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p13^{suc1} of *Schizosaccharomyces pombe* Regulates Two Distinct Forms of the Mitotic cdc2 Kinase

GABRIELE BASI† AND GIULIO DRAETTA*

Differentiation Programme, European Molecular Biology Laboratory, 69012 Heidelberg, Germany

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***suc1* is an essential gene initially identified for its ability to rescue certain temperature-sensitive alleles of *cdc2* in *Schizosaccharomyces pombe*. The role of suc1 in the regulation of the cdc2 kinase is not well understood. In our study, we have characterized the biochemical effect of loss of *suc1* function on specific cdc2-cyclin complexes. We show that the cig1 cyclin is associated with cdc2 and that the cdc2-cig1 kinase is activated at mitosis, with kinetics similar to those of the cdc2-cdc13 kinase. We provide evidence that loss of suc1 function affects the kinase activity of the two distinct mitotic forms of the cdc2 kinase. We also show that a dramatic increase in the level of the cdc13 protein is associated with loss of suc1. These results suggest that mitosis cannot be properly completed in the absence of suc1, possibly because of an increase in the level of cdc2-cdc13 complex, and support the idea of a role for suc1 in the regulation of multiple forms of the cdc2 kinase.**

The cdc2 protein kinase is a key regulator of the eukaryotic cell cycle. In the yeasts *Saccharomyces pombe* and *Saccharomyces cerevisiae*, the *cdc2*/*CDC28* gene is required for progression through the cell cycle at two transition points, in G_1 before Start and later in the cell cycle at the onset of mitosis (34, 36). Increasing evidence indicates that in higher eukaryotes, cell cycle transitions are regulated by a family of cdc2-like kinases, the cyclin-dependent kinases (37, 44). Cyclins are proteins which activate the cdk subunits at different phases of the cell cycle. The cdc2 kinase is activated at mitosis by B-type cyclins. In *S. cerevisiae*, four different cyclins (CLB1 to -4) which activate Cdc28 at mitosis have been identified. Two of these cyclins also function earlier in the cell cycle (18, 39). Other cyclins which function in S phase (16, 43) and in G_1 (20, 40) are known. In fission yeast species, three different B-type cyclin genes have been reported, *cdc13* (5, 21), *cig1* (9), and *cig2* (10). *cdc13* is an essential gene, required for entry into mitosis (5). *cig1* and *cig2* are not essential and are required in late mitosis for nuclear separation (11). A role for cig1 and cig2 during the G_1/S transition has also been suggested (9, 11; however, see reference 9 erratum). cdc2 also associates with a smaller subunit encoded by the *suc1* gene (8, 12). *suc1* was identified as a gene capable of rescuing some temperature-sensitive alleles of *cdc2* in fission yeast species (23, 24). Homologous genes in *S. cerevisiae* and higher eukaryotes have been identified (19, 41). However, despite the experimental efforts, a clear picture has not emerged concerning the function of *suc1*. Experiments with *Xenopus* extracts have suggested an inhibitory role for suc1 in the tyrosine dephosphorylation of cdc2. Addition of 2 μM suc1 to *Xenopus* oocyte extracts can effectively inhibit entry into M phase, as monitored by nuclear disassembly (15), cdc2 tyrosine dephosphorylation, and kinase activation (14). The suc1 inhibitory effect can be counteracted by the addition of cdc25 to the cell-free system (27). Consistent with an inhibitory effect of suc1 at the onset of mitosis, in *S. pombe*, *suc1* overexpression causes a delay of the cell cycle (23) or a cell cycle block in G_2 (2), depending on the levels of expression. Microinjection experiments with mammalian cells have indicated an inhibitory role for suc1 in mitosis (38). However, microinjection of suc1 causes the formation of multiple micronuclei rather than simply inhibiting entry into mitosis, an effect that is quite difficult to interpret. In *S. pombe*, after germination of *suc1*-disruptant spores, cell growth is arrested, with condensed chromosomes, mitotic spindles, and a threefold increase in the total histone H1 kinase activity (32). For *S. cerevisiae*, studies performed with strains carrying temperature-sensitive alleles of *cks1* (the budding yeast *suc1* homolog), indicate that Cks1 is required at the G_1/S and the G_2/M transition points (45).

In our study, we have investigated the effect of loss of *suc1* function in a fission yeast strain expressing *suc1* under the control of a thiamine-conditional promoter. We demonstrate the existence of two distinct cdc2-cyclin complexes active at mitosis, and we show that the loss of suc1 function affects the activity of both complexes. Our data support the idea that *suc1* is required for M-phase progression and indicate that suc1 is involved in the regulation of multiple forms of the cdc2 kinase.

MATERIALS AND METHODS

Growth of *S. pombe* cells and molecular genetic techniques. The strains used were wild-type 972 (h^{-S}), Sp202 (h^{-S} *leu1-32*), temperature-sensitive mutants Sp25 (h^{-S} *cdc10-129*), Sp26 (h^{-S} *cdc13-117*), Sp27 (h^{-S} *cdc1-7*), Sp28 (h^{-S} *cdc6-23*), Sp29 (h^{+N} *cdc20-M10*), Sp30 (h^{+N} *cdc22-M45*), Sp31 (h^{+N} *cdc17-K42*), Sp32 (h^{-S} *cdc25-22*), and Sp33 (h^{+N} *cdc21-M68*) and the cold-sensitive strain h^{-S} *nda3*-KM311 (26). For the construction of the suc1-conditional strain, the integrative plasmid pRIP89-*suc1*, carrying a modified version of promoter *nmt1* (*nmt1-T89* [4, 31]) was transformed in the diploid strain PN536 (h^-/h^+ *suc1*$^+$/*suc1*$^+$::*suc1*Δ*ura4*$^+$ *leu1-32*/*leu1-32 ura4-D18*/*ura4-D18*), a generous gift of J. Hayles (23). After sporulation of the transformants and digestion of the vegetative cells with Glusulase (DuPont), spores were plated in thiamine-free minimal medium and incubated until colonies formed. Colonies were replica plated in minimal medium with or without 4 μM thiamine and were screened for the ability to grow in the absence but not in the presence of thiamine. One such colony of each type was selected for further studies. Integration of the plasmid was verified by the plasmid instability test (1). For experiments involving induction of the *nmt1* promoter, cells were grown in minimal medium (33) with or without 4 μM thiamine. The concentration of thiamine used in experiments involving repression of the *nmt1-T89* promoter was 10 μM. For all the other experiments, the medium used was, unless otherwise indicated, YEA (3% glucose, 0.5% yeast extract, 75 mg of adenine per liter). The temperature for growth was 30°C, except for temperature- and cold-sensitive strains, which were grown at 25 and 36°C, respectively. Transformation of *S. pombe* was carried out by the lithium acetate procedure (33).

