The Cdk-associated protein Cks1 functions both in G₁ and G₂ in *Saccharomyces cerevisiae*

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The *CKS1* gene of *Saccharomyces cerevisiae* encodes a small essential protein shown to interact genetically and physically with the Cdc28 protein kinase. To investigate the specific functions of the *CKS1* gene product, conditional temperature-sensitive mutant alleles were generated. The mutations were found to impair the ability of cells to undergo both the G₁/S-phase and G₂/M-phase transitions of the cell cycle, as well as the ability to bud. Mutants were not defective, however, in their ability to activate Cdc28 kinase as assayed in vitro on the substrate histone H1. It is likely, therefore, that Cks1 mediates a more specialized function of the Cdc28 kinase such as its ability to form specific multimeric complexes or to localize properly in cellular compartments.

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The two principal cell cycle transitions of eukaryotic cells, that from G₁ to S-phase and that from G₂ to M-phase, are controlled by a specialized class of protein kinases, known as cyclin-dependent kinases (Cdks) (for recent reviews, see Pines and Hunter 1991; Myerson et al. 1992; Reed 1992). This has been demonstrated most convincingly in the two yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, where a single protein kinase catalytic subunit, Cdc28 (Lorincz and Reed 1984; Reed et al. 1985) and cdc2 (Hindley and Phear 1984; Simanis and Nurse 1986) in the respective organisms, is activated sequentially in the cell cycle by different classes of positive regulatory subunits known as cyclins (for reviews, see Xiong and Beach 1991; Lew and Reed 1992; Reed 1992). Vertebrate cells appear to have adapted a slightly more complex version of this scheme, where multiple protein kinase catalytic subunits of the Cdk family combine with different classes of cyclins to generate many combinatorial possibilities (for reviews, see Pines and Hunter 1991; Xiong and Beach 1991; Lew and Reed 1992; Meyerson et al. 1992; Reed 1992). Over the past several years, many details of the activation of Cdks have been elucidated. Although it has been shown that the minimum requirement for protein kinase activity is a molecule of cyclin and a Cdk catalytic subunit (Arion et al. 1988; Gautier et al. 1988; Labbe et al. 1988; Desai et al. 1992), the compositions of the Cdk complexes as they exist in vivo have remained largely uncharacterized. It is clear, however, that these complexes

are significantly larger than the minimum bimolecular heteroligomer (Draetta and Beach 1988; Wittenberg and Reed 1988). It is possible that properties such as substrate specificity or affinity and intracellular location may depend on components other than either cyclin or Cdk.

Genetic analysis in both fission and budding yeast has led to identification of a protein likely to be a component of cyclin-Cdk complexes. The gene encoding *suc1* was identified in fission yeast as a suppressor of temperature-sensitive *cdc2* mutations (Hayles et al. 1986a,b; Hindley et al. 1987). Likewise, the *CKS1* gene of budding yeast was identified as a suppressor of *cdc28* mutations (Hadwiger et al. 1989). Such genetic interactions often are attributable to physical interaction between gene products. Both of these genes encode small homologous proteins that can bind cdc2/Cdc28 complexes in vitro and are likely to associate with at least some fraction of active complexes in vivo (Brizuela et al. 1987; Hadwiger et al. 1989). In addition, *suc1* protein has been shown to suppress the thermolability of cdc2 kinase derived from temperature-sensitive mutant strains in vitro, implying that the in vivo suppression is mediated through physical interaction as well (Brizuela et al. 1987). Finally, in addition to yeasts, homologous proteins have been found in vertebrates and in *Drosophila*, suggesting a universal and highly conserved function (Draetta et al. 1987; Richardson et al. 1990; H.E. Richardson, and S.I. Reed, unpubl.).

The nature of this function, however, has been elusive. Elimination of gene function by constructing null mutations in both yeasts has revealed that *suc1* and *CKS1* are

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essential genes (Hayles et al. 1986b; Hindley et al. 1987). However, phenotypic analysis has been hampered by the lack of conditional loss-of-function alleles. As a result, the consequences of eliminating function have had to be evaluated in the context of gradual titration of the protein after genetic segregation or termination of synthesis. If the protein is stable and the titration gradual, interpretation of phenotype may be difficult. The lack of consensus emerging from the yeast mutational studies may therefore be attributable to experimental design. Heterozygous *suc1* disruption mutants put through meiosis yield spores that, by some criteria, are blocked in mitosis—unable to exit (Moreno et al. 1989). *cks1* disruption mutants that segregated a plasmid containing *CKS1* yielded some cells that appeared, based on morphology, to be in G₁ (Hadwiger et al. 1989). However, termination of synthesis of *CKS1* by use of a conditional promoter yielded a mixed population that appeared, again based primarily on morphology, to consist of G₁ and G₂ cells (Richardson et al. 1990). Additionally, overexpression of both *suc1* and *CKS1* was found to be deleterious, causing a delay (in budding yeast; Richardson et al. 1990) or arrest (in fission yeast; D. Beach, pers. comm.) in G₂. Consistent with these observations, it was found that *suc1* protein inhibited activation of *Xenopus* pre-maturation promoting factor (pre-MPF) by interfering with dephosphorylation of tyrosine 15 of Cdc2 in cell-free lysates (Dunphy and Newport 1989). This dephosphorylation has been shown to be essential for mitotic activation of the Cdc2 kinase (Gould and Nurse 1989; Morla et al. 1989).

