Identification of Functional Domains within the Septation Initiation Network Kinase, Cdc7*

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The septation initiation network (SIN) serves to coordinate cytokinesis with mitotic exit in the fission yeast Schizosaccharomyces pombe. SIN components Spg1 and Cdc7 together play a central role in regulating the onset of septation and cytokinesis. Spg1, a Ras-like GTPase, localizes to the spindle pole bodies (SPBs) throughout the cell cycle. It is converted to its GTP-bound (active) state during mitosis, only to become inactivated at one SPB during anaphase and at both SPBs as cells exit mitosis. Cdc7 functions as an effector kinase for Spg1, binding to Spg1 in its GTP-bound state, and therefore is present at both SPBs during mitosis and asymmetrically at only one during anaphase. Interestingly, the kinase activity of Cdc7 does not vary across the cell cycle, suggesting the possibility that Cdc7 kinase activity is independent of Spg1 binding. Consistent with this, we found that Cdc7 associates with Spg1 only during mitosis. To learn more about the essential role of Cdc7 kinase in the SIN and its regulation, we undertook a structure/function analysis and identified independent functional domains within Cdc7. We found that a region adjacent to the kinase domain is responsible for Spg1 association and identified an overlapping but distinct SPB localization domain. In addition Cdc7 associates with itself and exists as a dimer in vivo.

Cell division is a fundamental biological event that underlies the growth and development of all organisms. Critical events in the process of cell division include DNA replication, mitosis (where genetic material is faithfully segregated), and cytokinesis (which results in the actual separation of daughter cells). In a normal cell cycle, cytokinesis takes place only after chromosome segregation has been completed, thus safeguarding genomic integrity. A failure to temporally and spatially regulate this event is catastrophic for the cell, resulting in aneuploidy, cell death, or cancer (1).

Conserved GTase-driven signaling pathways have emerged that serve to coordinate mitotic exit with cytokinesis (2, 3). In the fission yeast Schizosaccharomyces pombe, this network, referred to as the septation initiation network (SIN),2 controls actomyosin ring construction and septum deposition after mitosis. Loss of function mutations in sin genes result in elongated multinucleate cells due to continuation of nuclear division and cell growth in the absence of deposition of septal material (2, 3). In contrast, hyperactivation of the SIN (caused by mutations in inhibitors of SIN activity) leads to formation of multiple septa without cell separation. SIN components include a GTase Spg1 (4, 5), a two-component GTase-activating protein for Spg1 composed of Byr4 and Cdc16 (6), and four protein kinases: Plo1 (7), Cdc7 (8), Sid1 and its regulatory subunit Cdc14 (9), and Sid2 and its regulatory subunit Mob1 (10–12). All SIN components reside on the SPB either constitutively or transiently at some stage during the cell cycle. Two proteins, Sid4 and Cdc11, function together as a platform to assemble the SIN and its regulators on the SPB (13–16). FRAP (fluorescence recovery after photobleaching) analysis of SIN components demonstrate that Sid4 and Cdc11 are stable proteins, as opposed to the regulatory molecules Spg1 and Sid2, which are extremely dynamic (16). These findings further support the role for Sid4-Cdc11 in serving as a scaffold to which other SIN components associate dynamically.

Cdc7 is a member of the STE11/MEKK family of kinases (17–20). Most kinases belonging to the MEKK family share a similar structure with the kinase domain at the C terminus and a regulatory domain at the N terminus (21). They also have been shown to bind to small G-proteins (21). Cdc7 differs from the typical member of the family in that the kinase domain of Cdc7 resides at its N terminus. However, like other family members, it binds to and is an effector for a GTase, in this case Spg1 (5, 22). Activation of the SIN pathway is triggered by activation of Spg1, and increased production of Spg1 leads to the formation of septa at any point in the cell cycle (5). Interestingly, Spg1 activation at the SPB is asymmetric. It exists in the GDP-bound state during interphase at the single SPB, and during metaphase it is GTP-bound at both SPBs only to be “inactivated” or GDP-bound at one SPB during anaphase B (22). Cdc7 is only recruited to the pole that is occupied by GTP-bound Spg1; hence it also is asymmetrically localized during anaphase. The asymmetric localization of SIN components appears to be important for proper SIN regulation, because Cdc7 is associated with both SPBs during anaphase in cells with deregulated septum formation (7, 23).

