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The Cak1p Protein Kinase Is Required at G_1/S and G_2/M in the Budding Yeast Cell Cycle

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ABSTRACT

The *CAK1* gene encodes the major CDK-activating kinase (CAK) in budding yeast and is required for activation of Cdc28p for cell cycle progression from G_2 to M phase. Here we describe the isolation of a mutant allele of *CAK1* in a synthetic lethal screen with the Sit4 protein phosphatase. Analysis of several different *cak1* mutants shows that although the G_2 to M transition appears most sensitive to loss of Cak1p function, Cak1p is also required for activation of Cdc28p for progression from G_1 into S phase. Further characterization of these mutants suggests that, unlike the CAK identified from higher eukaryotes, Cak1p of budding yeast may not play a role in general transcription. Finally, although Cak1 protein levels and *in vitro* protein kinase activity do not fluctuate during the cell cycle, at least a fraction of Cak1p associates with higher molecular weight proteins, which may be important for its *in vivo* function.

PROGRESSION through two key transition points in the cell cycle, from G_1 to S and from G_2 to M, is regulated by the activation and inactivation of cyclin-dependent kinases (CDKs). Only one CDK, Cdc28p, is necessary for normal cell cycle progression in the budding yeast *Saccharomyces cerevisiae*, and in the fission yeast *Schizosaccharomyces pombe* the cdc2 protein kinase is required. Higher eukaryotes have multiple CDKs; different CDKs function at different points in the cell cycle (reviewed in NASMYTH 1993; NORBURY and NURSE 1993; SHERR 1993). The Cdc28p/cdc2/CDK monomer is inactive; its activity is controlled in the cell cycle through a program of inhibitory and activating phosphorylation events and through the association with different classes of regulatory molecules including cyclins and inhibitors (reviewed in SOLOMON 1993; COLEMAN and DUNPHY 1994; ELLEDGE and HARPER 1994; PETER and HERSKOWITZ 1994b).

In *S. cerevisiae*, exit from G_1 into S is controlled at a point late in G_1 called Start and requires the association of Cdc28p with G_1-specific cyclins, Cln1p, Cln2p and Cln3p (NASH *et al.* 1988; HADWIGER *et al.* 1989; RICHARDSON *et al.* 1989; CROSS and BLAKE 1993). Association of Cdc28p with B-type cyclins (Clbs) is necessary for cell cycle progression after Start. Six B-type cyclins (Clb1-6p) have been identified, which fall into three classes based on sequence similarity and patterns of RNA accumulation during the cell cycle. Of these, Clb2p is the most important for progression from G_2 into M; strains deleted for *CLB2* have a growth defect and a G_2/M delay (SURANA *et al.* 1991; FITCH *et al.* 1992; RICHARDSON *et al.* 1992). Clb1,2p/Cdc28p kinase activity has also been proposed to promote the switch in G_2 from apical, tip-directed bud growth to isotropic bud growth. As a consequence, cells that are defective in the activation of Clb2p/Cdc28p show hyperpolarized bud growth and an elongated cell morphology (LEW and REED 1993).

In *S. pombe*, progression from G_2 to M depends on the dephosphorylation of cdc2 on tyrosine 15 and phosphorylation of threonine 167 in the T-loop (GOULD and NURSE 1989; DUCOMMUN *et al.* 1991). These phosphorylation/dephosphorylation events are also required for the function of Cdc28p in budding yeast (AMON *et al.* 1992; SORGER and MURRAY 1992; LEW and REED 1993; CISMOWSKI *et al.* 1995; DESHAIES and KIRSCHNER 1995) and the CDKs of higher eukaryotes (KREK and NIGG 1991; NORBURY *et al.* 1991; DESAI *et al.* 1992; SOLOMON *et al.* 1992), although their role in the regulation of cell cycle progression is not entirely clear. While phosphorylation on threonine 167 (threonine 161 in human cdc2, threonine 169 in Cdc28p) is required for entry into M phase, evidence from higher eukaryotes suggests that dephosphorylation of cdc2 on threonine 161 is required for anaphase and exit from M (LORCA *et al.* 1992). Recent studies in *S. cerevisiae* raise the possibility that dephosphorylation of Cdc28p on threonine 169 may be necessary for Start (LIM *et al.* 1996). The protein kinase responsible for the activating threonine phosphorylation is named CAK (CDK-activating kinase) and has been identified in higher eukaryotes using a biochemical approach (FESQUET *et al.* 1993; POON *et al.* 1993; SOLOMON *et al.* 1993). CAK from these systems is itself a CDK (called Cdk7 or MO15) whose *in vitro* activity requires association with a cyclin (cyclin H) (FISHER and MORGAN 1994; MAKELA *et al.* 1994) and either phos-

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phorylation on threonine 170 or an assembly factor of 36 kD (DEVAULT *et al.* 1995; FISHER *et al.* 1995; TASSAN *et al.* 1995). Recently, these proteins have also been identified as components of the transcription factor TFIIH and shown to phosphorylate the C-terminal repeat domain (CTD) of the largest subunit of RNA polymerase II *in vitro* (ROY *et al.* 1994; SERIZAWA *et al.* 1995; SHIEKHATTAR *et al.* 1995). The Cdk7/cyclin H protein complex may therefore have a role in both transcription and in cell cycle progression. Kin28p is a close homologue of Cdk7 in budding yeast, and it interacts with a homologue to cyclin H (VALAY *et al.* 1993). However, although Kin28p appears to be involved in transcription (CISMOWSKI *et al.* 1995; VALAY *et al.* 1995), is associated with TFIIH (FEAVER *et al.* 1994) and has CTD kinase activity, it does not have detectable CAK activity (FEAVER *et al.* 1994; CISMOWSKI *et al.* 1995). This result suggests that in *S. cerevisiae*, in contrast to higher eukaryotes, CTD kinase and CAK are encoded by different genes.

Sit4p is a protein phosphatase in *S. cerevisiae*, which, like Cdc28p, is required for progression from G_1 into S phase (SUTTON *et al.* 1991). The Sit4p protein phosphatase is involved in several aspects of cell cycle control in *S. cerevisiae* (SUTTON *et al.* 1991; FERNANDEZ-SARABIA *et al.* 1992). Sit4p is required late in G_1 for the normal accumulation of *CLN1* and *CLN2* RNA, and consequently, temperature-sensitive *sit4* strains arrest in late G_1 at the nonpermissive temperature (SUTTON *et al.* 1991). However, Sit4p has additional function(s) essential for bud emergence and cell cycle progression (SUTTON *et al.* 1991; FERNANDEZ-SARABIA *et al.* 1992; DOSEFF and ARNDT 1995). In an effort to identify the additional function(s) for Sit4p in cell cycle progression, we undertook a genetic approach. This approach was based on the fact that the phenotypes of *sit4* strains are dependent on an additional locus *SSD1*. *SSD1* is polymorphic in laboratory strains; Δ*sit4, ssd1-d or* Δ*sit4,* Δ*ssd1* cells are inviable, while Δ*sit4, SSD1-v* cells are viable, but with a growth defect and a G_1 delay (SUTTON *et al.* 1991). The function of Ssd1p in cells is unknown; however, it has been implicated in a wide variety of cellular processes, including growth control (CVRCKOVA and NASMYTH 1993), morphogenesis (COSTIGAN *et al.* 1992; LEE *et al.* 1993) and RNA metabolism (STETTLER *et al.* 1993). The screen that we devised was designed to identify genes that, when mutated, cause a requirement for *SIT4* for viability in an *SSD1-v* background. This synthetic lethal screen was expected to identify genes whose products have functions in the cell cycle that overlap those of Sit4p.