Flow cytometry and microscopy. Cells were fixed in 70% ethanol, stained with

* Corresponding author. Present address: Mitotix, 1 Kendall Sq., Bldg. 600, Cambridge, MA 02139. Phone: (617) 225-0001. Fax: (617) 225-0005.

† Present address: Department of Genetics, Harvard Medical School, Boston, MA 02115.

propidium iodide, and analyzed by using a FACScan flow cytometer (Becton Dickinson), as described in reference 42. 4′,6-Diamidino-2-phenylindole (DAPI) staining was carried out as described in reference 30. Phase-contrast and fluorescence micrographs were obtained with a Zeiss Axioskop microscope.

***S. pombe* extract preparation.** For preparation of *S. pombe* cell extracts, 5×10^9 exponentially growing cells were collected by centrifugation and pooled in a single Eppendorf tube. After centrifugation and aspiration of the supernatant, 250 μl of lysis buffer (50 mM Tris-HCl [pH 8], 250 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 1 mM dithiothreitol) containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 10 μg of tolylsulfonyl phenylalanyl chloromethyl ketone [TPCK] per ml, 10 μg of tosyl-lysine chloromethyl ketone [TLCK] per ml, 1 μg of aprotinin per ml, and 10 μg of soybean trypsin inhibitor per ml) were added to the cell pellet. Cell breakage was obtained by vortexing the mixture for 5 min at 4°C in the presence of 500 μl of glass beads. After centrifugation for 3 min at 13,000 rpm in an Eppendorf Microfuge at 4°C, the soluble fraction was transferred into a new Eppendorf tube and then was centrifuged twice for 15 min at 4°C. The protein concentration of the supernatant was determined by the Bradford assay (7).

Antibody production and purification. The preparation of the anti-suc1 and G8 (anti-Spcdc2) antisera has been previously described (8, 13). The anti-cdc13 antiserum was generated by injecting rabbits with the peptide MTTRRL-TRQHLLANT corresponding to the amino terminus of cdc13 (5). The anti-cig1 antiserum was generated by injecting rabbits with the peptide MDVSTQTRHA-TYFQDEN corresponding to the amino terminus of cig1 (9). The G3 antiserum was prepared against the peptide CNYLRDFH corresponding to the carboxy terminus of *S. pombe* cdc2 (25). The peptides were coupled to the keyhole limpet hemocyanin by methods described in reference 22. Rabbits were injected with 200 μg of keyhole limpet hemocyanin-peptide conjugate mixed with complete Freund's adjuvant. Subsequently, they were injected with the same amount of protein in incomplete Freund's adjuvant at 4-week intervals until significant immunoreactivity was detected. For affinity purification of the G3 antibodies, 11 mg of the peptide CNYLRDFH was covalently coupled to 2 g of CNBr-activated Sepharose 4B (Pharmacia) according to the instructions of the manufacturer. Twenty milliliters of immune serum was incubated batchwise with 6 ml of the peptide CNYLRDFH coupled to Sepharose and 20 ml of lysis buffer. After overnight incubation at 4°C in a rotator, the Sepharose was poured into a column and washed with 40 ml of lysis buffer and 20 ml of the same buffer containing 1.5 M NaCl. Ten milliliters of glycine-HCl (pH 2.3) in 1-ml aliquots was applied to elute the antibodies. To neutralize the acidic pH, 36 μl of 1 M Tris (pH 8.8) was added to each 1-ml fraction.

Immunoprecipitations and immunodepletions. Immunoprecipitations were performed by addition of 5 μl of antiserum to 1 ml of protein extract (0.5 mg/ml for kinase assays and 5 mg/ml for Western blots [immunoblots], unless otherwise indicated) and incubation for 1 h at 4°C. For peptide competition experiments, 25 μM peptide was added during this incubation. After centrifugation for 3 min at 13,000 rpm in a Microfuge, the soluble fraction was transferred to an Eppendorf tube containing 30 μl of protein A-Sepharose, vortexed briefly, and incubated for 30 min at 4°C in a rotator. The beads were then washed three times with cold lysis buffer. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on acrylamide gradient gel (7.5 to 17.5% for experiments involving suc1 immunodetection and 10 to 15% for experiments involving cig1 immunodetection) and were transferred to nitrocellulose membranes by semidry blotting (Millipore) as described in reference 22. For immunodepletions, 50 μl of protein A-Sepharose was coupled to the antiserum or preimmune serum for at least 1 h, and the beads were washed two times with lysis buffer. Extracts were incubated three times with the prepared immunopellets for 1 h at 4°C on a rotator and were recovered after centrifugation for 10 s in a Microfuge.

Histone H1 kinase assays. For kinase assays, immunoprecipitations were carried out as described above. After the immunoprecipitations, pellets were washed once in kinase assay buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol) and were incubated at 30°C in the presence of 40 μl of kinase assay buffer containing 40 μM ATP, 25 μCi of [γ-^{32}P]ATP, and 0.2 mg of histone H1 per ml, for 10 min. Samples were analyzed by SDS–12.5% PAGE, and the phosphorylated histone H1 was quantified with a Beckman scintillation counter.

In vitro translation of *cig1*. In vitro transcription and translation of *S. pombe cig1* cloned in the pT7F1A vector (28) were performed with the TnT T7 Coupled Reticulocyte Lysate System (Promega) in accordance with the instructions of the manufacturer.

PAP treatment of cig1. Anti-cig1 immunoprecipitates were incubated at 25°C for 1 h with potato acid phosphatase (PAP, Boehringer; 0.1 U/100 μl) in 0.1 M sodium-PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.0] buffer with or without phosphatase inhibitors (100 mM NaF, 3 mM Na_2MO_4, 10 mM β-glycerophosphate, 25 mM *p*-nitrophenyl phosphate [pNPP], and 3 mM $NaVO_4$). The control sample was incubated in the same buffer in the absence of PAP and protease inhibitors.

