Analysis of the Role of Phosphorylation in Fission Yeast
Cdc13p/CyclinB Function*

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The Cdk1p-cyclin B complex drives entry into mitosis in all eukaryotes. Cdc13p is the single essential cyclin in Schizosaccharomyces pombe and a member of the cyclin B family. Cdc13p abundance rises during G2-phase and falls as cells progress through mitosis and G1. Cdc13p degradation, mediated by the anaphase-promoting complex, is an important mechanism of Cdk1p inhibition and mitotic exit. Cdk1p-cyclin B1 complexes shuttle between the nucleus and cytoplasm, and preventing nuclear accumulation of Cdk1p-cyclin B1 in mammalian cells appears to be one mechanism of preventing entry into mitosis during a DNA damage-induced checkpoint delay. In vertebrates, phosphorylation plays a key role in regulating the intracellular distribution of cyclins. Previous mass spectrometric analysis identified sites of Cdc13p phosphorylation. Here, we have confirmed that these sites are the sole in vivo Cdc13p phosphorylation sites and have studied the role that phosphorylation plays in Cdc13p localization and function. Our data indicate that Cdc13p accumulates in the nucleolus in response to G2 checkpoint delays, rather than in the cytoplasm, and that phosphorylation plays no role in Cdc13p localization or function.

The Cdk1p-cyclin B complex drives entry into mitosis in all eukaryotes (1). In the fission yeast, Schizosaccharomyces pombe, progression through the cell cycle requires the function of its Cdk1p (encoded by the cdc25) gene both in G1 before the initiation of S-phase and at the G2-M boundary (2). The activity of S. pombe Cdk1p oscillates throughout the cell cycle, peaking as cells enter M-phase (reviewed in Ref. 3). This periodicity of Cdk1p is dependent on its cell cycle-specific association with various cyclins, its phosphorylation state, and the activity of an inhibitor of Cdk1p-cyclin function, Rum1p (reviewed in Ref. 1).

Unlike many other organisms, S. pombe cells produce relatively few cyclin partners for Cdk1p. Cdc13p is the single essential cyclin in S. pombe (4–6) and a member of the cyclin B family (reviewed in (7)). Cdc13p abundance rises during G2-phase and falls slowly as cells progress through mitosis and G1 (8). It functions both to drive the events of mitosis and prevent re-initiation of S-phase (9).

The degradation of Cdc13p as cells exit mitosis is an important mechanism of Cdk1p inhibition and occurs through ubiquitin-triggered proteolysis mediated by the anaphase-promoting complex ubiquitin ligase. The motif specifying Cdc13p degradation has been mapped to its N terminus, similar to other cyclin Bs (10). Truncation of the first 70 amino acids or deletion of the 9 amino acid destruction box stabilizes the protein in an in vitro assay for ubiquitin-mediated protein degradation. Overproduction of stable Cdc13p fragments in S. pombe cells results in high Cdk1p activity, an accumulation of cells in anaphase and a block to septation (10). These stable fragments of Cdc13p retain a conserved stretch of 150 amino acids that is a hallmark of cyclin proteins and is the region through which cyclins bind Cdk's and promote their activation (1). Transiently expressed anaphase-promoting complex activators are probably involved in the recognition of the "destruction box" motif within the N terminus of Cdc13p. In S. pombe, five such anaphase-promoting complex activators, members of the CDC20 protein family, are predicted by the genome sequence and three have been studied as follows: Slp1p (present in mitosis) (11, 12); Srw1p/Ste9p (present during G1) (13, 14); and Mfr1p/Fzr1p (present during meiosis and sporulation) (15, 16). Indeed, Ste9p/Srw1p is required for Cdc13p degradation when cells arrest in G1 in preparation for mating (13, 14, 17).

For some time, it has been appreciated that mitotic Cdk1p-cyclin B1 complexes shuttle between the nucleus and cytoplasm, accumulating in the nucleus during mitosis (18). Preventing nuclear accumulation of Cdk1p-cyclin B1 appears to be one mechanism of preventing entry into mitosis during a DNA damage-induced checkpoint delay (19–21). Cdk1p/Cdc13p accumulates in the nucleus during S- and G2-phases and also can be detected at the nucleolus, at the spindle pole body, and along the mitotic spindle (22–25). Interestingly, the ability of Cdk1p to enter the nucleus is Cdc13p-dependent (23). In other organisms, phosphorylation plays a key role in regulating the intracellular distribution of cyclins. Cyclin B1, as an example, has a cytoplasmic retention sequence (26) that contains a nuclear export sequence (27, 28). Phosphorylation of a single serine within the nuclear export sequence is sufficient to inhibit nuclear export (29). However, this is insufficient to explain the kinetics of mitotic entry. Additional phosphorylations within the cytoplasmic retention sequence are important to create a nuclear import signal that is essential for the timely onset of mitosis (29–31). At least some of the phosphorylation sites within the cytoplasmic retention sequence are probably auto-phosphorylation sites of Cdk1-cyclin B (32–34).