We have recently shown that one of the genes that we identified in the *SIT4* synthetic lethal screen encodes the major CAK from budding yeast (KALDIS *et al.* 1996). Using a combination of genetics and biochemistry, we demonstrated that Cak1p from *S. cerevisiae* is a protein kinase with unusual structural features, only distantly related to Cdk7/MO15 and other CDKs. In contrast to Cdk7/MO15, Cak1p can function as a monomer. Cak1p phosphorylates and activates Clb2p/Cdc28p *in vitro* and *in vivo* (KALDIS *et al.* 1996). Analysis of a temperature-sensitive allele of *CAK1*, *cak1-22,* showed that the majority of *cak1-22* cells arrest at the nonpermissive temperature at G_2/M. Further, *cak1* mutations showed genetic interactions with mutations in *CLB2,* which encodes the major mitotic cyclin in budding yeast. The genetic interactions and phenotypic defects of the *cak1-22* mutants are consistent with an essential role for Cak1p in the activation of Cdc28p for the G_2 to M transition (KALDIS *et al.* 1996). The identification of Cak1p as the major CAK in budding yeast has also recently been described by others (ESPINOZA *et al.* 1996; THURET *et al.* 1996). In this paper, we describe our further characterization of the properties and functions of Cak1p of budding yeast. Our analyses of the phenotypes of two different *cak1* mutants and strain backgrounds show that although the G_2 to M transition is most severely affected by loss of Cak1p function, Cak1p also has an essential role in activation of Cdc28p for the G_1 to S transition. Analysis of these mutants provides no evidence that Cak1p is required for activity of other CDKs in budding yeast.

MATERIALS AND METHODS

Strains and media: Table 1 shows the genotypes of the yeast strains used in this study. Unless indicated otherwise, all strains were grown in YPD medium (1% yeast extract, 2% Bacto-peptone and 2% glucose), which was supplemented with adenine at 0.3 mM and tryptophan at 0.1 μg/ml. YP-gal contained 2% galactose instead of glucose. YP-ethanol contained 3% ethanol instead of glucose. For experiments in which cells were incubated with ^{35}S-methionine, the synthetic minimal medium was yeast nitrogen base (Difco) supplemented with adenine at 0.3 mM, tryptophan at 0.1 μg/ml and leucine at 0.2 μg/ml, with 2% galactose as carbon source. For the acid phosphatase assays, cells were grown in SC (ARNDT *et al.* 1989).

Isolation of *pdl* mutants: Strains CY1231 and CY1234 were grown to stationary phase in YP-gal. Cells were incubated for 70 min at room temperature with 30 μl/ml EMS (Sigma). After stopping the mutagenesis with sodium thiosulfate, cells were diluted and plated onto YP-gal and subsequently screened for growth on YP-gal, but no growth on YPD.

Cloning of *CAK1*: The *CAK1* gene was cloned from a YCp50 library containing yeast genomic inserts (ROSE *et al.* 1987) by transformation of the *pGAL:SIT4 cak1-1* mutant and selection on SC-uracil medium. Plasmids that allowed growth on glucose were recovered and grouped by restriction pattern analysis. Two different groups with overlapping restriction fragments were obtained. The chromosomal locus of one cloned insert was marked with the *URA3* gene in strain CY1231. After crossing to the *cak1-1* mutant (SY5), linkage analysis was used to show that the Ura$^+$ phenotype segregated 100% (17 tetrads tested) with ability to grow on glucose.

A 3.9-kilobase (kb) region of DNA containing the complementing region of *CAK1* was inserted into pUC118 in both orientations and sequenced. Subsequent to our sequencing of the *CAK1* gene, the DNA sequence was released as part of the *S. cerevisiae* genome project. *CAK1* corresponds to open reading frame (ORF) YFL029C of the Chromosome VI sequence.

TABLE 1

***Saccharomyces cerevisiae* strains**

Strain	Genotype	Source
CY182 background[a]		
CY182	*MATa SIT4 his3Δ200 leu2-3 ura3-52 lys2 SSD1-v*	K. ARNDT
CY1143	*MATα*	K. ARNDT
CY1231	*MATa pGAL:SIT4*	K. ARNDT
CY1234	*MATα pGAL:SIT4*	K. ARNDT
SY5	*MATα pGAL:SIT4 cak1-1*	This study
SY6	*MATa SIT4 cak1-1*	This study
W303 background[b]		
W303-1A	*MATa ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1 ssd1-d*	R. ROTHSTEIN
W303-1B	*MATα*	R. ROTHSTEIN
CY6	*MATa/MATα ade2/ade2 his3/his3 leu2/leu2 can1/can1 ura3/ura3 trp1/trp1 ssd1-d/ssd1-d*	W303-1A crossed with W303-1B
SY8	*MATa/MATα cak1::HIS3/CAK1 ade2/ade2 his3/his3 leu2/leu2 can1/can1 ura3/ura3 trp1/trp1 ssd1-d/ssd1-d*	This study
SY9	*MATa/MATα cak1::LEU2/CAK1 ade2/ade2 his3/his3 leu2/leu2 can1/can1 ura3/ura3 trp1/trp1 ssd1-d/ssd1-d*	This study
SY50	*MATa cak1::HIS3* (*pGAL:CAK1* in YCp50)	KALDIS *et al.* (1996)
SY80	*MATa cak1::HIS3* (*CAK1* in YCp50)	KALDIS *et al.* (1996)
SY86	*MATa cak1::HIS3* (*CAK1-[HA]$_3$* in YCp50)	This study
SY89	*MATa cak1::HIS3* (*CAK1* in YEp24)	KALDIS *et al.* (1996)
SY101	*MATa cak1::HIS3* (*cak1-2-[HA]$_3$* in YEp24)	This study
SY106	*MATa cak1::HIS3* (*CAK1-[HA]$_3$* in YEp24)	This study
SY112	*MATa cak1::HIS3* (*CAK1* in *LEU2/CEN*)	This study
SY123	*MATa cak1::HIS3* (*cak1-2-[HA]$_3$* in YCp50)	This study
SY132	*MATa cak1::HIS3* (*cak1-22* in *LEU2/CEN*)	KALDIS *et al.* (1996)[c]
SY151	*MATa cak1::HIS3* (*cak1-22* in YCp50)	This study
SY162	*MATa cak1::HIS3* (*CAK1* in *LEU2/CEN*)	KALDIS *et al.* (1996)
SY190	*MATa cak1::HIS3* (*CAK1-[HA]$_1$* in YEp24)	This study
SY191	*MATa cak1::HIS3* (*CAK1-[HA]$_2$* in YEp24)	KALDIS *et al.* (1996)
SY207	*MATa cak1::HIS3* (*CAK1* in *TRP1/CEN*)	This study
SY208	*MATa cak1::HIS3* (*cak1-22* in *TRP1/CEN*)	This study
SY213	*MATa cak1::HIS3* (*pGAL:CAK1-[HA]$_3$* in YCp50)	This study
SY263	*MATα cak1::LEU2 cln1::HIS3 cln3::URA3* (*CAK1* in *TRP1/CEN*)	This study
SY264	*MATα cak1::LEU2 cln1::HIS3 cln3::URA3* (*cak1-22* in *TRP1/CEN*)	This study
SY266	*MATα cak1::HIS3 cln2::TRP1* (*CAK1* in YCp50)	This study
SY267	*MATα cak1::HIS3 cln2::TRP1* (*cak1-22* in YCp50)	This study
SY326	*MATa cak1::HIS3 cdc15-2* (*CAK1-[HA]$_3$* in YCp50)	This study
SY1031	*MATa cdc15-2*	A. B. FUTCHER
SY1046	*MATa clb2::LEU2*	A. B. FUTCHER
Additional strains		
SY258	*MATa pho85::LEU2 trp1Δ63 ura3-52 lys2-801 ade2-107^0 his3Δ200 leu2-Δ1*	A. NEIMAN
SY301	*MATa cak1-1 cdc15-2 his3 leu2 ura3*	This study[d]
SY304	*MATα cak1-1 his3 leu2 ura3*	This study[e]