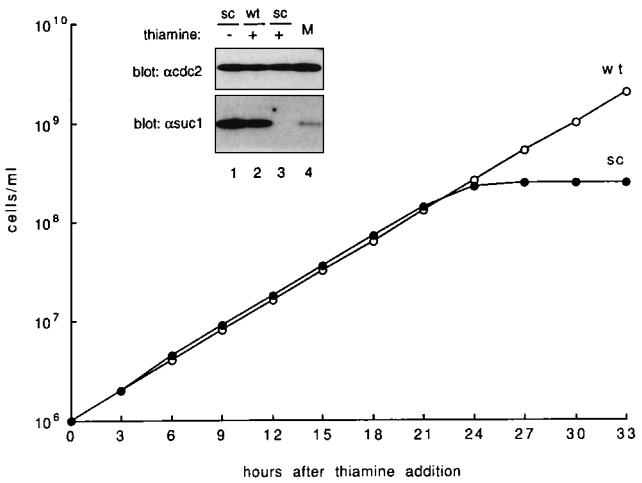

FIG. 1. Characterization of the suc1-conditional strain. Population growth curves of a wild-type (wt) strain and of the suc1-conditional strain are shown. Cells were grown in minimal medium at 30°C. At time zero, 10 μM thiamine was added to the medium. Serial dilution of the cells was used in order to count exponentially growing cells ($<10^7$ cells per ml) during the experiment. The number of cells at each point was calculated with a hemocytometer; that number was then multiplied for the factor of dilution used at time zero. (Inset) Levels of cdc2 and suc1 proteins in the wild-type and suc1-conditional strains. Equal amounts of total protein extracts (100 μg) were resolved by SDS-PAGE. After transfer by semidry blotting to a nitrocellulose membrane, the filter was probed with affinity-purified anti-cdc2 antibodies (αcdc2; upper blot) or with anti-suc1 polyclonal antiserum (αsuc1; lower blot). Lane 1, suc1-conditional strain grown in thiamine-free medium. Lanes 2 and 3, wild-type strain (lane 2) and suc1-conditional strain (sc) (lane 3) grown for 24 h in minimal medium containing 10 μM thiamine. Lane 4, bacterially expressed cdc2 and suc1. M, markers.

RESULTS

Construction of a *suc1* thiamine-conditional strain. An *S. pombe* diploid strain heterozygous for *suc1* disruption (see Materials and Methods) was transformed in minimal medium with a plasmid carrying the *suc1* gene downstream of a weak thiamine-repressible promoter (4). Diploid transformants were sporulated under conditions of nitrogen starvation. After germination in minimal medium, spores disruptant for endogenous *suc1* and containing the RIP89-*suc1* vector gave rise to colonies that were replicated in medium supplemented with thiamine. As judged by microscopic examination, addition of thiamine to the medium caused a change in cellular morphology which became fully evident after 24 h of incubation. Several different colonies showed a similar behavior. A representative isolate was selected for further studies and is referred to herein as the suc1-conditional strain. The growth kinetics of this strain were followed during a time course of 33 h after the addition of thiamine to the medium (Fig. 1). The generation time of a wild-type strain in minimal medium, at 30°C, is about 3 h. The generation time for the suc1-conditional strain grown in the absence of thiamine was similar to that for the wild type. After thiamine addition, cells continued to divide during the first 24 h and then growth was arrested. The block persisted over time, and the cells were completely unable to form colonies when plated in medium containing thiamine. The inset panels of Fig. 1 show an immunoblot of cdc2 and suc1 from extracts of wild-type and suc1-conditional cells grown in the absence (lane 1) or presence (lanes 2 and 3) of thiamine. While similar amounts of cdc2 were detected in all extracts, suc1 was undetectable upon promoter repression by thiamine. Fluorocytometric analysis revealed that growth of cells lacking suc1 was arrested with a G_2 DNA content (data not shown). Coincident with the cell cycle arrest after 24 h, a change in cell

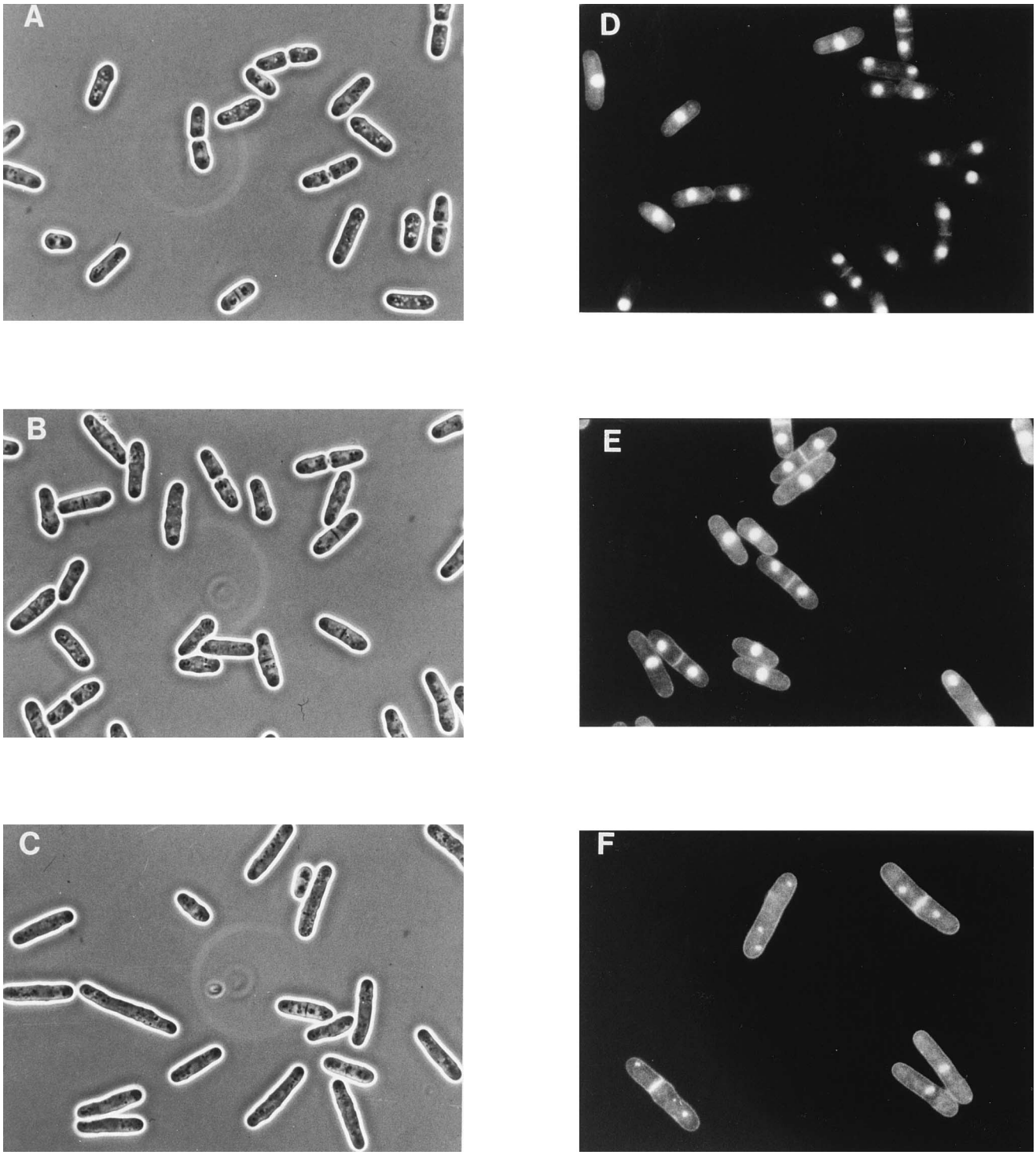

FIG. 2. Loss of suc1 function causes a block of cell cycle progression which is associated with chromosome condensation. (A to C) Phase-contrast micrographs of a wild-type strain (A) grown for 24 h in minimal medium with 10 μM thiamine and of the suc1-conditional strain grown for 24 h in minimal medium with (C) or without (B) 10 μM thiamine. (D to F) Fluorescence micrographs of DAPI-stained cells of the wild-type strain grown for 24 h in minimal medium with 10 μM thiamine (D) and of the suc1-conditional strain grown for 24 h in minimal medium with (F) or without (E) 10 μM thiamine.