In this study, we have examined the role of Cdc13p phosphorylation in its function and localization. We have confirmed the assignment of phosphorylation sites identified by mass spectrometry (35) and present evidence that Cdc13p phosphorylation

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tion is not important for its function in normal cell cycle progression or in the cell response to checkpoint signals that monitor DNA damage or the completion of DNA replication.

**EXPERIMENTAL PROCEDURES**

**Yeast Methods—** *S. pombe* strains used in this study are listed in Table I. Strains were constructed by random spore analysis or by tetrad dissection when necessary. *S. pombe* strains were grown in minimal medium with the appropriate supplements in the presence or absence of 5 μg/ml thiamine or in standard yeast extract medium (36). All of the temperature shift experiments were carried out by growing cells to mid-log phase at 25 °C and shifting to 36 °C for 4 h. Hydroxyurea was added to medium at a final concentration of 12 mM. Camptothecin was added at a final concentration of 40 μM.

**Antibodies, Cell Labeling, and Immunoprecipitation—** Rabbit polyclonal antibodies to Cdc13p were generated against the entire Cdc13 protein produced in *Escherichia coli*. The protein was purified from *E. coli* as inclusion bodies and recovered from contaminants by SDS-polyacrylamide gel electrophoresis. Serum from two bleeds (fifth and sixth) of one of the rabbits was subjected to ammonium sulfate precipitation and resuspension and is used throughout this study (GJG56).

**Phosphorylation of the Fission Yeast Cyclin B, Cdc13p**

**RESULTS**

**Cdc13p Is a Phosphoprotein—** To test whether Cdc13p is a phosphoprotein, wild-type *S. pombe* cells were labeled in vivo with [32P]orthophosphate and a denatured protein lysate was prepared. A single phosphoprotein of the expected size was immunoprecipitated specifically by anti-Cdc13p serum but not preimmune serum (Fig. 1A). Phosphoamino acid analysis of [32P]labeled Cdc13p indicated that it was phosphorylated exclusively on serine residues (Fig. 1B). Only a small percentage of [32P]Cdc13p was ever released from PDVF or nitrocellulose membranes or eluted from gel slices (data not shown). However, better recovery was obtained with chromatographic digestion than with tryptic digestion and separation of Cdc13p chymotryptic phosphopeptides revealed a complex pattern (Fig. 1C), raising the possibility that Cdc13p might be phosphorylated on several sites (Fig. 1C). Cdc13p is phosphorylated throughout the cell cycle—Cdc13p levels vary during the cell cycle (8, 48). To determine whether the extent of Cdc13p phosphorylation varied independently of its abundance, which would be an indicator of a possible regulatory role, different cell cycle mutants, wild-type cells, wild-type cells treated with drugs that block cell cycle progression, and wild type cells overproducing Cdc13p were labeled with [32P]orthophosphate and Cdc13p was immunoprecipitated from denatured cell lysates of each. The following mutants and drugs were chosen: a cdc10–129 mutant (G1-phase arrest), hydroxyurea (S-phase arrest), cdc15–22 (G2 arrest) and cdc25–25 cells that had been arrested in G2 and then released for 20 min into nocodazole (M-phase arrest). In large measure, the level of Cdc13p phosphorylation paralleled its abundance (Fig. 2A). This was confirmed by quantitation of three such experiments (Fig. 2B). Specifically, the amount of [32P] incor-