Cdc7 kinase plays a crucial role in propagation of the SIN, as it serves to relay the activation signal from Spg1-GTP to its downstream effectors. To understand how Cdc7 is regulated we have carried out a detailed structure/function analysis of Cdc7. We have mapped the regions within Cdc7 required for SPB localization as well as binding to Spg1. Additionally we found that Cdc7 self-associates via an intermolecular interaction.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Genetic Methods—S. pombe strains used in this study (Table 1) were grown in yeast extract or minimal medium with appropriate supplements (24). DNA transformations were done by electroporation (25) or lithium acetate transformation. Regulated expression of genes from various strengths of the nmt promoter in the pREP series of vectors was achieved by growth in the presence of thia-
mine (promoter repressed) and then removing the thiamine by washing into medium lacking thiamine (promoter nonrepressed) (26). *Saccharomyces cerevisiae* strain P69-4A was used for two-hybrid analysis (27) and transformed by a lithium acetate method (28). 

**TABLE 1**

| Strain      | Genotype                                      | Ref. no./source |
|-------------|-----------------------------------------------|-----------------|
| KGY246      | $h^-$ ade6-M210 ura4-D18 leu1-32              | Paul Nurse*     |
| KGY4271     | $h^-$ spg1-myc::KanR ade6-M210 ura4-D18 leu1-32 | This study     |
| KGY3842     | $h^-$ cdc7-myc::KanR ade6-M210 ura4-D18 leu1-32 | This study     |
| KGY2061     | $h^-$ cdc7-24 ade6-M21X ura4-D18 leu1-32       | Paul Nurse*     |
| KGY2678     | $h^-$ spg1-GFP::KanR ade6-M210 ura4-D18 leu1-32 | 15             |
| KGY2628     | $h^-$ sid4-GFP::KanR ade6-M210 ura4-D18 leu1-32 | 15             |
| KGY2945     | $h^-$ sid2-GFP::KanR leu1-32                  | 11              |
| KGY2167     | $h^-$ spg1-3XHA::URA4 ura4-D18 ade6-M210 leu1-32 | 13             |
| KGY5401     | $h^-$ cdc7-myc::KanR ura4-D26-M210 leu1-32 ura4-D18 | This study     |
| KGY5426     | cdc7-myc::KanR/sid2-GFP::KanR ade6-M210 ura4-D18 leu1-32 | This study     |
| KGY5781     | spg1-myc::KanR/spg1-3XHA::URA4 ade6-M210 ura4-D18 leu1-32 | This study     |
| KGY3498     | $h^-$ cdc7-3XHA::URA4 ura4-D18 ade6-M21X leu1-32 | 13             |

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**FIGURE 1.** Cdc7 associates with Spg1 only during mitosis. A, the cdc7-myc$_{13}$ spg1-ha$_{13}$ strain (KGY5401) was grown to mid-log phase and synchronized in G$_2$ by centrifugal elutriation. Samples were taken at regular time intervals and processed for staining with 6,4-diamidino-2-phenylindole (DAPI) and septation indexing as a measure of cell cycle progression. B, protein lysates were prepared from each sample, and anti-HA antibodies were used to immunoprecipitate Spg1 from lysates that were normalized to contain equal amount of protein as determined by immunoblotting for Cdc2 with anti–PSTAIRE antibodies (top panel). The amount of Spg1-HA$_3$ immunoprecipitated was detected by immunoblotting with 12CA5 antibodies (second panel), * indicates a background band that is present in 246 control; arrow, points to Spg1-HA$_3$ band. Cdc7-Myc$_{13}$ (indicated by the arrow) in Spg1-HA$_3$ immunoprecipitates was detected by immunoblotting with 9E10 antibodies (third panel), * indicates background band that is present in Spg1-HA$_3$. KGY2167 and wild-type KGY246 controls. The amount of Cdc7-Myc$_{13}$ in the lysates was detected by immunoblotting with 9E10 antibodies (bottom panel).
Amino acids 250–535 of Cdc7 are sufficient to bind Spg1. A, spg1 bait plasmid was cotransformed with various cdc7 or control prey plasmids. Transformants were grown on medium lacking histidine and adenine to score for positive interactions. cdc7 constructs; their ability to grow with the spg1 construct is indicated. B, approximately equal amounts of fusion proteins GST-Spg1Q69L and GST-Spg1T42A were incubated with recombinant Hist6-Cdc7-(250–535) or control protein Hist6-Prp19-(1–73). Hist6-Cdc7-(250–535) associated with GST-Spg1Q69L, but not GST or GST-Spg1T42A, when pulled down with glutathione-agarose beads, as visualized by Coomassie staining; Hist6-Prp19-(1–73) did not associate with either. C, the spg1-myc13 strain (KG4071) was transformed with pREP1 vectors producing GFP-Cdc7-(250–535). Transformants were grown in the presence (lane 1) and absence of thiamine (lane 2), and protein lysates were subjected to immunoprecipitation (IP) with polyclonal anti-GFP serum. Immunoprecipitates were run on a 4–12% bis-Tris gel and immunoblotted with polyclonal anti-GFP antibody (left panel) or 9E10 monoclonal anti-Myc antibody (right panel). wt, control GFP immunoprecipitates from untagged wild type (KG246) cells; D, wild-type cells (KG246) expressing GFP-Cdc7-(250–535) expressing GTP-Cdc7-(250–535) under the control of the nmt1 promoter (left panel) or the nmt1 promoter (right panel) were grown to mid-log phase in the absence of thiamine at 32 °C for 18 h. Cells were fixed in methanol and stained with 4,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei (left panel). As Cdc7-GFP loses its fluorescence upon fixation, GFP-Cdc7-(250–535) images of live cells were captured (right panel). E, Spg1-GFP (KG2628), Sid4-GFP (KG2628), and Sid2-GFP (KG2945) cells expressing either empty vector (pREP1NTAP) or NTAP-Cdc7-(250–535) under the control of the nmt1 promoter in pREP1 were grown to mid-log phase in the absence of thiamine at 32 °C for 18 h. Images of live cells were captured to visualize GFP-tagged proteins.

