc7-HA::ura4⁺ cdc14-118 ura4-D18 leu1-32 ade6-210 h⁺</i>	this study
YDM542	<i>cdc7-HA::ura4⁺ sid1-125 ura4-D18 leu1-32h⁻</i>	this study
YDM646	<i>GFP-cdc14::leu1⁺ cdc11-123 ura4-D18 leu1-32h⁻</i>	this study
YDM647	<i>GFP-cdc14::leu1⁺ spg1-106 ura4-D18 leu1-32 ade6-210 h⁺</i>	this study
YDM633	<i>GFP-cdc14::leu1⁺ sid1-125 ura4-D18 leu1-32 ade6-210 h⁺</i>	this study
YDM632	<i>GFP-cdc14::leu1⁺ sid4-A1 ura4-D18 leu1-32 ade6-210 h⁻</i>	this study
YDM631	<i>GFP-cdc14::leu1⁺ sid2-250 ura4-D18 leu1-32 ade6-210 h⁻</i>	this study
YDM636	<i>GFP-cdc14::leu1⁺ cdc7-24 ura4-D18 leu1-32 ade6-210 h⁻</i>	this study
YDM629	<i>GFP-cdc14::leu1⁺ 13Myc-sid1::ura4⁺ ura4-D18 leu1-32 ade6-210 h⁻</i>	this study
YDM626	<i>GFP-sid1::ura4⁺ 13Myc-cdc14::leu1⁺ ura4-D18 leu1-32 ade6-210 h⁻</i>	this study
YDM637	<i>13Myc-cdc14::leu1⁺ sid1-239 ura4-D18 leu1-32 ade6-210 h⁺</i>	this study
YDM666	<i>GFP-sid1::ura4⁺ nda3-km311 ura4-D18 leu1-32 ade6-210 h⁺</i>	this study
YDM555	<i>GFP-sid1::ura4⁺ nda3-km311 mad2Δ ura4-D18 leu1-32 ade6-210 h⁺</i>	this study
YDM667	<i>cdc7-GFP::ura4⁺ nda3 km311 ura4-D18 leu1-32 ade6-210 h⁺</i>	this study
YDM651	<i>cdc7-HA::ura4⁺ GFP-sid1::ura4⁺ cdc2-33 leu1-32h⁻</i>	this study
YDM168	<i>cdc7-24 h⁻</i>	P.Nurse

Evolutionary differences in sid pathway function

The *S.pombe* genes *plol*, *cdc16*, *byr4*, *spg1*, *cdc7* and *sid2* all have counterparts in *S.cerevisiae* (*CDC5*, *BUB2*, *BFA1*, *TEM1*, *CDC15* and *DBF2*, respectively). As in *S.pombe*, the products of these genes appear to define a signal transduction pathway required for late mitotic events and have been termed the mitotic exit network (Shou *et al.*, 1999). Evidence from budding yeast suggests that this pathway is required for inactivation of cyclin-dependent kinase (Cdk) activity at the end of anaphase and acts through Cdc14 phosphatase to signal cyclin proteolysis and stabilization of the Cdk inhibitor, Sic1; however, a definitive link to cytokinesis has yet to be identified (Visintin *et al.*, 1998; Alexandru *et al.*, 1999; Shou *et al.*, 1999; Visintin *et al.*, 1999). The spindle checkpoint pathway, acting through Bub2, can block cyclin proteolysis and mitotic exit by inhibiting the mitotic exit network genes (Alexandru *et al.*, 1999; Fesquet *et al.*, 1999; Fraschini *et al.*, 1999; Li, 1999). It has been unclear whether the sid pathway plays a role in cyclin proteolysis in fission yeast. Our results showing that Cdc13p (cyclin) destruction is unaffected in *sid* mutants are consistent with the sid pathway not contributing to cyclin proteolysis during anaphase. In fact, this study suggests that in *S.pombe*, the sid pathway itself may be regulated by Cdk inactivation. Some evidence suggests that the spindle checkpoint may inhibit the sid pathway directly in