[a] Strains below are isogenic to CY182 except as indicated.
[b] Strains below are isogenic to W303-1A except as indicated.
[c] Essentially identical to SY143 (KALDIS *et al.* 1996).
[d] Obtained from cross of SY304 with SY1031.
[e] Obtained from sixth back-cross of SY6 to W3031B.

Mutagenesis of *CAK1*: *Deletion of CAK1:* A 685-base pair (bp) *Xba*I-*Nsi*I fragment from the *CAK1* ORF was replaced with a 1.8-kb *Bam*HI fragment containing the *HIS3* gene to create plasmid SB14. SB14 was digested with *Sph*I (site is in vector, adjacent to cloned insert) and *Eco*RI and the Δ*cak1::HIS3* fragment transformed into diploid strain CY6. The diploid transformants were sporulated and tetrads dissected. Each tetrad (>40 tested) gave rise to two viable His$^-$ colonies and two microcolonies (50–100 cells) presumed to be His$^+$. To create *cak1::LEU2*, the *Xba*I-*Nsi*I fragment of *CAK1* was replaced with the 2.2-kb *Sal*I-*Xho*I fragment of *LEU2*.

Epitope tagging of Cak1p: Oligonucleotide-directed mutagenesis (KUNKEL 1985) was used to create a *Not*I restriction site immediately upstream of the termination codon for *CAK1* cloned in plasmid pUC118. To create *CAK1-[HA]$_3$*, a *Not*I restriction fragment encoding three tandem copies of the hemagluttinin antigen (HA) epitope (TYERS *et al.* 1992) was inserted in frame into the *Not*I site (SB27). To create *CAK1-[HA]$_1$* and *CAK1-[HA]$_2$*, duplex oligonucleotides encoding the epitope sequence YPYDVPDYA were placed into the *Not*I site. Clones that had either one or two tandem copies of the oligonucleotides were obtained.

Oligonucleotide-directed mutagenesis was used to create the mutant cak1-2 protein in which lysine (K) at position 31 was changed to arginine (R). The HA epitope-tagged version of cak1-2p (cak1-2p-HA) was obtained by replacing a 830-bp *Eco*RV fragment of SB27 (see above) with an *Eco*RV fragment containing the K31R mutation. The constructs were confirmed by DNA sequencing.

The *CAK1* wild-type and mutant genes were cloned into the *Bam*HI site of plasmids YCp50, YEp24, *LEU2/CEN* or *TRP1/CEN* as described (KALDIS *et al.* 1996). Plasmids were transformed into diploid strain SY8 (*CAK1/cak1::HIS3*) or SY9 (*CAK1/cak1::LEU2*) followed by sporulation and dissection to obtain *cak1::HIS3* or *cak1::LEU2* haploid cells containing the appropriate plasmids.

Preparation of cellular extracts and Western immunoblotting: Cell extracts were prepared as previously described (SUTTON *et al.* 1991). Preparation of samples for Western analysis was as described (SUTTON *et al.* 1991). Following electrophoresis, samples were transferred to Immobilon-P membrane (Millipore) using a Milliblot-SDE system (see Figure 5) or to nitrocellulose using a BioRad Trans-Blot SD Electrophoretic Cell (see Figure 6). Proteins were detected by ECL (Amersham) according to instructions from the manufacturer.

Immunoprecipitation and *in vitro* kinase assay: For ^{35}S labeling of proteins, cells were grown as previously described (SUTTON *et al.* 1991). For the experiments described in Figure 5, cell extracts were prepared from ^{35}S-labeled cells grown in synthetic minimal medium with galactose or from cells grown to early-logarithmic phase (A_{600} = 0.5) in YPD and immunoprecipitation carried out essentially as described (SUTTON *et al.* 1991). For each sample, 300 μl of extract (8 mg/ml protein) were incubated with 2 μl of 12CA5 monoclonal antibody (MAb) ascites for 1 hr and subsequently mixed with 30 μl of a suspension of protein A-Sepharose beads (Pharmacia). After rocking for 1 hr at 4°, the beads were washed four times as described (SUTTON *et al.* 1991). The samples from the ^{35}S-labeled cells were then prepared for electrophoresis as described (SUTTON *et al.* 1991). The beads for the samples to be analyzed for kinase activity were washed an additional two times with kinase reaction buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) and then all remaining liquid was removed by aspiration. For the *in vitro* kinase reaction, 4 μl of 2× kinase reaction buffer, 1 μl γ^{32}P-ATP (3000 Ci/mM), 2 μl ATP (3 μM) and 1 μl H_2O was added to the washed beads, which were then incubated for 15 min at 37°. The reaction was stopped by adding 20 μl of 2× gel sample buffer (SILHAVEY *et al.* 1984). The samples were heated to 95° for 5 min, centrifuged at 16,000 × *g* for 3 min and loaded onto an SDS-polyacrylamide gel. Following electrophoresis, the gel was fixed in 25% methanol, 10% acetic acid, dried and visualized by autoradiography. Phosphatase inhibitors were found not to affect the results of the protein kinase assay and were therefore omitted.