It has been impossible to incorporate the diverse observations outlined above into a coherent model for *suc1/CKS1* function. It is for this reason that we set out to create a conditional temperature-sensitive mutant allele of *CKS1* that could be studied in the context of the extensive body of cell cycle analysis performed on budding yeast. We reasoned that only through conditional loss of function could the function of Cks1 be clearly delineated. Here, we report the analysis of several temperature-sensitive alleles of *cks1* produced by in vitro mutagenesis.

Results

PCR mutagenesis of *CKS1*

The *CKS1* gene was subjected to mutagenesis by polymerase chain reaction (PCR) amplification (Lee et al. 1988; Saiki et al. 1988) under error-prone conditions (see Materials and methods). A library of $\sim 3 \times 10^5$ mutant alleles was constructed. Sequence analysis of random alleles from the mutant library was performed to assess the degree of mutagenesis. On the basis of complete sequence analysis of five random *CKS1* alleles from the library, we were able to conclude that, typically, 1.7 substitutions were contained in 100 nucleotides, or 7.7 substitutions in each coding region.

To screen for alleles conferring conditional loss of

function, the library, constructed in a yeast single-copy vector carrying the selectable *TRP1* marker, was introduced into a host strain carrying a genomic deletion of *CKS1* and a plasmid expressing wild-type *CKS1* under control of the conditional *GAL1* promoter (Richardson et al. 1990). In this recipient strain, *CKS1* is expressed when cells are grown on galactose medium but not when cells are grown on glucose medium. Upon a shift to glucose, cells are capable of a number of divisions owing to residual protein but eventually cease division (Richardson et al. 1990). The library was introduced into the galactose-conditional strain after a shift to glucose, and *TRP*⁺ colonies were screened for temperature sensitivity by replica-plating to 37°C. A surprising number of mutagenized alleles were found to confer temperature sensitivity ($\sim 25\%$). This was assumed to be a consequence of the intensity of mutagenesis and the likelihood that many null alleles in the population would confer inviability of transformant cells and not give rise to colonies. A number of mutants that exhibited good growth at 23°C but no growth at 37°C were chosen for further study.

Conditional loss of *CKS1* function suggests roles in G₁ and G₂

To facilitate analysis, mutant strains were cured of the plasmid containing the chimeric *GAL1::CKS1* allele. Of 20 temperature-sensitive mutants briefly investigated, three, Ts35, Ts38, and Ts42, were chosen for detailed analysis. Because similar results were obtained for all three, we present below only data for one, Ts38. A liquid culture of mutant strain Ts38, grown to mid-logarithmic phase at 23°C, was shifted to 37°C and analyzed for cell cycle progression by flow cytometric analysis of nuclear DNA content (Fig. 1). We first noticed that the mutant strain as well as a wild-type control (wild-type *CKS1* maintained on the same plasmid as the mutant alleles) exhibited an excess of G₂/M (2N) cells (Fig. 1) at 23°C relative to what is normally observed for wild-type cells in this genetic background. This is assumed to result from impaired expression of plasmid-borne alleles resulting in *CKS1* expression becoming limiting and suggests an essential function in G₂ or M-phase.

When an asynchronous culture of mutant cells is shifted to 37°C, less than a doubling of cell number is observed, suggesting that most cells arrest within one cell cycle (Fig 1, top). Flow cytometric analysis suggests that initially cells accumulate both in G₁ and in G₂/M (Fig. 1, first column). The data also suggest that some cells late in the cell cycle at the time of the temperature shift complete mitosis and form part of the G₁ population. Prolonged incubation at 37°C over the course of several hours, however, results in all cells accumulating with a 2N DNA content (Fig. 1, first column, bottom frame). These data suggest that even though Cks1 might have a function in G₁ or at the G₁/S-phase boundary, this function is either not absolutely essential or the mutant allele tested is not sufficiently restrictive to confer a

Figure 1. Comparison of asynchronous cultures of *cks1* mutant cells shifted to 37°C or 38.5°C. Wild-type and Ts38 mutant cells were shifted from 23°C to 38.5°C or 37°C (for the mutant). At hourly intervals cells were counted to determine cell density (top) or analyzed for nuclear DNA content by flow cytometry (bottom). (□) Wild type at 38.5°C; (○) Ts38 at 37°C; (●) Ts38 at 38.5°C. Arrows indicate cells with replicated (2N) nuclear DNA content.

tight block. On the other hand, the Ts38 mutant was capable of permanently blocking cells with a 2N nuclear DNA content, consistent with an essential role in G_2 or M-phase. Because the leakiness of the *cks1* phenotype in terms of G_1 arrest at 37°C could be attributable to either the lack of an absolute requirement or incomplete inactivation of the gene product, we repeated the cell cycle analyses described above at 38.5°C. This constitutes the maximum temperature at which cells of the genetic background employed (BF264-15D; Reed et al. 1985) grow normally. Note that cells carrying the wild-type *CKS1* allele grow exponentially under these temperature conditions (Fig. 1, top). Asynchronous mutant cells shifted to 38.5°C were analyzed by flow cytometry (Fig. 1, second column). Under these circumstances, cells terminally arrest with both 1N and 2N complements of nuclear DNA, suggesting that a permanent G_1 block has been achieved in some cases (Fig. 1, compare first col-

umn (37°C) with second column (38.5°C). Microscopic observation, however, suggested a significant degree of morphological heterogeneity. Consequently, mutant cells were presynchronized to analyze the *cks1* conditional phenotype with more precision.