S.pombe through the Cdc16p GAP (the *S.cerevisiae* BUB2 homolog), which is required for the spindle checkpoint pathway (Fankhauser *et al.*, 1993). Given what is known about the sid pathway, this result implies that response to activation of the spindle checkpoint may cause Cdc16p to inactivate Spg1p, in turn blocking Cdc7p recruitment to the SPB. However, we found that Cdc7p is present at the SPB in cells with the spindle checkpoint activated. Thus, if Cdc16p does function in the spindle checkpoint pathway in *S.pombe*, it seems unlikely that it functions by inactivating the sid pathway through Spg1p. Perhaps the different roles of the sid pathway in *S.pombe* and *S.cerevisiae* reflect the evolutionary divergence between the two organisms. It will be important in future studies to identify components of the sid pathway in higher eukaryotes and determine how they function.

Materials and methods

Yeast methods and strains

The *S.pombe* strains used in this study are listed in Table III. Fission yeast media and genetic manipulations were as previously described (Moreno *et al.*, 1991). DNA sequencing was performed at the UMass Medical School Nucleic Acid Facility (Worcester, MA). Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA) or from Operon Inc. (Alameda, CA).

Cloning of the *sid1* gene

sid1-239 (YDM75) was transformed with a genomic library constructed in pUR19 (Barbet *et al.*, 1992). Transformants were selected at 25°C, and then replica plated to 36°C. Four plasmids containing overlapping inserts were identified that were capable of rescuing the *sid1* temperature-sensitive growth defect at 36°C. Integration mapping showed that *sid1* mapped to the same region of the genome as the cloned gene, demonstrating that the cloned gene was *sid1* and not a multicopy suppressor. The ends of the genomic inserts of two of the rescuing plasmids were sequenced. A search of the Sanger Center *S.pombe* genome sequencing project database revealed that these genomic clones corresponded to sequenced regions of the *S.pombe* genome.

Construction of *sid1* deletion and epitope-tagged strains

A *sid1* deletion strain was constructed using the one-step gene replacement strategy (Grimm *et al.*, 1988). The knockout construct was made by first generating by PCR an 1100 bp *SacI*–*NotI* fragment containing the 5′-flanking region of *sid1* that ends at the *sid1* start codon. A *sid1* 3′-flanking region that contained the 1000 bp immediately downstream of the *sid1* stop codon was also amplified by PCR as a *NotI*–*Sall* fragment and the two fragments were cloned in a three piece ligation into *SacI*–*Sall*-cut pSK to generate plasmid pDM356. A fragment containing the *S.pombe ura4⁺* gene as a *NotI* fragment was then cloned into the *NotI* site of this plasmid to generate the *sid1* deletion construct, plasmid pDM357. The *SacI*–*Sall* fragment of this plasmid was purified and transformed into a diploid strain (YDM105 crossed with YDM108) using the lithium acetate method. PCR analysis of DNA prepared from individual transformants identified a strain bearing the *sid1* deletion (YDM579). The *sid1* deletion strain was induced to sporulate, and tetrads were dissected, which resulted in only two viable *ura4⁻* progeny per tetrad. The viability of the *sid1* deletion strain was rescued by a plasmid containing the *sid1* coding region expressed from the *nmt1* promoter (pDM321).

Single copy gene replacement strains expressing GFP and 13Myc-tagged versions of *sid1* were constructed using the following strategy. PCR was used to amplify the first 660 bp of the *sid1* coding region and to add a *NotI* site immediately after the start ATG. This fragment was cut with *NotI*–*EcoRI* and ligated, along with the *SacI*–*NotI*-cut *sid1* promoter fragment from pDM356, into the integrating vector pJK210 that had been cut with *SacI*–*EcoRI*, generating plasmid pDM336. Then either the 13Myc or GFP tags bearing *NotI* sites at their ends were cloned into pDM336 to generate plasmids pDM360 and pDM339, respectively. Each of these plasmids was linearized at the *NdeI* site in the coding region and transformed into strain YDM105 to generate strains expressing a single copy of GFP-tagged (YDM558) and 13Myc-tagged (YDM587) *sid1* expressed from its own promoter, as well as a truncated (non-functional) copy of *sid1*. Correct integration of each construct was confirmed by PCR.