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**Table I. Strains used in this study**

| Strain | Genotype | Source |
|--------|----------|--------|
| KGY2   | h*       | leu1–32 | Paul Nurse |
| KGY28  | h*       | 97 ∆ wild type | Paul Nurse |
| KGY155 | h*       | cdc10–129 | Paul Nurse |
| KGY170 | h*       | cdc25–22 | Paul Nurse |
| KGY262 | h*       | cdc13–117 leu1–32 ura4–D18 ade6–M210 | This study |
| KGY274 | h*       | cdc13–34 | This study |
| KGY629 | h*       | cdc13–3D–GFP::kanR | This study |
| KGY630 | h*       | cdc13–3A–GFP::kanR | This study |
| KGY843 | h*       | cdc13–3D–GFP::kanR | This study |
| KGY974 | h*       | cdc13–3A–GFP::kanR | This study |
| KGY976 | h*       | cdc13–3D–GFP::kanR | This study |
| KGY5049| h*       | cdc13–3D–GFP::kanR | This study |

1 The abbreviations used are: GFP, green fluorescent protein; PVDF, polyvinylidene difluoride.
sites and to assess their importance to Cdc13p function, they were particularly encouraging given that Ser177, Ser180, and 183 were predicted to be contained in large hydrophobic regions of the protein unanalyzed (35). Given the lack of other phosphorylation sites within Cdc13p, at least under normal growth conditions. This was a possibility given that the 91.7% sequence coverage of Cdc13p by mass spectrometry left no other phosphorylation sites, wild-type and cdc13-3A strains were labeled with [32P]orthophosphate and Cdc13p was immunoprecipitated from each (Fig. 3B). As before, [32P]-labeled Cdc13p was detected in wild-type cells. In contrast, no labeled Cdc13p was detected from cdc13-3A. This is not due to the absence of Cdc13p protein in the labeled cells (Fig. 3C). This result confirms that Ser177, Ser180, and/or Ser183 represent bona fide in vivo phosphorylation sites. Moreover, this result indicates that there are no other phosphorylation sites within Cdc13p, at least under normal growth conditions. This was a possibility given that the 91.7% sequence coverage of Cdc13p by mass spectrometry left some regions of the protein unanalyzed (35). Given the lack of effect, we have not determined which of the three serines becomes phosphorylated or whether the phosphorylation occurs at two or all three sites.

The Absence of Cdc13p Phosphorylation Does Not Affect Cdc13p Intracellular Localization—Phosphorylation of mammalian cyclin B1 plays an important role in its intracellular localization (reviewed in Refs. 18 and 49). As one response to genotoxic stress, cyclin B1 is also retained or shuttled into the cytoplasm (19–21). To determine whether phosphorylation or the lack thereof affected Cdc13p localization, cdc13+ and cdc13-3A were tagged at their endogenous locus with sequences encoding GFP. Live cell imaging of these strains indicated that Cdc13p-3A localized normally (Fig. 4, upper right panel). Similar to wild-type Cdc13p (Fig. 4, upper left panel), Cdc13p-3A was present in the nucleus with nucleolar concentration at the spindle pole bodies and also decorated the mitotic spindle. To determine whether checkpoint activation and cell cycle delay affected its localization, wild-type and cdc13-3A cells were incubated for 4 h in hydroxyurea and the localization of Cdc13 proteins was examined. After this treatment, it was clear that cells had arrested in interphase and were somewhat elongated (Fig. 4, lower panels). Moreover, Cdc13p and Cdc13p-3A had both accumulated at the spindle pole bodies and in the nucleus with nucleolar localization being particularly evident (Fig. 4, lower panels). We conclude that the absence of Ser177, Ser180, and/or Ser183 phosphorylation does not adversely affect the ability of Cdc13p to gain entry into the nucleus or localize to the nucleolus either normally or upon activation of the DNA replication checkpoint.

Because we did not detect an alteration in cell growth or checkpoint response when Cdc13p was not phosphorylated, we considered the possibility that dephosphorylation rather than phosphorylation of Cdc13p might play a regulatory role. To test this theory, we altered the cdc13+ cDNA by site-directed mutagenesis to encode proteins in which the serine phosphorylation sites were replaced with aspartic acid residues (S177D, S180D, and S177D, S180D, and S183D) in the hope that at least one and possibly all three serines were integrated into the genome in the place of wild type cdc13+. These mutant strains all divided at normal rates and at a normal size. No cell cycle defect could be discerned (data not shown).
Given the apparent lack of functional changes, we examined the localization of only the triple mutant that we named Cdc13p-3D. cdc13–3D was tagged at its endogenous locus with sequences encoding GFP. Live cell imaging of this strain indicated that it localized normally (Fig. 5 D). Furthermore, cdc13–3D cells arrested normally in hydroxyurea and the Cdc13–3Dp accumulated in the nucleus, particularly the nucleolus (Fig. 5 E). To determine whether DNA damage precluded Cdc13p concentration in the nucleus and whether this was altered by phosphorylation, the cdc13-GFP, cdc13–3A-GFP, and cdc13–3D-GFP strains were treated with camptothecin for 3 h (50). In each case, the Cdc13 protein accumulated in the nucleus, particularly the nucleolus (Fig. 5, F–H). These results indicate that neither phosphorylation nor dephosphorylation of Cdc13p influences its intracellular localization.