**RESULTS**

**Cdc7 Associates with Spg1 Only during Mitosis—Cdc7** has been shown to associate with GTP-bound but not GDP-bound Spg1 and is presumed to function as its effector. Most G-protein effector kinases are activated upon binding to their cognate G-proteins (31, 32). However, the kinase activity of Cdc7 has been shown to remain constant throughout the cell cycle despite the observation that Spg1 is in its GTP-bound form at the SPB only during mitosis (22). Given this anomaly, we decided to address whether Cdc7 is always associated with Spg1 even when not at the SPB. We generated a synchronously population of cells by centrifugal elutriation that produced endogenously tagged Cdc7-Myc13 and Spg1-HA3. Samples were collected at regular intervals and examined for seption and nuclear division as indicators of the cell-cycle stage (Fig. 1A). Protein lysates were prepared, and the presence of Cdc7-Myc13 in Spg1 immunoprecipitates was assessed by immunoblotting (Fig. 1B). A control spg1 HA3 strain lacking endogenously tagged Cdc7 and a wild type untagged strain were used as controls. A small percentage of Cdc7-Myc13 was present in Spg1-HA3 immunoprecipitates prepared from mitotic cells (at minutes 30, 45, and 60) but not from interphase cells. This is also the stage at which Spg1 is in its GTP-bound form (22). In unincellules cells that exited from mitosis (at minutes 90, 105 and 120), no Cdc7-Myc13 was detectable. Lack of Spg1-HA3/Cdc7-Myc13 association was not due to lower amounts of Spg1-HA3 in immunoprecipitates; higher concentrations of Cdc7-Myc13 in mitotic samples (Fig. 1B, bottom panel) or other general protein levels (Fig. 1B, top panel). Taken at face value, these results indicate that unlike in other G-protein-associated kinases, the binding to Spg1 does not influence Cdc7 kinase activity.

The **N Terminus of Cdc7 Contains an Spg1 Binding Site**—Given that the association of Cdc7 with Spg1 at the SPB is a likely SIN-activating step, we addressed what regions of Cdc7 are responsible for binding to Spg1. We first analyzed a series of cdc7 fragments in a directed two-hybrid assay. Residues 250–535 were found to contain a strong interaction domain (Fig. 2A). Because Cdc7 only associates with Spg1 in its GTP-bound form (22), we tested whether Hist6-Cdc7-(250–535) bound Spg1 in a nucleotide-dependent manner. For this we examined the ability of bacteria produced Hist6-Cdc7-(250–535) to interact with recombinant Spg1 mutants in vitro. Hist6-Cdc7-(250–535) was able to bind GST-Spg1Q69L, a constitutively active mutant (5), but not GST-Spg1T42A, an effector domain mutant (5) (Fig. 2B). We also tested...
whether Cdc7-(250–535) and Spg1-Myc13 could co-immunoprecipitate from S. pombe proteins lysates. To this end, GFP-tagged Cdc7-(250–535) was overproduced in a spg1-1-myc strain. Anti-GFP immunoprecipitates contained Spg1-Myc13 (Fig. 2C), confirming that residues 250–535 of Cdc7 were sufficient to interact with Spg1.