morphology consisting of a heterogeneous elongation of the suc1-conditional cells was observed (Fig. 2A to C). The phenotype of the cells was further characterized by DAPI staining (Fig. 2D to F). Under permissive conditions, the suc1-conditional strain produced staining similar to that of the wild type, and spherical or hemispherical nuclei were clearly visible (Fig. 2D and E). In contrast, after *suc1* repression the conditional strain showed nuclei with heterogeneous morphology. Most of the cells showed condensed chromatin, and in some instances individual chromosomes were visible (Fig. 2F). The septum

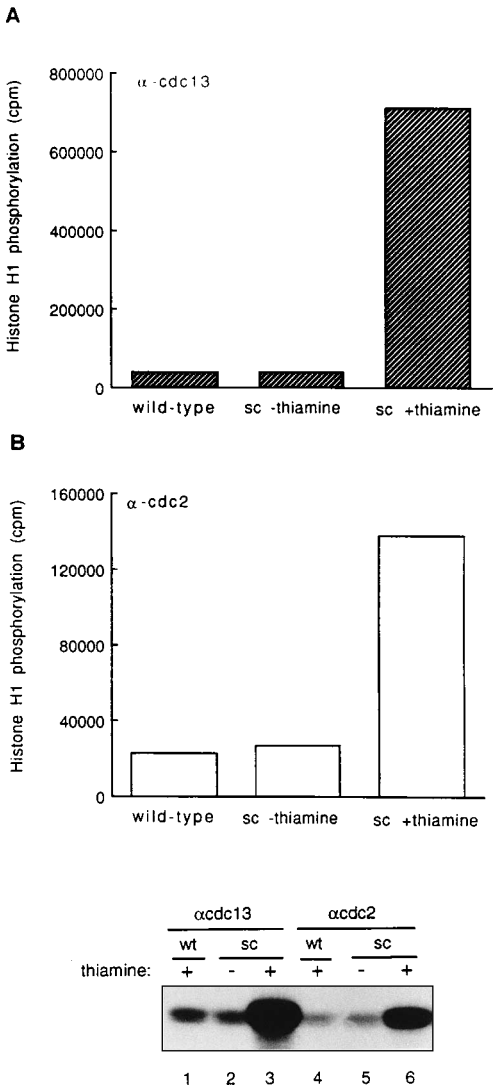

FIG. 3. High cdc2 kinase activity level associated with loss of suc1 function. Cells were grown as described in the legend for Fig. 2. Cell extracts were subjected to anti-cdc13 (α-cdc13) (A) or anti-cdc2 (α-cdc2) (B) immunoprecipitations and assayed for histone H1 kinase activity. Reaction products were analyzed by SDS-PAGE and autoradiography (bottom panel) and were quantified by scintillation counting. sc, suc1-conditional strain; wt, wild type.

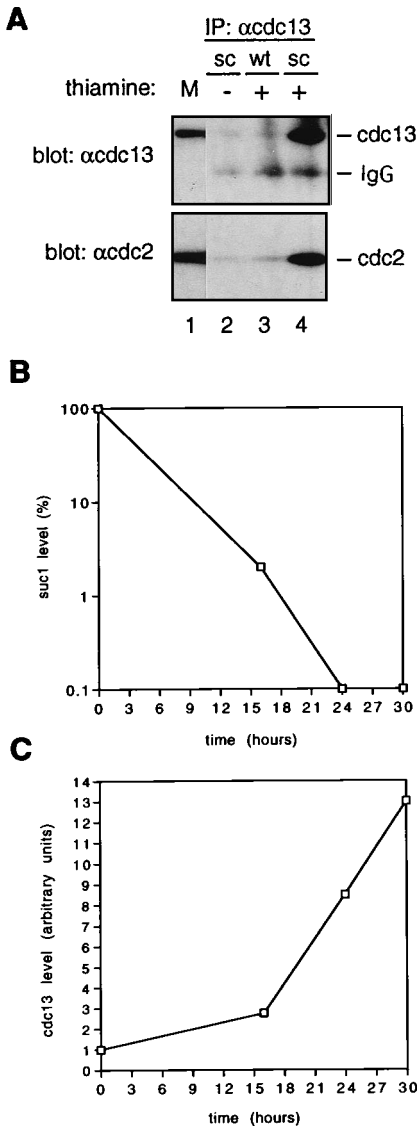

FIG. 4. An increase of the cdc13-cdc2 complex is associated with loss of suc1 function. (A) Immunoblots of cells grown for 24 h in minimal medium with (+) or without (−) 10 μM thiamine. Anti-cdc13 immunoprecipitates were separated by SDS-PAGE and transferred by semidry blotting to nitrocellulose membranes. The filter was probed with anti-cdc13 polyclonal antiserum (top blot) or with anti-cdc2 affinity-purified antibodies (bottom blot). Lane 1, markers (M) (bacterially expressed cdc13 and cdc2); lane 2, suc1-conditional strain grown in thiamine-free medium; lanes 3 and 4, wild-type (wt) strain (lane 3) and suc1-conditional strain (lane 4) grown for 24 h in minimal medium containing 10 μM thiamine. sc, suc1-conditional strain. (B and C) Time courses for anti-suc1 (B) and anti-cdc13 (C) precipitations of extracts prepared 0, 16, 24, and 30 h after thiamine addition. Samples were subjected to immunoblot analysis, and the levels of suc1 and cdc13 were quantified by autoradiographic scanning. The strain used in these experiments is described in the text.

was present in only some cells. Although at later time points most cells became very elongated, some cells did not elongate and others became misshapen or swollen (not shown). These results, obtained in cycling cells, are in agreement with a previous study performed on spores disruptant for suc1. When such spores were germinated, after two or three rounds of cell division, cells were arrested with a heterogeneous morphology and several cells showed condensed chromosomes and a mitotic spindle (32).

Effect of loss of *suc1* function on the cdc2 kinase. We investigated the effect of loss of suc1 function on the cdc2 kinase. The cdc2-cdc13 histone H1 kinase activity was measured after immunoprecipitation of cdc2 or cdc13. Extracts were prepared from a wild-type strain or from the suc1-conditional strain grown under permissive or restrictive conditions (Fig. 3). In the suc1-conditional strain grown under restrictive conditions, the mitotic cdc2-cdc13 kinase activity was found to be increased by approximately 15-fold (Fig. 3A) and the total cdc2 kinase activity was increased by approximately 6-fold (Fig. 3B) over wild-type levels.