Strains expressing a single copy of GFP- and 13Myc-tagged versions of *cdc14* from the *cdc14* promoter were constructed using a strategy similar to that described above for *sid1*. PCR was used to generate a 960 bp *SacI*–*NotI* fragment containing the 5′-flanking region of *cdc14*, as well as the entire *cdc14* coding region as a *NotI*–*BamHI* fragment. These two fragments were cloned into the integrating vector pJK148 that had been cut with *SacI*–*BamHI* to generate plasmid pDM369. Then either the 13Myc or GFP tags bearing *NotI* sites at their ends were cloned into pDM369 to generate plasmids pDM374 and pDM370, respectively. Each of these plasmids was linearized at the *NdeI* site in the *leu1⁺* gene and transformed into strain YDM105 to generate strains expressing a single copy of GFP-tagged (YDM627) and 13Myc-tagged (YDM617) *cdc14* expressed from its own promoter, as well as a second, untagged copy of *cdc14*. Correct integration of each construct was confirmed by PCR.

Construction of *sid1* kinase-dead plasmids

Triple HA-tagged (pRep41HA-Sid1) *sid1* was constructed by adding *BamHI* sites to the ends of the *sid1* coding region by PCR followed by cloning into the *BamHI* site of vector pRep41HA (Craven *et al.*, 1998). Kinase-dead *sid1* was created by site-directed mutagenesis with a QuikChange kit (Stratagene) using pRep41HA-Sid1 directly as a template. A single nucleotide change generated by the following oligonucleotides was sufficient to create the conservative lysine to arginine mutation at residue 38: 5′-GTGTCTGGCGACATTATCGCT-ATTAGACAGGTAAGACTAATTAATTTTTAC-3′ and 5′-GTAAAA-ATTAATTAGTCTTACCTGTCTTACCTGTCTAATAGCGATAATGTCGCCAGACAC-3′. Changes were confirmed by sequencing.

Microscopy

Indirect immunofluorescence staining was performed as previously described (Balasubramanian *et al.*, 1997). The tubulin antibody TAT-1 (Woods *et al.*, 1989) was a generous gift from K.Gull (University of Manchester, UK). Cdc13 and HA antibodies (BABCO, Richmond, CA) were diluted 1:200. Primary antibodies were detected with anti-rabbit or anti-mouse Texas red or Alexa 594–IgG (Molecular Probes). DNA was visualized with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) at 20 µg/ml. Images were captured using a Nikon Eclipse E600 microscope with a cooled CCD camera (Dage 300, MVI, Avon, MA) and IPLab Spectrum software (Signal Analytics Corporation, Vienna, VA).

Protein methods and kinase assays

Sid1 kinase assays were performed as previously described (Sparks *et al.*, 1999) except that a different kinase assay buffer was used (10 mM MgCl₂, 10 mM Tris pH 7.4). For co-immunoprecipitation studies, lysates were incubated with anti-GFP antibody, immune complexes were recovered with protein G–Sepharose beads and resolved on 7% polyacrylamide gels, and Western blots were probed with Myc antibody.

For block and release experiments, cells were incubated at restrictive temperatures (6 h at 19°C and 3.5 h at 36°C for *nda3-km311* and *cdc25-22* strains, respectively), then released by shifting to permissive temperature. For other synchronous cell experiments, cells were grown to mid-log phase at 25°C in YE medium, and G₂ cells were isolated by centrifugal elutriation. Cells were collected on a filter, resuspended in pre-warmed YE medium, incubated for 30 min at 36.5°C, and then samples were collected every 20 min for fluorescence-activated cell sorting (FACS) analysis, nuclei count, septation count and cell lysates. H1 kinase assays were performed as previously described (Gould *et al.*, 1991).

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