Analysis of cks1 mutants presynchronized in G_1 or S-phase

Wild-type and mutant (Ts38) cultures were first synchronized by incubation with the mating pheromone α -factor (Fig. 2). After treatment with mating pheromone for 2 hr,

Figure 2. Comparison of *cks1* mutant cells prearrested using mating pheromone and released to 37°C or 38.5°C. Wild-type and Ts38 mutant cells were prearrested using mating pheromone and then released to 38.5°C or 37°C (for the mutant). Cells grown at 23°C were incubated in medium with the mating pheromone α -factor for 2 hr after which cultures were shifted to 37°C for an hour prior to removal of mating pheromone. After removal of mating pheromone (hour 1), aliquots were taken for flow cytometric analysis of nuclear DNA content at hourly intervals. The top panel corresponds to culture cell density; bottom panels correspond to flow cytometric analysis of nuclear DNA content. (□) Wild type at 38.5°C; (○) Ts38 at 37°C; (●) Ts38 at 38.5°C. Arrows correspond to cells with replicated (2N) DNA content.

when most cells had accumulated in G₁, cultures were shifted to 37°C or 38.5°C for an additional hour in the presence of mating pheromone. The cells were then shifted to 37°C or 38.5°C medium without pheromone and analyzed for cell cycle progression by flow cytometry (Fig. 2). As expected, wild-type cells recovered quickly after removal of pheromone (Fig. 2, third column). On the other hand, mutant cells at 37°C delayed for more than an hour before entering S-phase (Fig. 2, first column) and finally arrested with a 2N complement of DNA (Fig. 2, first column). Microscopic analysis of the mutant cells during this sequence indicated that they assumed an aberrant morphological phenotype [Fig. 3, cf. d–f (mutant) with a–c (wild type)]. Terminally arrested cells were extremely large, with at least one extended process (Fig. 3f). Often this process exhibited a curvature that eventually was directed back toward the main portion of the cell (Fig. 3f). Of note was the fact that these cells were not budded even though they had completed DNA replication, on the basis of flow cytometric analysis (Fig. 2). Thus, even though loss of Cks1 function under these semirestrictive conditions could only delay the onset of DNA replication, it could completely block the formation of a bud.

Figure 3. Morphology of *cks1* mutant cells after presynchronization in G₁ or S-phase. Mutant Ts38 (d–l) and wild-type (a–c) cells were photographed using differential interference contrast (Nomarski) optics. Micrographs correspond to 1 hr after shift to restrictive temperature but before removal of mating pheromone (a,d,g) or hydroxyurea (j); 2 hr after removal of mating pheromone (b,e,h) or hydroxyurea (k), or 4 hr after removal of mating pheromone (c,f,i) or hydroxyurea (l). Rows correspond to wild type presynchronized with mating pheromone and released to 37°C (a–c); Ts38 presynchronized with mating pheromone and released to 37°C (d–f); Ts38 presynchronized with mating pheromone and released to 38.5°C (g–i); Ts38 presynchronized with hydroxyurea and released to 38.5°C (j–l).

As expected, cells presynchronized and arrested at 38.5°C behaved differently. Under these conditions, mutant cells did not replicate genomic DNA, indicating that a tight G₁ block was achieved (Fig. 2, compare second column to first column). Morphologically, although these arrested cells continued to grow in an asymmetric manner, they did not form bulbous processes, as was the case at 37°C (Fig. 3, cf. i with f). The phenotype observed is reminiscent of *cdc28* mutant cells arrested in G₁ (Reed 1980).

It has been reported that loss of the homologous protein *sucl* in fission yeast by meiotic segregation causes cells to arrest in mitosis. Therefore, it was of interest to determine whether the terminally arrested cells having a replicated genome (37°C) were blocked in G₂ or in M-phase. In budding yeast, cells arrested in G₂ have a short, thick intranuclear microtubule structure and a nonelongated nucleus, whereas mitotic cells have an extended microtubule spindle and a stretched or divided nucleus. Therefore, terminally arrested *cks1* mutant cells 4 hr after release from the mating pheromone block were stained with DAPI to reveal nuclear morphology and with anti-tubulin antibodies to reveal the state of the spindle (Fig. 4, b and e, and c and f, respectively). Based on examination using a fluorescence microscope, the *Cks1* mutants at 37°C were clearly arrested in G₂. In spite of their unusual exterior morphology, these mutants contained nonextended nuclei and short intranuclear microtubule structures typical of G₂ (Fig. 4b,e). Additionally, also consistent with G₂ arrest, most cells contained a single bundle of extranuclear microtubules extending into the process described above (Fig. 4e). In a normal G₂ cell, these cytoplasmic microtubules would be extended into the bud (Byers and Goetsch 1975). Thus, in contrast to what has been reported for fission yeast, budding yeast cells deprived of *CKS1* function arrest in G₂.