**DISCUSSION**

Numerous studies have determined that cyclin B phosphorylation in vertebrate cells is an important influence on its intracellular localization. As observed in other organisms, we have shown here that Cdc13p, the only essential B-type cyclin of *S. pombe*, is a phosphoprotein. It is phosphorylated on one to three sites in close proximity to one another that lie N-terminal to the cyclin box and C-terminal of the destruction box. However, our evidence suggests that phosphorylation of this region is unnecessary for the function or regulation of Cdc13p.

Although 32P incorporation into Cdc13p was detected by us earlier, advances in mass spectrometry were required to provide information on the residues involved (35). These residues lie next to one another at amino acid positions 177, 180, and 183, and it is still not clear whether a single amino acid or all three sites serve as the phosphoacceptor residues. In retrospect, it is clear why conventional approaches used successfully to identify phosphorylation sites in other proteins (45) failed to reveal the identity of Cdc13p phosphorylation sites. Sequences surrounding Ser177, Ser180, and Ser183 dictate that tryptic and

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and/or Ser180 are conserved within Cig2p, a closely related but non-essential S. pombe cyclin B. However, they are not general conserved among cyclin B molecules. One of the serines (Ser177) is followed by a proline residue and thus is a potential phosphorylation site. It appears from our data that cytoplasmic sequestration of the Cdc13p is not involved in the fission yeast cell response to DNA replication- or DNA damage-induced G2 arrest. This contrasts with the situation in vertebrate cells in which Cyclin B accumulates in the cytoplasm in response to DNA damage (19–21). Rather, our data suggests that Cdc13p is sequestered in the nucleolus when cells are delayed in G2-phase by activation of G2 checkpoints and that nucleolar accumulation does not require changes in Cdc13 phosphorylation state. The different strategy of sequestering Cdk1p activity within the nucleolus in S. pombe may obviate the need for controlling nuclear access through a checkpoint signaling pathway. Nucleolar localization of Cdc13p was first detected more than a decade ago (22, 23), but the sequences within Cdc13p responsible for this localization pattern have not been elucidated.

Whereas Cdc13p phosphorylation sites were identified by mass spectrometry, a substantial portion of the protein (>10%) was not accounted for in the mass spectrometric data and, even in the regions covered, it is possible to miss some sites of phosphorylation (35). Therefore, it remained possible that other Cdc13p phosphorylation sites existed in these unidentified parts of the protein. This possibility is strongly disfavored by the fact that a S177A, S180A, and S183A triple mutant protein was not accounted for in the mass spectrometric data and, even in the regions covered, it is possible to miss some sites of phosphorylation (35). Therefore, it remained possible that other Cdc13p phosphorylation sites existed in these unidentified parts of the protein. This possibility is strongly disfavored by the fact that a S177A, S180A, and S183A triple mutant protein fails to incorporate any 32P when cells are labeled with orthophosphate, and it is highly probable that these serines represent the only in vivo phosphorylation sites in the protein. Consistent with the lack of regulatory influence, our data indicate that the amount of Cdc13p phosphorylation parallels the

S-P Cdk1 autophosphorylation site. Although Cdk1p-Cdc13p complexes do undergo some autophosphorylation in vitro, this is not affected in quality or quantity by mutation of Ser180 to alanine. Thus, autophosphorylation sites are not related to the sites studied here and may be in vitro artifacts because the sites analyzed here are the only ones detected in Cdc13p molecules isolated from cells.

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accumulation of the protein whether it occurs in a prolonged G2 arrest induced by cdc25 mutation or G2 checkpoint activation. Although the lack of functional consequence of Cdc13p phosphorylation is unexpected, this study has illustrated the power of combining a mass spectrometric approach with site-directed mutagenesis and in vivo labeling to comprehensively analyze the potential functions of protein phosphorylation events.

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