Loss of suc1 function has no effect on the levels of cdc2 protein (Fig. 1, top inset panel). To investigate the effect of suc1 loss on the levels of cdc13 protein, cdc13 was immunoprecipitated from an extract of the suc1-conditional strain grown for 24 h in the presence of thiamine (Fig. 4A). The level of cdc13 in the suc1-conditional strain was dramatically in-

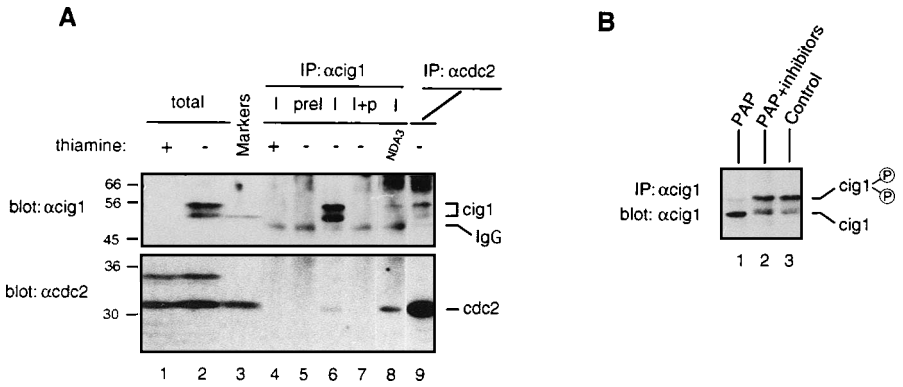

FIG. 5. Immunoblotting of the cig1 protein. (A) The Sp202 (*leu1-32*) strain transformed with the pREP1-*cig1* plasmid was grown in minimal medium with (+) or without (−) 4 μM thiamine for 16 h. Total extracts or immunoprecipitations (IP) were analyzed by SDS-PAGE and transferred by semidry blotting to nitrocellulose membranes. The filter was probed with anti-cig1 antiserum (αcig1; upper blot) or with anti-cdc2 affinity-purified antibodies (αcdc2; bottom blot). Lanes 1 and 2, total extracts (100 μg). Lane 3, in vitro-translated cig1 and bacterially expressed cdc2. Lanes 4 to 9, immunoprecipitations with anti-cig1 antiserum (lanes 4 and 6 to 8), with anti-cig1 preimmune serum (preI) (lane 5), and with anti-cdc2 antiserum (lane 9). The anti-cig1 antiserum was incubated in the presence of competing peptide (I+p) (lane 7), or the extract was prepared from a cold-sensitive *nda3*-KM311 strain grown in YEA medium at 36°C and then shifted at 22°C for 8 h (lane 8). Marker sizes (in thousands) are indicated on the left. (B) The Sp202 (*leu1-32*) strain transformed with the pREP1-*cig1* plasmid was grown in minimal medium without thiamine for 16 h. Anti-cig1 immunoprecipitates were incubated at 25°C for 1 h with PAP (lane 1), with PAP and phosphatase inhibitors (Ⓟ) (lane 2), and without PAP (control) (lane 3). Immunoprecipitates were then analyzed by immunoblotting with anti-cig1 antiserum. IgG, immunoglobulin G.

creased compared with the level for a wild-type strain or the same conditional strain grown under permissive conditions. Moreover, the additional cdc13 was found to be associated with cdc2.

The levels of suc1 and cdc13 were quantified by immunoprecipitating suc1 or cdc13 during a time course experiment (Fig. 4B and C). For this purpose, a strain expressing suc1 under the control of the *nmt1*-T4 promoter (4) was used. The levels of suc1 decreased rapidly after thiamine addition, and after 24 h suc1 was detected at less than 0.1% of the initial levels. In contrast, following thiamine addition an increase in cdc13 abundance estimated to be approximately ninefold was detected after 24 h. These data show that the cell cycle arrest caused by loss of suc1 function is correlated with an increase in cdc2-cdc13 kinase activity and also in the cdc13 protein levels.

cig1 associates with the cdc2 kinase. The experiments reported in the previous section did not allow us to establish whether suc1 specifically regulates the mitotic cdc2-cdc13 kinase or also has an effect on the activity of other cdc2-cyclin complexes. To address this point, we decided to study a cdc2-cyclin complex different from cdc2-cdc13 and to investigate the effect of loss of suc1 function on that complex. For this purpose, the cig1 cyclin was chosen. An initial biochemical characterization of this cyclin was therefore necessary. A polyclonal antiserum against a peptide representing a unique region of cig1 (the first 15 N-terminal residues) was generated. Lanes 1 and 2 of Fig. 5A show immunoblots of cig1 (top panel) and cdc2 (bottom panel) performed on a total cell extract from an *S. pombe* strain carrying the pREP1-*cig1*-inducible vector, grown in the presence or the absence of thiamine. After 16 h of promoter derepression, two bands with M_rs of 50,000 and 54,000 were detected by the anti-cig1 antiserum. The faster-migrating band had the same electrophoretic mobility as in vitro-translated cig1 protein (lane 3). Both bands were recognized by the anti-cig1 antiserum in immunoprecipitations (lanes 4 to 7). In addition, a coprecipitated band with an M_r of 34,000, recognized by affinity-purified anti-cdc2 antibodies, was also detected. The specificity of the immunoprecipitation was tested by using preimmune serum or immune serum in the presence of competing antigenic peptide (lanes 5 and 7). The endogenous level of cig1 was apparently too low to be detected by our anti-cig1 antiserum in wild-type asynchronous cells, and the protein also was undetectable in cells lacking suc1 (data not shown). For this reason, we could not determine whether loss of suc1 affected the levels of cig1 protein.