We noticed that when GFP-Cdc7-(250–535) was overproduced, it generated a SIN phenotype (Fig. 2D). This could be because it prevented endogenous Cdc7 from localizing to the poles and thus from binding Spg1 or because it titrated Spg1 from the SPBs. To distinguish between these two possibilities, we first examined the localization of GFP-Cdc7-(250–535) when it was overproduced. Inconsistent with the first possibility, GFP-Cdc7-(250–535) localized to the cytosol (Fig. 2D). Furthermore, overproduction of Cdc7-(250–535) displaced endogenous Spg1-GFP from the SPB (Fig. 2E). The SPB itself was intact under these conditions, because the localization of the SIN anchor Sid4-GFP and the downstream SIN component Sid2-GFP, which dynamically associates with Cdc11, were unaffected (Fig. 2E). These data taken together indicate that amino acids 250–535 of Cdc7 are sufficient for binding to Spg1 in vitro and in vivo and, when present in sufficient quantity, to sequester Spg1 away from SPBs. Further, these results indicate that Cdc7 residues outside of its Spg1 binding domain are required for Cdc7 localization to the SPB.

### Amino Acid Residues 360–870 Mediate SPB Localization of Cdc7—

To determine which region(s) of Cdc7 target it to the SPB, we expressed N- and C-terminal truncations of Cdc7 fused to GFP. Because Cdc7 is an essential gene we tested these truncations in cells that also contained endogenous Cdc7. Full-length GFP-Cdc7 localized to the SPB in a cell cycle–dependent manner. However, unlike endogenous Cdc7, it localized to the SPB symmetrically during anaphase, as has been noted previously when Cdc7 is overexpressed (22). A kinase-dead mutant of Cdc7, GFP-Cdc7K38R, and also GFP-Cdc7-(250–1062) and GFP-Cdc7-(360–1062) constructs lacking the kinase domain were capable of localizing to the SPB (Fig. 3). Hence, neither the kinase domain nor kinase activity is required for Cdc7 SPB localization. We next tested C-terminal truncations to narrow the region responsible for Cdc7 SPB localization. Although GFP-Cdc7-(360–870) could localize to the SPB, further loss of N- or C-terminal residues resulted in abrogation of SPB localization (Fig. 3B). Hence the region that is responsible for SPB association resides within residues Cdc7-(360–870).

To correlate the ability of Cdc7 to construct to localize and bind Spg1 with overall Cdc7 function, GFP-Cdc7 truncation mutants were tested for the ability to rescue growth of cdc7–24 cells at 36 °C (Fig. 3A). Full-length and C-terminally truncated GFP-Cdc7-(1–900) rescued this growth (Fig. 3A), and as expected, all Cdc7 fragments lacking the kinase domain did not. The minimal rescuing fragment, GFP-Cdc7-(1–900), contains both the SPB localization and Spg1-association domains. These results indicate that C-terminal residues Cdc7-(900–1062) are dispensable for Cdc7 function.

### Cdc7 Self-associates in Vivo—

It has been shown that Cdc15, the S. cerevisiae ortholog of Cdc7, interacts with itself. To determine whether Cdc7 associates with itself, co-immunoprecipitation analyses were performed on a diploid strain in which one allele of cdc7 was tagged with the Myc13 epitope and the other was tagged with the HA3 epitope. Anti-HA immunoprecipitates from the dually tagged diploid strain, but not the singly tagged strains, contained Cdc7-Myc13 and vice versa (Fig. 4A). These results established that Cdc7 is capable of associating with itself in vivo, suggesting that Cdc7 exists in cells as an oligomer. Because Spg1 associates with Cdc7, we asked whether it, also, was present in an oligomeric complex in vivo. We constructed a diploid strain containing both Spg1-Myc13 and Spg1-HA3 and performed immunoprecipitations using anti-HA and anti-Myc antibodies. In these experiments anti-HA immunoprecipitates from diploid strains did not contain Spg1-Myc13 and vice versa (Fig. 4B). Therefore Spg1, unlike Cdc7, does not appear to oligomerize in vivo.

The results obtained above left open the possibility that monomeric Spg1 associates with oligomers of Cdc7. To test this likelihood, lysates prepared from the diploid S. pombe strain cdc7–HA/ecd7–myc13 were incubated with recombinant GST-Spg1Q69L, a constitutively active mutant (5) of Spg1, or with GST alone bound to glutathione-agarose beads. Bacterially produced GST-Spg1Q69L, but not GST, bound to Cdc7 from lysates (Fig. 4C, top panel). Bound proteins were eluted off the beads, the eluate was subjected to immunoprecipitation with anti-HA antibodies, and the immunoprecipitates were blotted for the presence of Cdc7-Myc13. As would be predicted if monomeric Spg1 bound to an oligomer of Cdc7, anti-HA immunoprecipitates contained Cdc7-Myc13 (Fig. 4C, bottom panel).