For the protein kinase assays described in Figure 6, cell extracts were prepared and immuoprecipitation of Cak1p-HA was as described above except that for the *cdc15* synchronization experiments, 200 μl of extract (0.9 mg/ml protein) was used for each immunoprecipitation with 0.5 μl of 12CA5 MAb, and for the elutriation experiment, 100 μl of extract (0.4 mg/ml protein) was used with 0.5 μl of 12CA5 MAb. After incubation of the extracts with the antibody and subsequently with protein A beads, the beads were washed four times as described (SUTTON *et al.* 1991), two times in CAK assay buffer (80 mM Tris, pH 7.3, 20 mM EGTA, 15 mM $MgCl_2$, 10 mM DTT, 1 mg/ml ovalbumin, 1.25 μM each of leupeptin, antipain, chymostatin and pepstatin) and then remaining liquid was removed by aspiration. For the *in vitro* kinase assay, 10.5 μl of CAK assay buffer, 1 μl γ^{32}P-ATP (3000 Ci/mM), 1.6 μl ATP (100 μM), 0.4 μl 1 M $MgCl_2$ and 1 μl Cdk2 (KALDIS *et al.* 1996) was added to the beads. After incubation for 30 min at 24°, the reactions were stopped with 20 μl 2× gel sample buffer and processed as described above.

Liquid acid phosphatase assays: Quantitative liquid acid phosphatase assays were done essentially as described in O'NEILL *et al.* (1996). Cells were grown in high phosphate (SC) medium to A_{600} of between 0.4 and 1.2.

Northern analysis and probes: Northern analysis was performed as described (FERNANDEZ-SARABIA *et al.* 1992). The probes used were the 750-bp *Xho*I-*Hin*dIII fragment of *CLN2*, the 2.7-kb *Eco*RI fragment of *RNR1*, the 2-kb *Hin*dIII-*Xho*I fragment of *CLB2*, the 600-bp *Eco*RI-*Hin*dIII fragment of *ACT1*, a 1.2-kb fragment from YEp24 for *URA3* and a 1.1-kb *Xho*I-*Hin*dIII fragment of *CDC28*. The probe for *CLB3* RNA was a 1.2-kb *Bam*HI fragment that was generated from restriction digestion of a *CLB3* gene that has a naturally occurring *Bam*HI site at the 5′ end of the ORF and a genetically engineered *Bam*HI site near the 3′ end of the ORF (provided by B. FUTCHER).

Immunofluorescence: 4′-6′-diamidino 2-phenylindole (DAPI) staining was carried out as previously described (SUTTON *et al.* 1991). Cells were viewed with a Zeiss Axioskop microscope with a ×100 objective and Kodak Tmax p3200 film was used for photography.

Cell cycle synchronization: Cells were synchronized with α-factor as described (FERNANDEZ-SARABIA *et al.* 1992) using α-factor at a final concentration of 0.012 mM. Cells with a temperature-sensitive *cdc15-2* allele were synchronized by shifting an exponentially growing culture from 24 to 37° for 3.3 hr. Cells were then released from the block by shifting cultures to a 24° water bath. Centrifugal elutriation of cells was as described (SUTTON *et al.* 1991) except that the cells were grown in YP-ethanol. Approximately 10^{10} cells from an exponential culture were loaded into the elutriator. Cells were prepared for flow cytometry as described (FERNANDEZ-SARABIA *et al.* 1992) and were analyzed with a Coulter EPICs Elite cytometer. Cell volume distributions were obtained using a Model ZM Coulter channelizer as described (NASH *et al.* 1988).

RESULTS

Identification of the *CAK1* gene in a synthetic lethal screen with *SIT4*: In an *ssd1-d* strain, *SIT4* is essential. In an *SSD1-v* background, loss of Sit4p function does not cause lethality (although cells grow slowly) (SUTTON *et al.* 1991). *SSD1-v* strains of opposite mating type in which *SIT4* was under control of the *GAL1* promoter (CY1231 = *MAT***a** *pGAL:SIT4 SSD1-v, CY1234 = MATα pGAL:SIT4 SSD1-v*) were created. In galactose medium, these strains grew well because *SIT4* was expressed (Figure 1A). In glucose medium, expression of *SIT4* was repressed. However, the strains were viable because of *SSD1-v*, but grew at a slow growth rate (Figure 1A). Strains CY1231 and CY1234 were grown to stationary phase in YP-gal. The cells were mutagenized with EMS. Following mutagenesis, cells were diluted and plated onto YP-gal medium. Approximately 40,000 colonies of each mating type were screened by replica plating for mutants that grew on YP-gal, but not on YPD. Mutants were classified into complementation groups by mating and testing for growth on glucose. Mutations in nine different complementation groups were obtained by