Interestingly, the 54K band could be detected after immunoprecipitation from an *nda3*-KM311 cold-sensitive mutant strain arrested at 20°C for 8 h (Fig. 5, lane 8). This mutant, defective in β-tubulin, when shifted to the restrictive temperature arrests its cycle in midmitosis with condensed chromosomes and a high level of cdc2 kinase activity (26, 32). The retarded mobility of the cig1 band found in *nda3* arrested mitotic cells suggested that it could correspond to a phosphorylated form. To test this hypothesis, a cig1 immunoprecipitation, prepared from a cig1-overexpressing strain, was treated with PAP. After this treatment, the electrophoretic mobility of the upper band increased to equal that of the lower band (Fig. 5B, lane 1). A control treatment used in the absence of PAP or in the presence of PAP and phosphatase inhibitors (lanes 2 and 3) confirmed that the change in mobility was due to dephosphorylation rather than to protein degradation. In the *nda3*-KM311 arrested cells, cdc2 coprecipitated with cig1, indicating that the cdc2-cig1 complex might play a role in mitosis. The hyperphosphorylated form of cig1 was found to associate with cdc2 in this mutant or in a wild-type strain overexpressing cig1 (Fig. 5A, lanes 8 and 9). It is possible therefore that either cig1 becomes rapidly phosphorylated by cdc2 as soon as the complex forms or its phosphorylation by a yet unknown kinase(s) is a prerequisite for its association with cdc2. Further investigation is required to address this point. We then investigated whether the cdc2-cig1 complex had histone H1 kinase activity. We could detect specific phosphorylation of histone H1 after immunoprecipitation of cig1 from a wild-type strain (Fig. 6A). In the cig1 overexpressing strain, after induction for 16 h in thiamine-free medium, we could detect a 20-fold increase in the cig1-associated histone H1 kinase activity (Fig. 6B). The cig1-associated histone H1 kinase activity precipitated from a temperature-sensitive *cdc2* (*cdc2-L7*) strain disappeared after incubation at 37°C, and the temperature sensitivity of the kinase was rescued by the addition of bacterially produced suc1 prior to the incubation, suggesting that cdc2 was the cig1-associated kinase (data not shown).

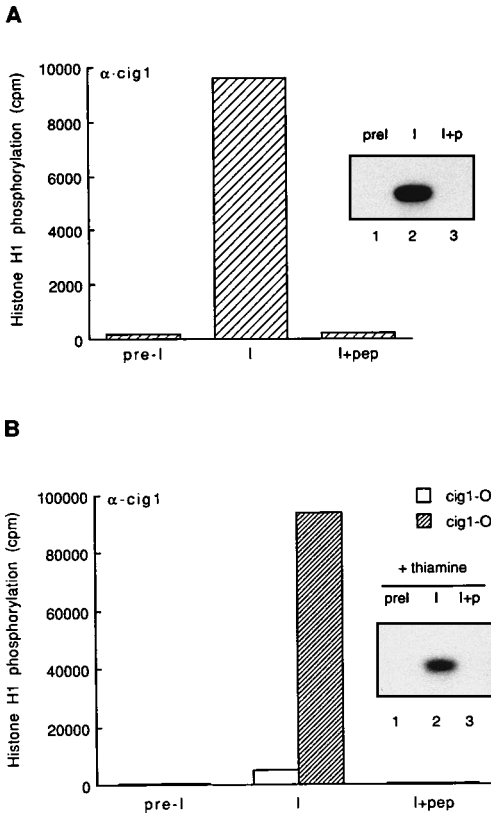

FIG. 6. cig1-associated histone H1 kinase activity. (A) cig1-associated histone H1 kinase activity in a wild-type strain. Anti-cig1 (α-cig1) immunoprecipitates were separated from an extract of the Sp972 (wild-type) strain and assayed for histone H1 kinase activity. Reaction products were analyzed by SDS-PAGE and autoradiography and were quantified by scintillation counting. Lane 1, preimmune serum (pre-I); lane 2, immune serum (I); lane 3, immune serum in the presence of competing peptide (I+pep). (B) cig1-associated histone H1 kinase activity in an *nmt1-cig1* strain. The Sp202 (*leu1-32*) strain transformed with the pREP1-*cig1* plasmid was grown in minimal medium with (lanes 1 to 3) or without (lanes 4 to 6) 4 μM thiamine for 16 h. Anti-cig1 immunoprecipitates were assayed for kinase activity, with histone H1 as the substrate. Lanes 1 and 4, preimmune serum; lanes 2 and 5, immune serum; lanes 3 and 6, immune serum in the presence of competing peptide. cig-OP, hyperphosphorylated cig1.

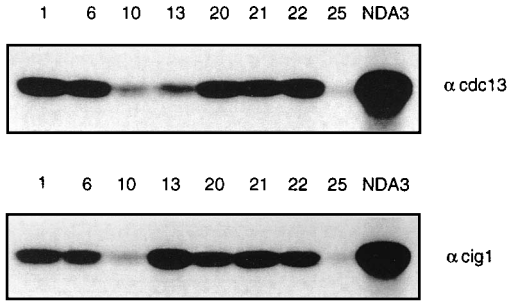

FIG. 7. Histone H1 kinase assay from cdc mutants. Anti-cdc13 (αcdc13; upper panel) or anti-cig1 (αcig1; lower panel) immunoprecipitations were performed with extracts of temperature-sensitive mutants arrested at 36°C for 4 h, with the only exception being the cold-sensitive *nda3*-KM311 mutant that was arrested at 22°C for 8 h. Immunoprecipitates were assayed for histone H1 kinase activity and analyzed by SDS-PAGE and autoradiography. Strains: 1, *cdc1-7*; 6, *cdc6-23*; 10, *cdc10-129*; 13, *cdc13-117*; 20, *cdc20-M10*; 21, *cdc21-M68*; 22, *cdc22-M45*; 25, *cdc25-22*; NDA3, *nda3*-KM311.

Activation of cdc2-cig1 kinase at mitosis and effect of loss of suc1 function. To investigate the regulation of the cdc2-cig1 complex during the cell cycle, we first assayed the kinase activity of the complex in conditional mutants of *S. pombe* arrested at different stages of the cell cycle (17). The kinase activity of the mitotic cdc2-cdc13 complex was also measured for comparison (Fig. 7). In a *cdc10-129* mutant, which arrests in G_1, the kinase activity level of both complexes was very low, and a similar result was obtained with a *cdc25-22* mutant, which arrests at the G_2/M transition with tyrosine-phosphorylated cdc2. In G_1/S-arrested mutants (*cdc20-M10*, *cdc21-M68*, and *cdc22-M45*) or in G_2-arrested mutants (*cdc1-7* and *cdc6-23*), the kinase activity level of both complexes is higher than that in G_1 but much lower than in the *nda3*-KM311 mutant, which arrests in mitosis with the two complexes showing their maximal level of activity. Note that the *cdc13-117* mutant, which arrests with characteristics of both G_2 and M phases (21), was the only mutant showing a difference between the two activities. While the cdc2-cig1 activity level in this mutant was higher than that in G_1/S or G_2 mutants, the cdc2-cdc13 activity level was much lower and was similar to the level found in the *cdc10* mutant. The *cdc13-117* mutant is indeed defective for the association with cdc2 (6) and, for this reason, the kinase activity level associated with cdc13 in this strain is very low. On the other hand, the relatively high kinase activity level associated with cig1 in the cdc13 mutant indicates that the anti-cig1 antiserum detects an active complex distinct from the cdc2-cdc13 complex. In order to confirm that cig1 and cdc13 formed two distinct complexes with cdc2 at mitosis, an immunodepletion experiment was performed (Fig. 8). After depletion of a mitotic extract with control serum, anti-cdc13 antiserum, or anti-cdc2 antiserum, the supernatant was used for immunoprecipitations using antisera against cig1 or cdc13 in the presence or the absence of competing peptides. The cdc2-cdc13 complex, which showed higher kinase activity levels than the cdc2-cig1 complex (lanes 1 to 4), was quantitatively depleted by the anti-cdc13 antiserum, while the activity of the cdc2-cig1 complex was not affected by this depletion (lanes 5 to 8). Depletion with anti-cdc2 antiserum eliminated both activities, proving that the kinase subunit associated with cig1 is cdc2 (lanes 9 to 12).