The nuclear and microtubule structure of *cks1* mutant cells arrested at the higher temperature (Fig. 4, c and f, respectively) was different. In this case, nuclei contained unseparated, probably single, microtubule-organizing centers [spindle pole bodies; Fig. 4f] comparable to what has been observed for G₁-arrested *cdc28* mutant cells (Ghiara et al. 1991). Note that the phenotype is also similar to what is observed in cells arrested in G₁ by mating pheromone (Fig. 4d, Byers and Goetsch 1975). The morphological differences observed in cells arrested at 38.5°C versus 37°C suggest that some morphological aspects of growth exhibited at 37°C may be a function of arrest in G₂, such as differences in microtubule structures (Fig. 4, cf. e and f).

As reported above, *cks1* mutant cells prearrested with mating pheromone were incapable of budding. It was conceivable, however, that this defect was specifically related to the interaction between the Cks1 deficiency and mating pheromone arrest. Another cell cycle synchrony experiment, however, suggests that this is not the case. Mutant (Ts38) and wild-type cells were prearrested in S-phase using the ribonucleotide reductase inhibitor hydroxyurea (Fig. 5; Slater 1973). After 3 hr, cul-

Figure 4. DAPI and anti-tubulin staining of *cks1* mutant cells. Ts38 mutant cells were synchronized at 23°C using mating pheromone (a,d) or hydroxyurea (g,i); shifted to restrictive temperature for an hour prior to removal of the drug, and incubated for an additional 4 hr at 37°C (b,e,h,k) or 38.5°C (c,f,i,l). (b,c,e,f) Cells presynchronized with mating pheromone; (h,i,k,l) cells presynchronized with hydroxyurea. In each case, the pairs of micrographs vertically juxtaposed, e.g., a and d or g and j, correspond to the same microscopic field viewed with different filters: (a–c, g–i) DAPI and phase contrast; (d–f, j–l) anti-tubulin.

tures were shifted to 37°C or 38.5°C for 1 hr, after which the hydroxyurea was removed. Unexpectedly, many of the mutant cells at 37°C completed one cell division after a delay, hesitated again in the subsequent G₁ interval, and finally replicated their DNA in the absence of budding (Fig. 5, first column and top). The terminal phenotype was equivalent to that exhibited by *cks1* mutants presynchronized using mating pheromone (Fig. 4h,k). We draw two conclusions from this experiment. First, mutant cells prearrested in S-phase may have a reduced requirement for Cks1 function to complete mitosis. The possible implications of this will be discussed below. Second, the *cks1*-mediated defect in bud formation is general and not dependent on prior treatment with mating pheromone.

Cells released from a hydroxyurea block at 38.5°C were capable of replicating genomic DNA, but few were capable of undergoing the G₂/M-phase transition and dividing (Fig. 5, compare two columns and respective cell number increases at top). This is in contrast to the situation at 37°C, where many cells complete one division before arresting. At the higher temperature, most cells maintain the morphology associated with hydroxyurea

arrest (dumbbell shape; Fig. 3k,l). Note also that whereas the terminal phenotypes at 37°C and 38.5°C are distinct externally as a result of the intervening division at the lower temperature (Fig. 4h,i), the G₂ spindle morphologies are quite similar (Fig. 4k,l).

Taken together, these data suggest that the leaky phenotypes observed at 37°C are a result of incomplete inactivation of Cks1 protein rather than the absence of an absolute requirement for the protein. However, we cannot rule out that Cks1 has functions that only become absolute at extreme elevated temperatures. Furthermore, it could be argued that the phenotype observed might not correspond to a true loss of function, in that Ts38 could

Figure 5. Comparison of *cks1* mutant cells presynchronized using hydroxyurea and released to either 37°C or 38.5°C. Ts38 mutant cells were presynchronized using hydroxyurea at 23°C and released to 38.5°C or 37°C. After 3 hr in hydroxyurea, cultures were shifted to 37°C or 38.5°C for an additional hour, after which hydroxyurea was removed (hour 1) and incubation continued at 37°C or 38.5°C. The top panel corresponds to culture cell densities; bottom correspond to flow cytometric analysis of nuclear DNA content. (○) Ts38 at 37°C; (●) Ts38 at 38.5°C. In frames with a single arrow, the arrow corresponds to cells with replicated (2N) nuclear DNA content; in the frame with two arrows, the left arrow corresponds to unreplicated (1N) nuclear DNA content and the right arrow corresponds to replicated (2N) DNA content.

be an atypical allele. However, the experiments described in this and the preceding sections were repeated for two other temperature-sensitive mutants, Ts35 and Ts42, with identical results (data not shown). Therefore, it is likely that the phenotype described corresponds to a loss of Cks1 function.

cks1 mutants are not defective in generation of Cdc28 kinase activity

The cell cycle defects of *cks1* mutants are consistent with a role in activation of the Cdc28 kinase. Previous genetic and biochemical studies indicate that Cks1 and its homologs in other organisms interact directly with Cdc28 and equivalent protein kinases. However, there is no evidence to date that in vitro activity requires the Cks1 protein. Nevertheless, one explanation for the inability of *cks1* mutants to progress from G₂ to M-phase is an inability conferred by Cks1 deficiency to activate mitotic levels of Cdc28 kinase. We therefore assayed Cdc28 protein kinase activity in mutant (Ts38) and wild-type cell extracts at intervals after release from a mating pheromone block at 37°C (Fig. 6). On the basis of histone H1 kinase activity, Cdc28 protein kinase activity is immunoprecipitable from both mutant and wild-type cells as they progress through the cell cycle. Most importantly, *cks1* mutant cells arrested in G₂ contain high levels of immunoprecipitable histone H1 kinase activity (Fig. 6B). Therefore, it is not likely that Cks1 simply facilitates the assembly of an active Cdc28 kinase complex. However, it should be pointed out that histone kinase activity as-

sayable in vitro is not necessarily equivalent to in vivo mitotic inductive activity, because the relevant substrates have not yet been identified and parameters other than kinase activity per se may be important.