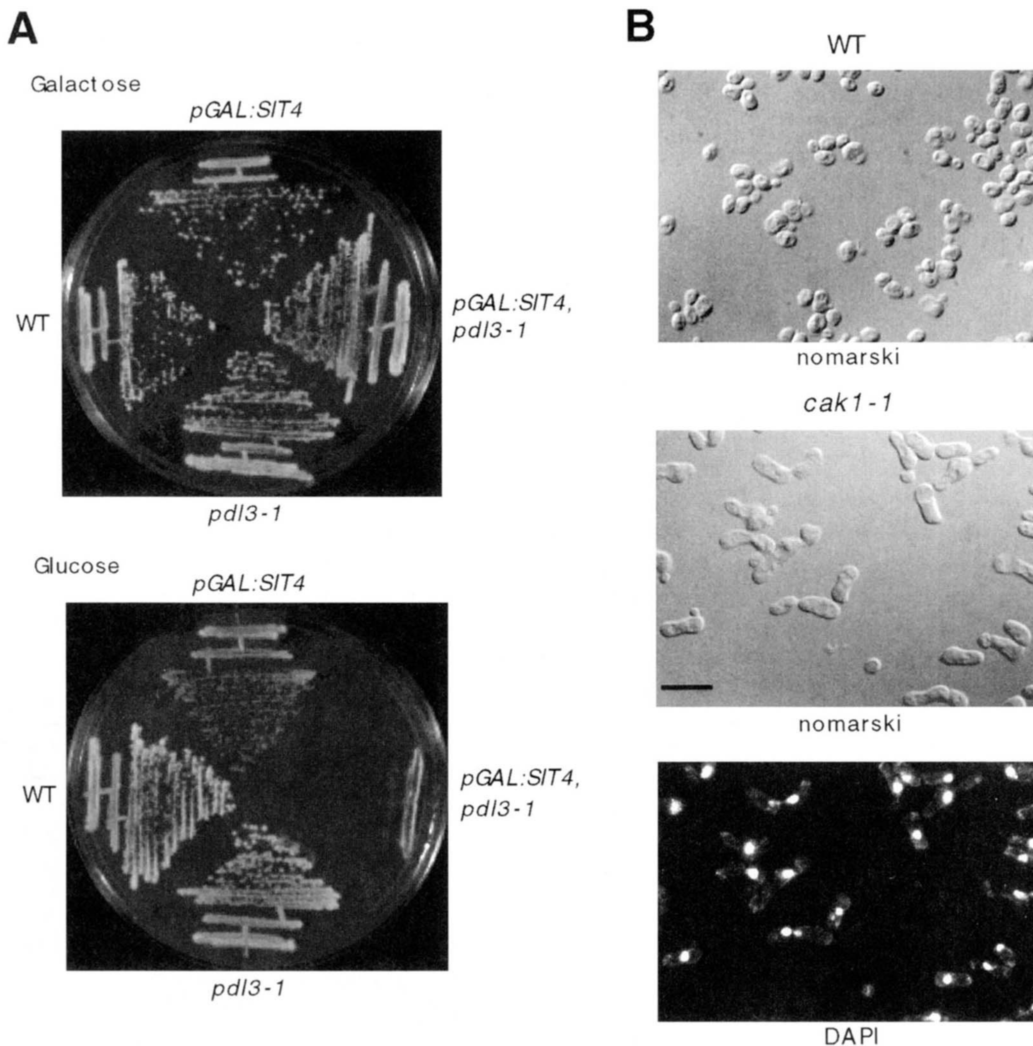

FIGURE 1.—*cak1-1* mutants require *SIT4* for viability. (A) A strain in which *SIT4* is under control of the *GAL1* promoter (*pGAL:SIT4* = CY1234) grows as well as a wild-type strain (WT = CY1143) on YP-gal medium (galactose), but also grows (slowly) on YPD (glucose). If the *pGAL: SIT4* strain also has a mutation in *PDL3/CAK1* (*pGAL: SIT4 pdl3–1* = SY5), it grows well on galactose, but does not grow on glucose. The *pdl3–1/cak1-1* mutation (*pdl3–1* = SY6) does not have a major effect on the growth rate of *SIT4* cells on either galactose or glucose. Cells were grown at 30° for 2 days (glucose) or 3 days (galactose). (B) *cak1-1* cells have an elongated morphology. Micrographs of wild-type (WT = CY182) and *cak1-1* cells (SY6) using Nomarski optics and DAPI staining of DNA. Bar, 8 μm.

this screen. The genes containing these mutations were named *PDL* for phosphatase deficient lethals. For most of the complementation groups, only one member was isolated, indicating that the screen was not saturating. One of the mutations chosen for further study (*pdl3-1*) caused a slight growth defect when *SIT4* was expressed, but no growth when *SIT4* was not expressed (Figure 1A). Because we have recently determined that *PDL3* encodes an unusual protein kinase that is the major CAK in *S. cerevisiae* (KALDIS *et al.* 1996), we have renamed this gene *CAK1*.

***cak1-1* cells are delayed in cell cycle progression late in G_2:** To study the phenotypes of *cak1-1* strains, we crossed the *pGAL:SIT4 cak1-1* strain (SY5) to the parental strain in which *SIT4* was under control of its own promoter (CY182). Following sporulation and dissection of tetrads, we obtained a *SIT4 cak1-1* strain (SY6). This *cak1-1* strain had a striking, elongated cell shape compared to wild-type strains (Figure 1B). Furthermore, an asynchronous population of *cak1-1* cells showed an enrichment in budded cells compared to wild type (69% compared to 44%). DAPI staining of the DNA for *cak1-1* cells showed that many of the budded cells had an undivided nucleus at the neck between the mother and bud (Figure 1B) and staining of microtubules with an antitubulin antibody revealed that these cells had a short spindle spanning this nucleus (data not shown). The phenotypes of the *cak1-1* mutants are very similar to those of strains deleted for the gene encoding the major mitotic cyclin *CLB2* (SURANA *et al.* 1991). This result suggests that the *cak1-1* mutation, like the *cak1-22* mutation (KALDIS *et al.* 1996), may primarily affect the ability of the cells to progress through G_2 into M.

The *cak1-1* mutation is not temperature sensitive, but caused a growth defect at 30° in a wild-type *SIT4* background compared to an isogenic *CAK1* strain (120 min doubling time in YPD compared to 80 min for wild type). Flow cytometry analysis of asynchronous *cak1-1* cells showed an enrichment in cells that have a 2N DNA content compared to the isogenic *CAK1* strain (Figure 2A). Such an enrichment in cells with a 2N DNA content could result from a shortened G_1 phase or an elongated G_2 or M phase in the *cak1-1* mutant. Flow cytometry analysis also revealed that the *cak1-1* cells are larger than wild-type cells (data not shown), which suggests