To estimate the oligomeric state of endogenous Cdc7, lysates from asynchronously growing Cdc7–Myc13 and control untagged strains were prepared under native conditions and sedimented on sucrose gradients. The presence of Cdc7–Myc13 in each fraction was determined by immunoprecipitating and immunoblotting for the Myc epitope. Molecular size standards were run in parallel on an identical sucrose gradient. The majority of Cdc7–Myc13 co-sedimented in fractions 9 and 10 with
the molecular mass marker phosphorylase B, which forms a trimer of 292.8 kDa. Because the predicted molecular mass of a Cdc7-Myc13 dimer is ~285 kDa, this suggests that Cdc7 exists primarily as a dimer. Some Cdc7-Myc13 sediments lower in the gradient, and this could represent Cdc7 in complex with other proteins (such as Spg1) and/or in higher order complexes with itself (Fig. 4D). Importantly, Cdc7-Myc13 was not observed in any fraction that would be consistent with a monomeric form (140 kDa) of Cdc7-Myc13.
Structure/Function Analysis of SIN Kinase Cdc7

To determine the domains required for Cdc7 self-interaction, we analyzed a series of cdc7 fragments in a two-hybrid assay. The best interaction was between the N-terminal and C-terminal portions of the protein (Fig. 4E). Further truncations of either the N-terminal or C-terminal constructs abolished the interaction in the yeast-two hybrid system. This suggests that Cdc7 is capable of interacting with itself and that this interaction is between the N and C termini. In light of the sucrose gradient analysis, this interaction is most likely an intramolecular one.

DISCUSSION

Cdc7 is a protein kinase essential for cytokinesis in fission yeast. It is an effector for the Ras superfamily GTPase Spg1, the activation of which is central to determining the timing of septation and cytokinesis in fission yeast. In the current model for SIN activation and signaling, Spg1 is converted to its GTP-bound state, which enables association of Cdc7 to the SPB. Hence it has been suggested that the regulation of Cdc7 kinase activity is mediated by its localization to a signaling competent SPB. In this study we demonstrate an association between Spg1 and a small fraction of Cdc7 during mitosis. Given that Spg1 associates with the SPB dynamically during both interphase and mitosis (16), it is not surprising that we could detect only a small fraction of Cdc7 association with Spg1. The mitosis-specific association of Cdc7 with Spg1 provides a means for Cdc7 to access its relevant targets at the SPB, and hence regulating that association would be a key step in regulating SIN signaling. To elucidate Cdc7 regulation, we have identified various functional domains within Cdc7. We have shown that a region adjacent to the kinase domain is responsible for association with Spg1 but insufficient for SPB localization. In addition we found that Cdc7 self-associates, adding another layer of complexity to SIN organization.

A region just adjacent to the kinase domain of Cdc7, Cdc7-(250–535), acts as an effector domain and interacts with a G-protein, in this case Spg1, in a nucleotide-dependent manner. It has been shown previously that Cdc7 fails to localize to SPBs in germinating spg1 null spores or spg1–8 cells at restrictive temperature (33). Although these data suggest that Spg1 is required for association of Cdc7 to the SPB, we found that amino acids Cdc7-(250–535) are capable of associating with Spg1 both in vitro and in vivo but fail to localize to the SPB. This suggests that additional residues and mechanisms exist that promote or maintain Cdc7 SPB localization. Indeed, the smallest region that could localize to the SPB, Cdc7-(360–870), is significantly larger than the Spg1-interacting region, although they overlap. Embedded within the Spg1 and SPB binding domain is a coiled-coil region; however, residues within this region are not sufficient to support binding to Spg1 or to localize to the SPB. Cdc7 has been shown to associate with the SIN complex anchor Cdc11. However, we were unable to detect any direct interaction between Cdc11 and the SPB binding domain of Cdc7. Interestingly, within Cdc15, the S. cerevisiae equivalent of Cdc7, the Ten1 binding domain, is also not sufficient for SPB localization. In fact, the relative organization of the Spg1 binding domain being adjacent to and including residues that are a part of a larger SPB binding domain is reminiscent of the domain structure found for Cdc15 (34).

In testing various fragments of Cdc7 for Spg1 and SPB interaction, we found that a GFP fusion of Cdc7-(1–900) localized efficiently to the SPB when produced at low level (Fig. 3A). This result is inconsistent with a previous report suggesting that the C-terminal 162 amino acids of Cdc7 are essential for SPB targeting (35). It is possible that by using the strong nmt1 promoter to drive expression at high levels, Lu et al. (35) were unable to discriminate specific SPB staining from background.

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