Mutant Cks1 proteins are defective in associating with Cdc28 kinase

Although it has been shown that Cks1 and its homologs can associate in vitro with Cdc28 kinase and genetic analysis supports a physical interaction between Cks1 and Cdc28, there was no evidence that the mutational defect observed for *cks1* temperature-sensitive mutants is related to interaction with CDC28. The observation that *cks1* mutants were capable of accumulating normal levels of Cdc28 kinase activity was at least consistent with a non-Cdc28-related role for Cks1. We therefore sought to determine whether the mutant gene products exhibited a defect in Cdc28 interaction in vitro. Mutant *cks1* alleles were therefore cloned into bacterial expression vectors, and mutant (Ts35) and wild-type protein purified and coupled to Sepharose beads. The rationale for choosing Ts35 rather than Ts38 was that the latter formed insoluble inclusion bodies when expressed in *Escherichia coli* whereas the former was soluble like wild-type Cks1. Ts35 and Ts38 exhibited identical phenotypes. Cks1 beads or the equivalent have been shown to bind Cdk/cyclin complexes efficiently in active form (Brizuela et al. 1987; Richardson et al. 1990). As expected, the wild-type Cks1 protein bound Cdc28 kinase efficiently as determined by histone kinase activity as well as by immunoblotting (Fig. 7). The mutant Cks1 proteins were much less efficient (20- to 50-fold) than wild type even though equivalent amounts of wild-type and mutant proteins were coupled to the beads (Fig. 7). Because these mutant proteins corresponded to temperature-sensitive alleles, we sought to determine whether the ability to bind Cdc28 kinase was thermolabile. However, we found that in vitro binding was equivalently defective at all temperatures (Fig. 7). The fact that beads prepared from wild-type Cks1 had a reduced efficiency at elevated temperature made it difficult to draw conclusions concerning in vitro thermolability of mutant beads. From these data, however, it appears that *cks1* mutations that confer a temperature-sensitive cell cycle defect also greatly reduce the ability of the mutant protein to interact with Cdc28 kinase in vitro at all temperatures. Whether the temperature-sensitive phenotype results from a general reduction of Cks1 function such as Cdc28 interaction or whether the function is specifically temperature-sensitive in vivo but more general in vitro remains to be determined. These data, however, are consistent with the function of Cks1 being mediated via interaction of Cdc28.

Mutations alter conserved residues

Sequence analysis of 16 temperature-sensitive alleles indicated that each contained multiple point mutations (Fig. 8). Some of these were silent with regard to amino

Figure 6. Cdc28 histone H1 kinase activity in arrested *cks1* mutant cells. Wild-type and mutant (Ts38) cells were presynchronized using mating pheromone for 2 hr and shifted to 37°C temperature for 1 hr, after which the mating pheromone was removed (hour 1) and the restrictive temperature incubation was continued. Lysates were prepared from cells at hourly intervals, and Cdc28 histone H1 kinase assays were performed. (A) Percentage of budded cells. (○) Wild-type; (●) Ts38. (B) Histone kinase assay for Ts38 mutant. (C) Histone kinase assay for wild type. Note that the wild-type time course contains two cell cycles.

Figure 7. Ability of beads prepared from wild-type and mutant Cks1 protein to bind Cdc28. (A) Histone kinase activity (cpm); (B) histone kinase activity (autoradiograph); (C) Cdc28 protein (Western blot). (Lanes 1–3) Wild type beads; (lanes 4–6) Ts35 mutant beads. (Lanes 1,4) Beads only (no cell lysate); (lanes 2,5) beads with yeast lysate incubated at 23°C and assayed at 23°C; (lanes 3,6) beads with lysate incubated and assayed at 37°C.

acid substitution. However, it will not be possible to make strong structure/function inferences until alleles containing single substitutions are constructed. Nevertheless, within the context of all of the Cks1 homologs sequenced, it is clear that some residues are highly conserved from yeast to man. It is interesting that some of the temperature-sensitive *cks1* alleles contain nonconservative substitutions at invariant residues (designated by asterisks in Fig. 8). These, therefore, are excellent candidates for mutations that confer structural/functional defects that are reflected in the mutant phenotype.