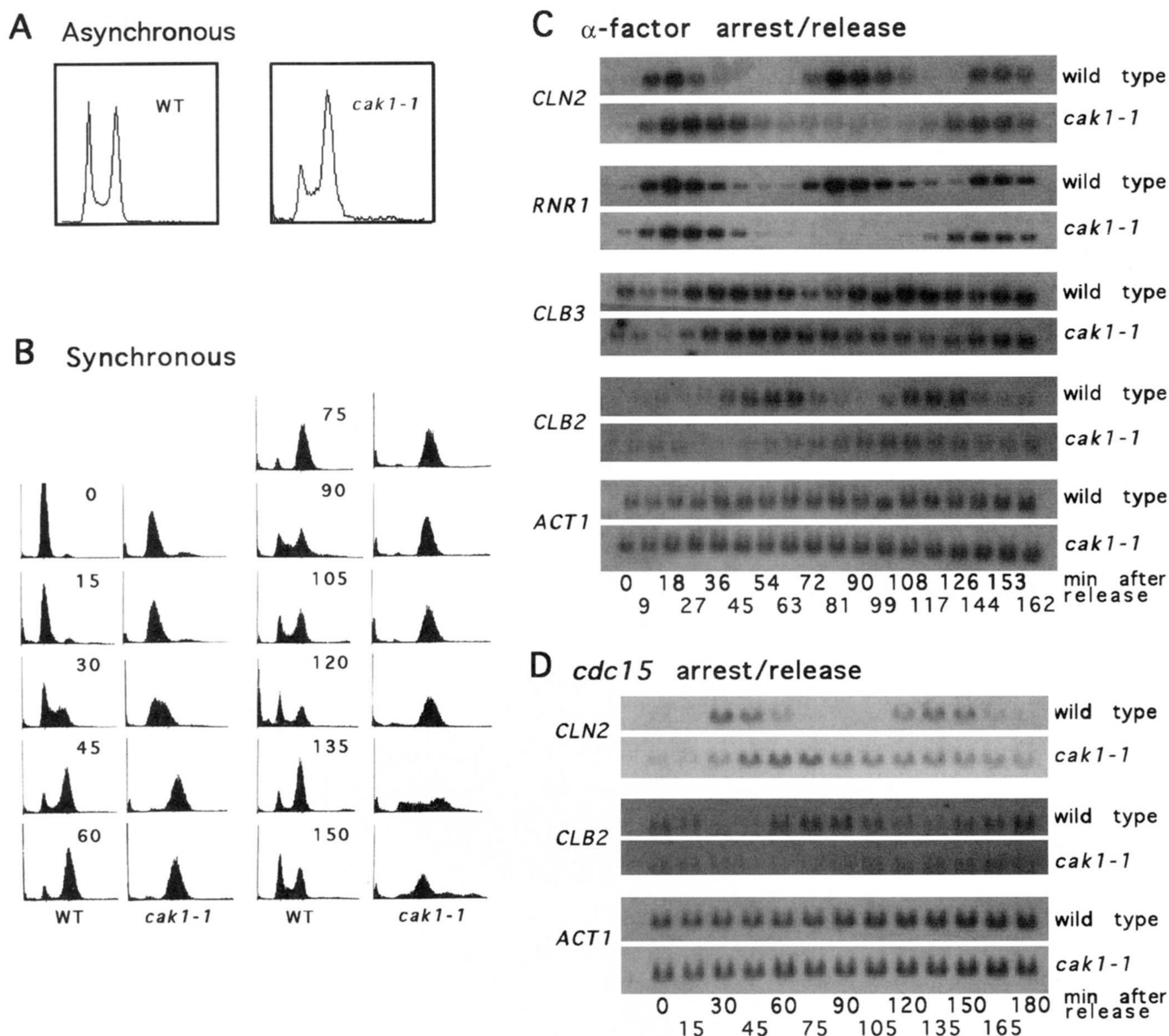

FIGURE 2.—The *cak1-1* mutation causes a delay at G_2/M. (A) Flow cytometry analysis of the DNA content of asynchronous wild-type (CY182) and *cak1-1* (SY6) cells. (B) Exponentially growing cultures of CY182 and SY6 were synchronized in G_1 by treatment with α-factor at 30° for 3.3 hr. The α-factor was removed by filtration and samples collected at the indicated times (minutes) for flow cytometry. (C) Northern analysis of RNA from CY182 and SY6 synchronized with α-factor as in B. At the indicated times after release from α-factor, samples were taken and RNA isolated. Five micrograms of RNA was loaded onto each lane, and wild-type and *cak1-1* samples were analyzed on the same gel. (D) Northern analysis of RNA from *cdc15* (SY1031) and *cdc15 cak1-1* (SY301) strains synchronized in M with a *cdc15*-induced arrest at 37°. At the indicated times after release to 24°, samples were taken and RNA isolated and analyzed as in (C). The G_1/S transitions (50% of cells have new buds) for *cdc15* cells were at 52 and 150 min and for *cdc15 cak1-1* cells was at 70 min.

that the mutants have an elongated G_2 or M, as a shortened G_1 phase would be expected to result in a smaller cell size. To confirm this, we synchronized wild-type and mutant cells by treatment with α-factor. Both strains arrested after 3 hr in α-factor with a 1N DNA content (Figure 2B). Upon release from α-factor, both strains completed S phase (had a 2N DNA content) within a few minutes of each other (Figure 2B, $t = 45$). However, the *cak1-1* strain exhibited an extensive delay after S phase; the wild-type strain progressed through almost two cell cycles during the time the mutant cells completed only one cycle (Figure 2B). These data show that the *cak1-1* mutation causes a cell cycle specific growth delay in G_2 and/or M phase.

To examine more precisely the effect of the *cak1-1* mutation on cell cycle progression, RNA was isolated from samples taken as wild-type and *cak1-1* cultures proceeded through the cell cycle following synchronization by release from an α-factor block. The RNA was examined by Northern analysis using probes for genes that

show periodic transcription during the cell cycle. The results of this analysis are presented in Figure 2C. RNA for the G_1 cyclin genes *CLN1, CLN2* and *HCS26,* as well as RNA for a number of genes required for DNA synthesis, including *RNR1,* accumulate to maximal levels late in G_1 (ELLEDGE and DAVIS 1990; WITTENBERG *et al.* 1990; CROSS and TINKELENBERG 1991). The rate of accumulation of RNA for *CLN2* and for *RNR1* after release from α-factor was similar in the *CAK1* and *cak1-1* strains (Figure 2C). The pattern of RNA accumulation for these transcripts also confirms that the wild-type strain progressed through two cell cycles while the mutant only completed one cycle during the course of this analysis. RNA for the B-type cyclins, Clb3p and Clb4p, accumulates in S phase and remains high until anaphase (FITCH *et al.* 1992; RICHARDSON *et al.* 1992). The *cak1-1* cells showed a slight delay in the accumulation of *CLB3* RNA compared to wild-type cells (Figure 2C). *CLB3* RNA began to accumulate at 27 min after release from the α-factor block in wild-type cells, but not until 36 min after release in the mutant cells. RNA for Clb1p and Clb2p accumulates late in G_2, peaks in early M and decreases in anaphase (SURANA *et al.* 1991; FITCH *et al.* 1992; RICHARDSON *et al.* 1992). The *cak1-1* strain showed a prolonged delay in the accumulation of *CLB2* RNA. The wild-type strain achieved maximal levels of *CLB2* RNA within 54 min after α-factor release, while *cak1-1* cells obtained maximal levels only after 108 min (Figure 2C). These results demonstrate that while the *cak1-1* mutant is unaffected in the timing of the G_1 to S transition, and is only slightly delayed in the rate of accumulation of *CLB3* RNA in S phase, it is very defective in the rate of accumulation of *CLB2* transcripts. The *cak1-1* mutation also causes a defect in the maximal level of *CLB2* RNA; wild-type cells showed higher levels of *CLB2* RNA than did mutant cells in this and other analyses (Figure 2C and data not shown). The pattern of accumulation of *CLB1* RNA paralleled that of *CLB2* RNA in these analyses (data not shown). The kinetics of accumulation and disappearance of RNA for *RNR1* was almost the same in the wild-type and *cak1-1* mutant cells, indicating that the apparent delay in G_2 in the *cak1-1* cells did not result from a loss of cell cycle synchrony of this strain upon release from α-factor. While *RNR1* RNA declined at about the same rate in the mutant and wild-type cells, *CLN2* RNA declined more slowly in the *cak1-1* cells. This observation is consistent with the finding that Clb2p is required for the repression of *CLN2* but not of *RNR1* transcription (AMON *et al.* 1993). The results from this analysis therefore demonstrate that the delay in progression through the cell cycle caused by the *cak1-1* mutation occurs primarily in late G_2, at or near the time at which *CLB1* and *CLB2* RNA begin to accumulate. This result is consistent with a role for Cak1p in the activating phosphorylation of Clb1,2p/Cdc28p for the G_2 to M transition. The transcription of *CLB1* and *CLB2* is dependent on a positive feedback loop, which requires activated Clb1,2p/Cdc28p for its function (AMON *et al.* 1993). The inability of *cak1-1* mutants to activate Cdc28p late in G_2 can therefore explain the reduced levels of *CLB2* RNA in this analysis as well as the delay in entry into M phase.

The experiment described above showed no effect of the *cak1-1* mutation on the timing of *CLN2* RNA accumulation. It is possible that lack of an effect on the timing of *CLN2* accumulation was an artifact of the method of synchrony used. Therefore, we repeated the Northern analysis using *cak1-1* and *CAK1* cells that were synchronized by release from a block in anaphase rather than by a block in late G_1. To do this, we constructed *cak1-1* and *CAK1* strains that carried a temperature-sensitive allele of the *CDC15* gene (*cdc15-2*). Both strains arrested synchronously as large budded cells after 3 hr at the nonpermissive temperature. Budding index and Northern analysis revealed that wild-type cells underwent two cell cycles while the *cak1* mutant progressed through less than one cycle following release from the *cdc15* block (Figure 2D). In this experiment, the *cak1-1* cells did exhibit a slight delay (~15 min) in the accumulation of *CLN2* RNA compared to *CAK1* cells, as well as a 15–20-min delay in the emergence of new buds at the G_1 to S transition (Figure 2D). This result suggests that the *cak1-1* mutation affects either the progression of cells out of M phase or progression through G_1. However, as in the α-factor experiment, the most severe defect was in the accumulation of *CLB2* RNA, which only begins to accumulate in the last time points of the experiment, 90 min after the time of maximal accumulation of *CLB2* RNA in the *CAK1* strain. This analysis confirms the conclusion that *cak1-1* mutations have the most profound effects on cell cycle progression at the G_2 to M transition.