We then investigated the effect of loss of suc1 on the cdc2-cig1 kinase activity. Loss of suc1 caused an increase in kinase activity of approximately threefold above wild-type levels (Fig. 9). Compared with the effect observed on the cdc2-cdc13 kinase activity, which in the absence of suc1 increased about 15-fold above wild-type levels (Fig. 3), the cdc2-cig1 kinase was relatively little affected by loss of suc1 function. This difference could reflect the fact that the two complexes are activated at mitosis with different kinetics. In order to examine this possibility, we monitored the kinetics of activation of the cdc13-cdc2 kinase and of the cig1-cdc2 kinase after release at the permissive temperature (25°C) of a $cdc25^{ts}$ mutant which had been blocked at 37°C for 4 h. During the first 40 min after the release, samples were taken every 10 min in order to monitor precisely the kinetics of activation of the two complexes, while the last three samples were taken at 20-min intervals (Fig. 10). We were not able to detect any significant difference in the kinetics of activation: both complexes reached the maximal activation level (corresponding to an increase of about fivefold above the initial level) after 20 min and declined to the basal level after 60 to 80 min. We conclude therefore that loss of suc1 function differentially affects the activities of the two mitotic forms of the cdc2 kinase.

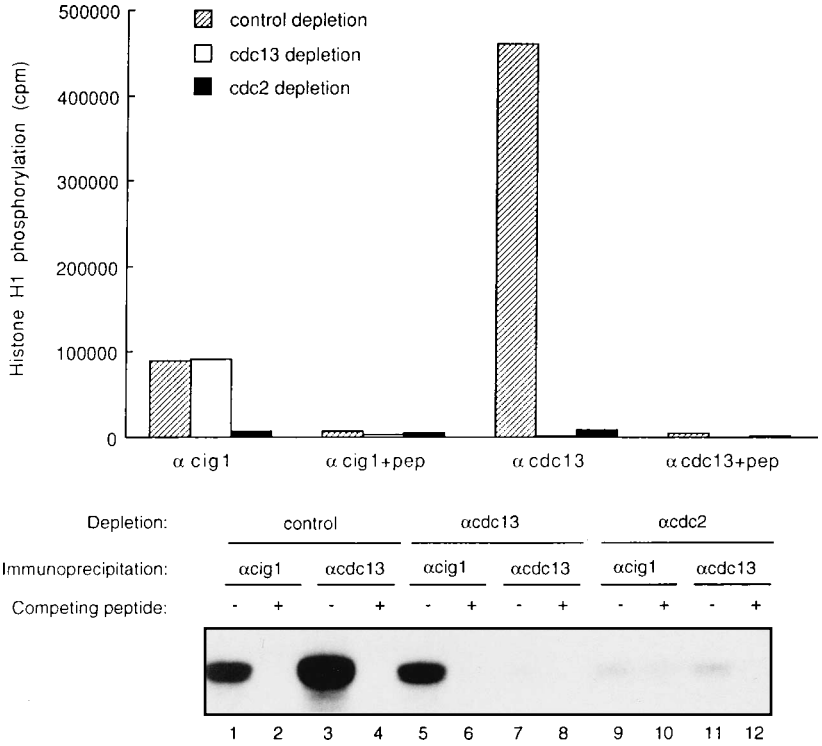

FIG. 8. cdc2 kinase associated with two different cyclins at mitosis. An extract prepared from an *nda3*-KM311 mutant arrested at 22°C for 8 h was used for three different immunodepletions performed by using a control serum (lanes 1 to 4), an anti-cdc13 antiserum (lanes 5 to 8), and an anti-cdc2 antiserum (lanes 9 to 12). In each case, after the first depletion, the supernatant was used for two successive depletions. Immunoprecipitations were performed with the depleted extracts by using either anti-cig1 (αcig1) or anti-cdc13 (αcdc13) antiserum in the presence (+) or absence (−) of competing peptide (pep), and then the precipitates were assayed for histone H1 kinase activity.

DISCUSSION

In this study, we have investigated the effect of loss of suc1 function in *S. pombe* and we have demonstrated the existence of a mitotic complex formed by cdc2 and the cig1 cyclin. The effect of loss of suc1 function on this complex and on the cdc2-cdc13 mitotic complex has been investigated.

We report that in cycling cells, with suc1 expression under the control of a thiamine-repressible promoter, loss of suc1 function causes an arrest of the cells with a heterogeneous phenotype and of most of the cells with condensed chromosomes. A previous study performed with germinating spores showed that arrest occurred in cells lacking suc1 with a heterogeneous morphology and in some cells with condensed chromosomes and a mitotic spindle (32). The significance of the heterogeneity of the phenotype is not clear at present.

By using specific anti-cig1 antibodies, we have shown that the cig1 cyclin associates with cdc2. The cdc2-cig1 kinase forms a complex which is active at mitosis, and it is distinct from the cdc2-cdc13 complex. We also show that the two complexes are activated at mitosis with the same kinetics. Genetic data have suggested that cig1 is required in late mitosis for nuclear separation (11). A role for cig1 for progression through G_1/S has also been proposed (9, 11; however, also see the reference 9 erratum). Our findings suggest a function for cig1 at mitosis. cig1 shares the greatest sequence similarity with cyclins CLB3 and CLB4, which have been shown to act in two phases of the *S. cerevisiae* cell cycle, in S and M phases (18, 39). It is possible that cig1 has a similar function in *S. pombe*, acting both in S phase and M phase, and that other cyclins with redundant functions exist. Further investigation will be required to establish the actual role of cig1 in mitosis and in other phases of the cell cycle.

Loss of suc1 function caused a 15- to 16-fold increase of the cdc2-cdc13 kinase activity but only a 2- to 3-fold increase of the cdc2-cig1 kinase activity over the respective wild-type levels. Since the two complexes are activated with the same kinetics, we propose that suc1 differentially affects the kinase activities of the two complexes.