Discussion

Cks1 function is required at multiple points in the cell cycle

Previous studies in a variety of organisms have led to a number of different models for Cks1 function. In fission yeast, nullizygous spores derived from heterozygous *suc1* disruptants have been reported to arrest in mid-mitosis (Moreno et al. 1989). Disruption of *cks1* in budding yeast yields a large number of unbudded arrested cells, suggestive of a G_1 arrest, although in light of the current study that assessment has to be taken with caution (Hadwiger et al. 1989). In both yeast cells and *Xenopus* extracts, excessive Cks1/suc1 has been found to be inhibitory (Hindley et al. 1987; Dunphy and Newport 1989; Richardson et al. 1990), although in none of these cases is it clear that this phenomenology reflects true functions of the protein. The present study constitutes the first use of conditional loss-of-function mutations to assess the role(s) of this protein directly. Based on anal-

ysis of temperature-sensitive *cks1* mutants shifted to the restrictive temperature either from asynchronous cultures or after presynchronization, one can conclude that Cks1 has multiple cell cycle functions in budding yeast. At a semirestrictive temperature, cells appear to be delayed at the G_1 /S-phase transition, based on flow cytometric analysis, but could achieve a 2N nuclear DNA content eventually. The mutants were more defective in forming a bud in that a shift to restrictive temperature during G_1 or late in the previous cell cycle blocked budding completely. The fact that both commitment to S phase and budding are thought to involve the form of Cdc28 kinase driven by G_1 cyclins (Clns; Richardson et al. 1989) suggests that Cks1 may interact with the same kinase differentially in the context of different functions. *cks1* mutant cells were also totally defective in the G_2 /M-phase transition at this semirestrictive temperature, and all cells eventually accumulated in a G_2 state except for the fact that they were not budded. At restrictive temperature (38.5°C), mutant cells arrested either in G_1 or G_2 , depending on their cell cycle position at the time of the temperature shift, indicating an absolute requirement for Cks1 at the G_1 /S-phase transition. The transition from G_2 to M-phase is mediated by the form of Cdc28 kinase driven by B-type cyclins (Ghiara et al. 1991; Surana et al. 1991; Fitch et al. 1992; Richardson et al. 1992), whereas, as stated above, the transition from G_1 to S-phase as well as bud formation is mediated by a different form driven by G_1 cyclins or Clns (Richardson et al. 1989). Thus, if Cks1 is functioning via interaction with Cdc28 kinase, it apparently does so in a global fashion, interacting with diverse forms of the kinase at several points in the cell cycle.

Possible functions for Cks1

On the basis of the data presented here and elsewhere, a number of hypotheses can be framed concerning Cks1 function. One possibility is that Cks1/suc1 contributes a chaperone function to the assembly of cyclin/Cdk complexes. The observation that some mutant cells first arrested in S-phase using hydroxyurea could then complete mitosis at the semirestrictive temperature (37°C) when released from the hydroxyurea block is consistent with this idea. It is known that cyclin/Cdk complexes accumulate during a hydroxyurea block and the prolonged incubation prior to the inactivation of Cks1 might allow accumulation of sufficient complexes for mitotic induction. However, there are several reasons for discarding this hypothesis. First, Cdc28 kinase accumulates readily, presumably by the assembly of cyclin/Cdc28 complexes, upon release from a pheromone block even though *cks1* cells become arrested in G_2 in this instance (Fig. 6). Second, overexpression of a B-type cyclin, expected to relieve the G_2 /M-phase defect of *cks1* mutants if cyclin/Cdc28 assembly were the impaired function, had no suppressive effect (data not shown). Finally, the ability of mutant cells prearrested by treatment with hydroxyurea to complete mitosis was severely curtailed by incubation at the restrictive temperature (38.5°C).

Figure 8. Predicted Cks1 proteins based on DNA sequence analysis of 16 temperature-sensitive *cks1* mutant alleles. The sequences of wild-type Cks1 (budding yeast), suc1 (fission yeast), and Cks1s1 and Cks1s2 (human) proteins are shown for comparison. Asterisks (*) indicate residues conserved for all members of the family. For mutant proteins, amino acids shown indicate a divergence from the wild-type sequence.

Alternatively, although a simple role for Cks1 in Cdc28 kinase activity would appear to be ruled out, a more subtle role remains an important possibility. Cks1 might, for example, modify substrate specificity or favor the stability of a subset of Cdc28 multimeric complexes. Alternatively, Cks1 might govern some aspect of intracellular localization of a subset of Cdc28 complexes. These models are attractive in that they can accommodate the apparent indifference of *in vitro* histone kinase activity to loss of Cks1 function. They also might explain why both loss of function and hyperexpression have cell cycle inhibitory effects. If a balance between Cks1-modified and Cks1-unmodified forms of Cdc28 kinase is necessary for proper distribution of kinase activity to essential targets, a predominance of either form over the other might leave complementary sets of substrates unphosphorylated. We cannot exclude the possibility that Cks1 has an important cell cycle role independent of the Cdc28 kinase, resulting in the mutant phenotypes observed.

Effects of *cks1* mutants on morphogenesis

Mutational loss of Cks1 function using temperature-sensitive mutations has several profound consequences for

cellular morphogenesis. As stated above, the most obvious is a severe impairment of budding even when DNA replication can occur. We have shown recently that polarization of the yeast cytoskeleton, a necessary prelude to bud emergence, depends directly on activation of the Cln-driven (G₁) form of the Cdc28 kinase (Lew and Reed 1993). This form of the kinase, then, based on current studies, would be one that is particularly sensitive to the requirement for Cks1. Although mutant cells do not bud, they establish long projections that usually exhibit a high degree of curvature. Thus, some form of polarity is established (to maintain process tip-directed growth), but this polarity becomes skewed from linear. Other cell types that form processes, such as wild-type cells responding to mating pheromone or *cdc28* temperature-sensitive mutant cells at the restrictive temperature, do not display this curvature. One possible explanation is the presence in the *cks1* cells of a very distinct extended bundle of cytoplasmic microtubules not normally seen. This microtubule bundle extends into, and usually the length of, the process. It is possible that the presence of such an organized extranuclear microtubule structure in an unbudded cell provides the curving forces. Such structures, albeit on a smaller scale, are usually only seen in budded cells. Another novelty observed in these termi-

nally arrested cells was the accumulation of a mass of mitochondrial DNA, usually encompassing all of the mitochondrial DNA in the cell, as determined by staining with DAPI, at the far end of the cytoplasmic microtubule bundle (see Fig. 5b,h). It is possible that the mitochondria have metabolic motors that drive them down this excessively developed microtubule structure. Normally this mechanism would be expected to drive mitochondria from the mother cell into the bud to assure proper segregation at cell division.