The temperature-sensitive *cak1-22* strain is defective at multiple points in the cell cycle: As previously described (KALDIS *et al.* 1996), we used alanine scanning mutagenesis to create a temperature-sensitive *cak1* allele, *cak1-22.* The majority of cells in this mutant arrest at the nonpermissive temperature late in G_2 as large cells with an elongated bud and a short spindle spanning an undivided nucleus (KALDIS *et al.* 1996). Thus, our analyses of two different *cak1* mutations show clearly that Cak1p is required for activation of Cdc28p for the G_2 to M transition. We also examined the terminal phenotype of Δ*cak1* cells. To do this, we replaced ~60% of the sequences encoding the *CAK1* ORF with the *HIS3* gene by single-step gene replacement techniques using a diploid strain. Sporulation of the resulting heterozygous diploid gave rise to two large His^- colonies and two microcolonies of ~50–100 cells (presumed His^+). Analysis of the microcolonies revealed that 11% of the cells are unbudded, 50% are large budded, often with elongated buds, 27% have two buds and 12% have more than two buds and a filamentous morphology. Thus, as with the two *cak1* mutations we analyzed, loss of *cak1*

function by deletion has the most profound effect on progression through G_2 or M phase. However, although the majority (65%) of *cak1-22* cells arrest at G_2/M at the nonpermissive temperature, 30% of the cells arrest as large unbudded G_1 cells (KALDIS *et al.* 1996), suggesting that in these cells the activation of Cdc28p at G_1/S is also defective. If Cak1p is required to activate Cdc28p for the G_1 to S transition as well as for G_2 to M, then there might be genetic interactions between mutations in *CAK1* and mutations in genes encoding the G_1 cyclins. We have already shown that *cak1-22* mutations caused lethality at the permissive temperature when combined with Δ*clb2* (KALDIS *et al.* 1996), providing further evidence for a function of Cak1p in activation of the mitotic form of the Cdc28p kinase. The results of similar analyses combining the *cak1-22* mutation with deletions of the genes encoding the G_1 cyclins are shown in Figure 3A. Possibly because of the functional redundancy of the G_1 cyclins, no additional phenotypic defects were detected when we combined the *cak1-22* mutation with deletion of either *CLN1* or *CLN3* alone (data not shown). However, we did see genetic interactions when we crossed the *cak1-22* strain with a strain containing deletion of both *CLN1* and *CLN3* (Figure 3A). The triple mutant was barely able to grow at 33°, while the Δ*cln1,3* strain and the *cak1-22* strain grew nearly as well as wild type at this temperature (Figure 3A). Further, when we crossed the *cak1-22* strain with a strain containing deletion of *CLN2*, we found that the double mutant was nearly inviable at 30° (Figure 3B). These results strongly argue for a role of Cak1p in the activation of the G_1 cyclin/Cdc28p complexes. The growth defects caused by combining *cak1-22* with *cln* mutations are not as severe as that seen for the *cak1-22* Δ*clb2* strain (KALDIS *et al.* 1996), consistent with the fact that the *cak1* mutants we have analyzed are more defective in the G_2 to M transition than in the G_1 to S transition. That the *cak1-22* mutant is most defective in activation of the mitotic cyclin/Cdc28p kinase is further supported by the fact that while the temperature sensitivity of *cak1-22* strains is partially suppressed by expression of *CLB2* from the cell cycle-independent *ADH* promoter (KALDIS *et al.* 1996), expression of *CLN2* from this same promoter is unable to suppress the temperature sensitivity of *cak1-22* strains. Expression of *CLN2* from the *ADH* promoter was also unable to augment the growth rate of *cak1-22 ADH:CLB2* cells (data not shown).

Additional support for a role of Cak1p in the activation of G_1 cyclin/Cdc28p protein kinase complexes comes from the observation that *cak1* mutant cells are more sensitive to growth arrest by α-factor than are wild-type cells (Figure 3C). At the semipermissive temperature of 30°, the *cak1-22* strain showed strong growth inhibition by concentrations of α-factor that did not affect the growth of the isogenic wild-type strain (Figure 3C). This effect was not a result of the fact that the *cak1-22* strains grow more slowly than the wild-type strains; a Δ*clb2* strain, which has a growth rate similar to that of *cak1-22*, showed no increased sensitivity to α-factor (Figure 3C). The *cak1-1* strain is also more sensitive to α-factor than its isogenic parent (data not shown). Because α-factor arrests growth in G_1 through inhibition of the Cln1,2P/Cdc28p complexes (PETER *et al.*

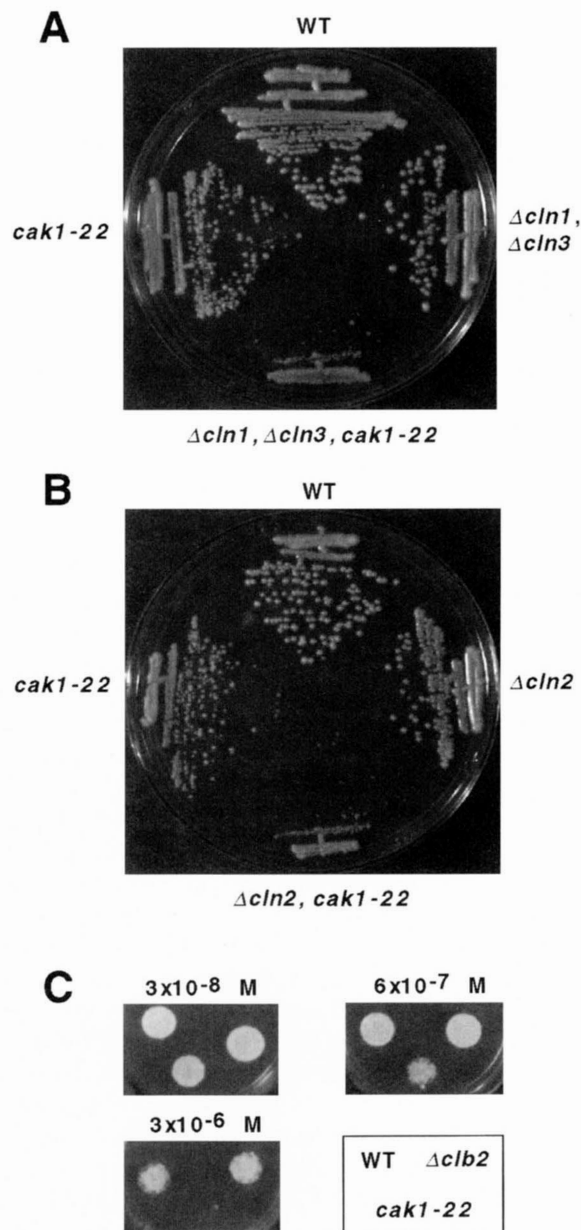

FIGURE 3.—*cak1* mutants are defective in Cln/Cdc28p function. (A) Wild-type (SY207), Δ*cln1* Δ*cln3* (SY263), Δ*cln1* Δ*cln3 cak1-22* (SY264), and *cak1-22* (SY208) strains were grown at 33° for 2 days on YPD. (B) Wild-type (SY80), Δ*cln2* (SY266), Δ*cln2 cak1-22* (SY267) and *cak1-22* (SY151) strains were grown at 30° for 2 days on YPD. (C) Wild-type (SY112), Δ*clb2* (SY1046) and *cak1-22* (SY132) strains were tested for sensitivity to growth arrest by α-factor by spotting 10 μl of an exponentially growing culture (3×10^3 cells) on YPD plates containing the indicated concentrations of α-factor. Plates were incubated at 30° for 40 hr.