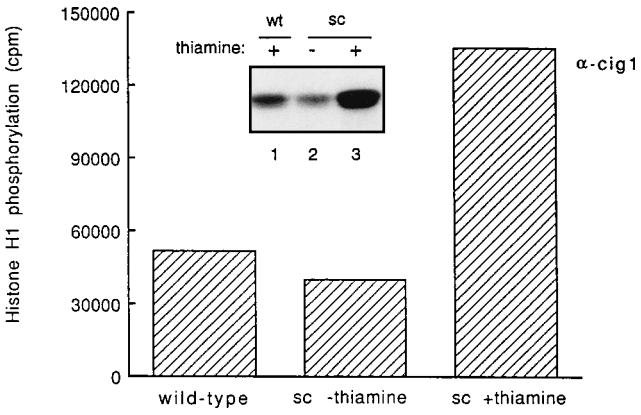

FIG. 9. cig1-associated histone H1 kinase activity in wild-type and suc1-conditional strains. Wild-type (wt) (lane 1) and suc1-conditional strains (lanes 2 and 3) were grown in minimal medium with (+) or without (−) 10 μM thiamine. Extracts were subjected to anti-cig1 (α-cig1) immunoprecipitation (inset), and the immunoprecipitates were assayed for histone H1 kinase activity. sc, suc1-conditional strain.

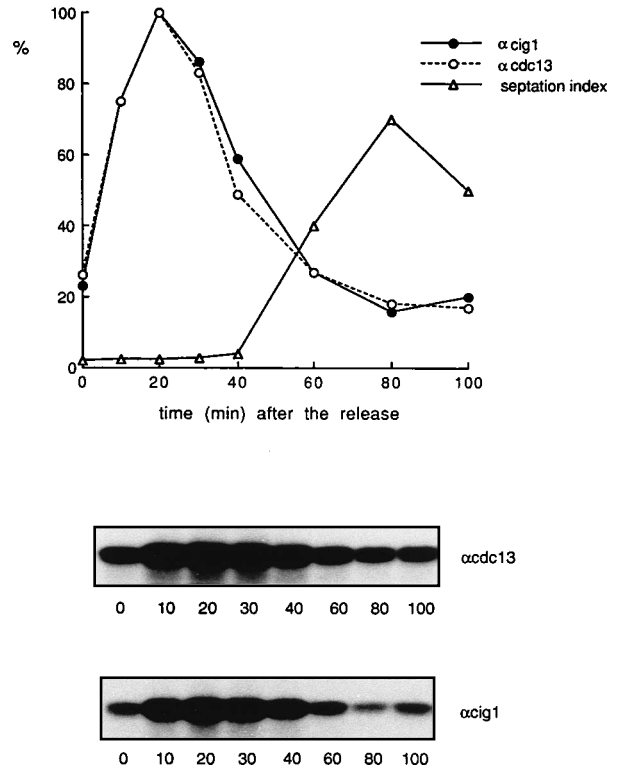

FIG. 10. Activation of the cig1-cdc2 kinase after the release of a *cdc25-22* arrested mutant. A *cdc25-22* temperature-sensitive mutant was arrested at 36°C for 4 h and released (time zero) by cooling to 25°C quickly in an ice bath. Samples were collected at the indicated times. Immunoprecipitations (shown below the activity graph) were performed with anti-cig1 (αcig1) and anti-cdc13 (αcdc13) antisera and assayed for histone H1 kinase activity. Values are expressed as a percentage of the maximum level of kinase activity of each complex.

These and previous findings (32) support the idea that suc1 is required for M-phase progression. In contrast, experiments with *S. cerevisiae* indicate that Cks1 (the budding yeast suc1 homolog) is required at the G_1/S and G_2/M transition points, and its loss does not apparently affect the activity of cdc28 kinase (45). This conclusion is based on the analysis of three temperature-sensitive *cks1* mutant strains, which behave as leaky temperature-sensitive mutants: i.e., the cell cycle is blocked in G_2 when the temperature is shifted to 37°C. However, if the same cells are shifted to an environment with the highest temperature at which they normally grow (38.5°C), the cycle also stops in G_1. We have not been able to detect an effect on G_1 progression associated with loss of suc1 function (data not shown). Therefore, suc1 might differ from Cks1 in this respect. However, it is formally possible that suc1 has a G_1 function also in *S. pombe* and this cannot be detected by our experimental conditions.

We have been unsuccessful in attempts to isolate a temperature-sensitive mutant of suc1, and this might reflect the great stability of the *S. pombe* protein. Another possible interpretation is that the G_1 block reported for *S. cerevisiae* by Tang and Reed (45) is due to a stabilizing function of Cks1 on cdc28 at a high temperature. In vitro experiments have shown that in *S. pombe* suc1 has a stabilizing effect on $cdc2^{ts}$ mutants at 37°C (6, 29, 32). A stabilizing effect on the wild-type cdc2 kinase activity has also been observed at this or higher temperatures (3, 6). This observation suggests that at high temperatures loss of suc1 function affects the stability of cdc2, hence affecting all the restriction points at which the cdc2 function is required. In this respect, it is not surprising that suc1 was initially isolated from *S. pombe* as a suppressor of temperature-sensitive strains of *cdc2* defective in their G_1 and G_2 functions (24). A quantitative study of the effect of loss of *CKS1* function on the G_1 and G_2 forms of the cdc28 kinase in *S. cerevisiae* would be informative. We show that in the absence of suc1 not only the activity of the cdc2-cdc13 kinase but also the abundance of the cdc13 protein increases dramatically (Fig. 4).

Since suc1 is a component of the cdc2 complex (8), it is possible that it is required for degradation of the mitotic cyclin cdc13 and that in its absence cell cycle arrest occurs in mitosis because cdc13 cannot be degraded. This could be due, for example, to a failure to activate the ubiquitin degradation pathway in the absence of suc1. It will be interesting to investigate whether suc1 is required for cyclin degradation in a reconstituted system in which the molecular components involved in cyclin degradation can be studied more directly.

Alternatively, in the absence of suc1, the cdc2-cdc13 complex might be unable to properly execute mitosis. Crystallization and biochemical studies of Ckshs2 (a human suc1 homolog) have suggested the existence of dimers and hexamers of suc1 (35). On the basis of these studies, it has been proposed that suc1 could have a docking function required for the multimerization of cdc2-cyclin heterodimers and that formation of higher order complexes containing multiple cdc2-cyclin heterodimers would be essential for proper progression through mitosis (35). If this model is correct, cdc2-cyclin multimers could not be assembled in the absence of suc1, leading to the activation of a form of cdc2 kinase which would perform only some of the functions required for proper execution of mitosis. Failure to complete some critical mitotic events could result in the activation of a feedback control mechanism which blocks cyclin degradation and leads to the accumulation of mitotic cyclins. If this model is correct, accumulation of cdc13 would be not the cause but a consequence of the arrest in M phase. In an *nda3* arrested mutant defective in microtubule polymerization, mitosis cannot be properly completed and this causes a block in cyclin degradation. In those cells, the levels of cdc13 and cig1 (Fig. 5) are high and readily detectable. It is interesting that in the absence of suc1 there is a dramatic increase in the levels of cdc13, while we could not detect an increase in the levels of cig1. This difference might be important and should be considered in further studies investigating the function of suc1 and its molecular mechanism of action.

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