Cks1 homologs in other organisms

Homologs of Cks1 have been found in all eukaryotes where an effort has been made to detect them (Richardson et al. 1990; H.E. Richardson and S.I. Reed, unpubl.). So far, all are able to complement a deletion of the *CKS1* gene in yeast, suggesting a high degree of conservation in both structure and function. This is surprising in light of differences in size of the various Cks1 homologs. Both budding yeast and fission yeast forms are significantly larger than their metazoan counterparts, having internal insertions and flanking extensions. Cks1 from budding yeast particularly has a long carboxy-terminal extension. Nevertheless, all Cks1 homologs contain blocks of sequence that are universally highly conserved (see Fig. 8). It is interesting to note that all of the sequenced mutant alleles contained at least one amino acid substitution in one of these highly conserved blocks.

It is more difficult to dispose of the apparent experimental discrepancies obtained with Cks1 and its homologs in different organisms. In fission yeast, where *suc1*, the prototype of the family was found, loss of the *suc1* gene appeared to result in a mitotic arrest rather than a G_2 arrest (Moreno et al. 1989). However, an equally likely interpretation in light of the current results is that residual *suc1* protein (present owing to the experimental design) allows cells to enter mitosis but only partially in that all necessary substrates may not be phosphorylated. They would then be prevented from progressing farther by a form of checkpoint control (Hartwell and Weinert 1989), ostensibly arresting in mitosis. Alternatively, the homologous proteins have different functions in the heterologous organisms. Constructing conditional alleles of *suc1* is probably the best way to resolve this issue. Adding fission yeast *suc1* to *Xenopus* extracts containing pre-MPF inhibits activation of MPF apparently by inhibiting dephosphorylation of tyrosine 15 on Cdc2 (Dunphy and Newport 1989). It is not clear whether this is a biologically significant phenomenon. In budding yeast, where tyrosine dephosphorylation of Cdc28 does not appear to be important for entry into mitosis under normal circumstances (Amon et al. 1992; Sorger and Murray 1992), overexpression of Cks1 or other homologs is inhibitory (Richardson et al. 1990). Therefore, other possible inhibitory mechanisms must be entertained. A final issue is that vertebrates appear to have at least two isoforms of Cks1 (Richardson et al. 1990). Human types 1 and 2 are extremely similar at the primary structure level. However, different patterns of

mRNA accumulation through the cell cycle suggest that they may have different functions (Richardson et al. 1990). Whereas as one mRNA accumulates early in the cell cycle, the other accumulates late. This opens the possibility that one isoform is involved with interphase functions of Cdks, whereas the other regulates mitotic functions of Cdc2. Resolution of this issue awaits a better understanding of Cks1 function.

Materials and methods

PCR mutagenesis and construction of the mutant library

PCR mutagenesis was carried out according to the method described by Leung et al. (1989), with slight modification. A set of reactions was set up with different concentrations of DMSO and $MnCl_2$ (1–10% DMSO and 0.2 or 0.5 mM $MnCl_2$). The 5' primer, 5'-CTGCAGCTCGAGCCCTGAG-3', carrying an *XhoI* site (underlined), corresponds to sequence 228 bp upstream from the translational start, and the 3' primer, 5'-CGGGATCCAATTTCAGTAATTAGAG-3', containing a *BamHI* site (underlined), is 10 bp downstream from the stop codon. After amplification, all reactions were combined, digested with *XhoI* and *BamHI*, and ligated into plasmid pSE271 (*TRP1 CEN1*) linearized using *Sall* and *BamHI*. Supercompetent cells [*E. coli* XL1-BLUE; 5×10^8 transformants/ μ g of DNA] were prepared as described by Hanahan (1983). The ligation mixture was transformed directly into the supercompetent cells and grown up in 500 ml of selection medium for preparation of library DNA.

Yeast strains, genetic procedures, media, and growth conditions

All yeast strains used in this study were derivatives of BF264-15DaU: *MATa ade1 his2 leu2-3,112 trp1-1^a ura3Dns* (Richardson et al. 1989). The strains used in this study contained a genomic disruption of *CKS1* (*cks1::LEU2*; Richardson et al. 1990). The starting strain contained centromeric plasmid YCpG2[*CKS1*] (*URA3::LEU2::GAL1p::CKS1*), which places *CKS1* under control of the *GAL1* promoter. This strain can only grow on medium containing galactose. A library of mutagenized *CKS1* alleles in the *CEN::TRP1* vector pSE271 was introduced by transformation, selecting for tryptophan prototrophs on glucose medium. Temperature-sensitive mutants were then identified by replica-plating colonies to 37°C and screening for lack of growth. Temperature-sensitive mutant strains were then cured of YCpG2[*CKS1*] by replicating to medium containing 5-fluoro-orotic acid (5-FOA), which selects for *ura3* auxotrophy (Sherman et al. 1986). *bar1* derivatives of these strains were then constructed by replacing the endogenous gene with a *URA3* insertion mutant allele by the one-step gene replacement method of Rothstein (1983). The congenic wild-type strain was constructed in the same manner except that pSE271 with the wild-type *CKS1* gene was introduced instead of the mutant library.

Standard genetic procedures for yeast were used (Sherman et al. 1986). Yeast transformations were carried out by the alkali cation method (Schiestl and Gietz 1989).

Yeast cultures were grown in YEP (1% yeast extract, 2% Bacto-peptone, 0.005% adenine, 0.005% uracil) supplemented with glucose (2%) or galactose (2%). When plasmid selection was required, cells were then grown in minimal medium supplemented with required nutrients but lacking either uracil or tryptophan, as appropriate. Permissive temperature was 23°C,

and restrictive temperature was either 37°C or 38.5°C, as specified. Incubation with α -factor was at 30 ng/ml, and incubation with hydroxyurea was at 0.2 M.

Photomicroscopy, immunofluorescence staining, cell counting, and flow cytometry

Yeast cells were photographed live, using differential interference contrast (Nomarski) optics with a 100 $\times$ objective. Fluorescence photomicroscopy on fixed and stained cells was performed using a Zeiss Axiophot photomicroscope with a 100 $\times$ objective. DAPI and anti-tubulin staining was performed as described by Ghiara et al. [1991].

Cell numbers and budding percentages were determined using a hemacytometer and a Leitz SM-Lux phase-contrast microscope with a 40 $\times$ objective.

Cell cultures were analyzed for DNA content using flow cytometry by the method of Hutter and Eipel [1979]. Yeast cells were fixed in 70% ethanol, stained with propidium iodide, and analyzed for fluorescence using a Becton-Dickinson FACScan analyzer [Lew et al. 1992].

Bacterial expression of wild-type and mutant Cks1 and coupling to CNBr-activated Sepharose

The coding regions of wild-type and mutant *cks1* genes were placed under the control of the T7 promoter in plasmid pRK171 [Rosenberg et al. 1987] by PCR using the 5' primer 5'-GC-CATATGTACCATCACTATCAC-3' (*Nde*I and initiation codon are underlined), and the same 3' primer for mutagenesis described above. *E. coli* strain BL21(DE3) carrying plasmid pLysS [Studier et al. 1990] was used as the host for expression and protein preparation as described below. The cells carrying expression plasmids pRK171[wt*CKS1*] and pRK171[ts*CKS1*] were grown in 500 ml of selection medium and induced with 0.4 mM IPTG at A₆₀₀ of 0.5 for 3 hr at 37°C. Cells were pelleted and lysed by sonication in 2 ml of TEN buffer (50 mM Tris-HCl at pH 8.0, 0.5 mM EDTA, and 0.3 M NaCl) supplied with protease inhibitors (4 μ g/ml each of leupeptin, aprotinin, and pepstatin and 0.1 mM PMSF). It was observed that the expression levels were the same for both wild-type and mutant proteins. However, whereas the wild-type Cks1 protein was totally soluble in aqueous buffer, only a portion of the mutant protein (30–50%, depending on the allele) was soluble. Soluble protein was purified by FPLC using a Superose 12-gel filtration column and a Mono-Q ion exchange column (Pharmacia LKB). Protein concentrations were determined by the Bradford method using the Bio-Rad protein assay reagent. The coupling of Cks1 protein to CNBr-activated Sepharose 4B (Pharmacia) was performed as recommended by the manufacturer. The proteins were coupled at a density of 0.5 mg/ml, and efficiency was determined by estimating the remaining soluble protein by SDS-PAGE.

Histone H1 kinase assays and detection of Cdc28 protein

The congenic wild-type and temperature-sensitive *cks1* strains were presynchronized with α -factor at room temperature for 2 hr and at 37°C for 1 hr, and incubated at 37°C without pheromone for 4 hr. Aliquots of cells were collected at hourly intervals starting at the time of removal of α -factor. Preparation of protein extracts, immunoprecipitation of Cdc28 protein kinase, and histone H1 kinase assays were as described by Wittenberg and Reed [1988].

Cdc28 protein kinase assays with Cks1–Sepharose beads were carried out essentially as described by Richardson et al. [1990]. In these experiments, 50 μ l of Cks1–Sepharose beads was incu-

bated with 500 μ g of yeast protein extract prepared from exponentially growing wild-type yeast and preincubated at 37°C for 30 min, a treatment found to reduce nonspecific background in subsequent kinase assays. After incubation at room temperature for 1 hr, the beads were washed at either 0°C or 37°C for testing the temperature dependency of binding. The washed beads were then divided into two moieties, one which was used to assay protein kinase activity and the other of which was used for the detection of Cdc28 protein by Western blotting using antiserum directed against the amino terminus of the Cdc28 polypeptide [Mendenhall et al. 1987].

DNA sequence analysis of mutant cks1 alleles

The same 5' and 3' primers used for PCR mutagenesis mentioned above were used for sequencing the full length *cks1* genes from both ends with the Sequenase version 2.0 sequencing kit (U.S. Biochemical).

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