1993; TYERS and FUTCHER 1993; PETER and HERSKOWITZ 1994a), this result suggests that these complexes are already functionally impaired in the *cak1* mutant backgrounds.

The *cak1-22* mutation does not affect the functions of other CDKs in budding yeast: Our previous biochemical analyses showed that Cak1p is required for activation of the Cdc28p protein kinase through phosphorylation of threonine 169 in its T-loop (KALDIS *et al.* 1996). The phenotypes of the *cak1* mutants we have analyzed can all be explained by defects in Cdc28p functions. However, *S. cerevisiae* has other CDKs, which are closely related to Cdc28p and which may also require an activating phosphorylation by Cak1p. One of these CDKs is Kin28p, which is 38% identical to Cdc28p and has a putative phosphorylatable threonine (threonine 162) in a position analogous to threonine 169 of Cdc28p (SIMON *et al.* 1986). Kin28p most closely resembles vertebrate Cdk7/MO15, and, like Cdk7/MO15, is a component of the transcription factor TFIIH (FEAVER *et al.* 1994), phosphorylates the CTD of the large subunit of RNA Polymerase II *in vitro* (FEAVER *et al.* 1994; CISMOWSKI *et al.* 1995) and functions in general transcription (CISMOWSKI *et al.* 1995; VALAY *et al.* 1995). Unlike Cdk7/MO15, however, Kin28p does not have *in vitro* CAK activity (FEAVER *et al.* 1994; CISMOWSKI *et al.* 1995). In *S. cerevisiae, CAK1* was recently identified by others as a multicopy suppressor of a *kin28* mutant, and *cak1* mutations cause lethality when combined with *kin28* mutations (VALAY *et al.* 1995; THURET *et al.* 1996). Therefore, Cak1p may be required for the activation of Kin28p for its role in transcription as well as for the activation of Cdc28p for its role in cell cycle progression. The phenotypes of the *cak1* mutants analyzed to date, as well as the terminal phenotype of strains deleted for *CAK1,* are heterogeneous and therefore do not rule out an additional role of Cak1p in transcription. If Cak1p is required to activate Kin28p for its function in general transcription, then *cak1* mutants should also be defective in general transcription. To test this, we shifted the *cak1-22* strain to the nonpermissive temperature and isolated RNA from fractions taken at different times after the shift. We have previously shown that activation of Cdc28p by Cak1p is severely diminished within 1 hr after shift of *cak1-22* strains to the nonpermissive temperature and that <10% of Cdc28p activity remains after 5 hr at nonpermissive temperature (KALDIS *et al.* 1996). The RNAs were examined by Northern analysis for steady-state levels of transcripts (Figure 4). In the case of *URA3, CDC28* and *ACT1* RNAs, little or no difference in abundance between *CAK1* and *cak1-22* strains was detected (Figure 4). In contrast, in a *kin28* mutant, these transcripts dropped to <25% of their normal steady-state levels within 1.5 hr after shift to nonpermissive temperature (CISMOWSKI *et al.* 1995). Only in the case of *CLB2* was there a decrease in RNA levels in the *cak1-22* strain after shift

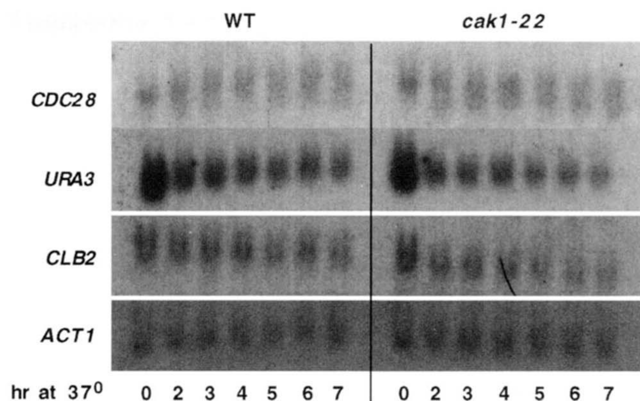

FIGURE 4.—Effect of *cak1* mutations on general transcription. Exponential cultures of wild-type (SY162) and *cak1-22* (SY132) strains were shifted to the nonpermissive temperature of 37° at $t = 0$. At the indicated times after shift, samples were collected, RNA was isolated and examined by Northern analysis with probes specific for the indicated genes. Each lane contained 5 μg of RNA.

to 37° (Figure 4). This effect on *CLB2* transcription is probably the indirect result of the fact that active Cdc28p is required for the maximal expression of *CLB2* (AMON *et al.* 1993). Our analysis of the pattern of RNA accumulation during the cell cycle in the *cak1-1* mutant (Figure 2C and D) also did not reveal any differences in the steady-state levels of RNAs examined, with the exception of *CLB2* RNA. Therefore, there is no evidence from analyses with two different *cak1* mutants to suggest that Cak1p is required for the activation of Kin28p for its role in transcription.

Another CDK in *S. cerevisiae* is Pho85p, which is >50% identical to Cdc28p (TOH-E *et al.* 1988) and has a serine in the site analogous to threonine 169 and a threonine just upstream. In the presence of high levels of inorganic phosphate, the Pho80p/Pho85p cyclin/CDK complex phosphorylates and inactivates the transcription factor Pho4p (HIRST *et al.* 1994; KAFFMAN *et al.* 1994). Inactivation of Pho4p prevents the expression of *PHO5,* which encodes the secreted acid phosphatase. If Cak1p is required to activate Pho80p/Pho85p, then inactivation of Cak1p should cause an increase in acid phosphatase levels. To test whether Cak1p is required for the activation of Pho85p, we examined the effect of a shift of *cak1-22* cells to nonpermissive temperature on the levels of acid phosphatase. Wild-type (SY162) and *cak1-22* (SY132) cells were grown in high phosphate medium (SC) at 24 or 37°. *cak1-22* cells grown either at 24° or 7 hr after shift to 37° had an activity of 1.1 relative to wild-type cells grown at 24°, wild-type cells grown for 7 hr at 37° had an activity of 0.95 relative to wild-type cells at 24°. A strain with a deletion of *PHO85* (SY258) grown at 30° had an activity of 7.3 relative to the wild-type cells in these assays. Therefore, under these conditions, inactivation of the *cak1-22* allele by shift to nonpermissive temperature appeared to have no sig-

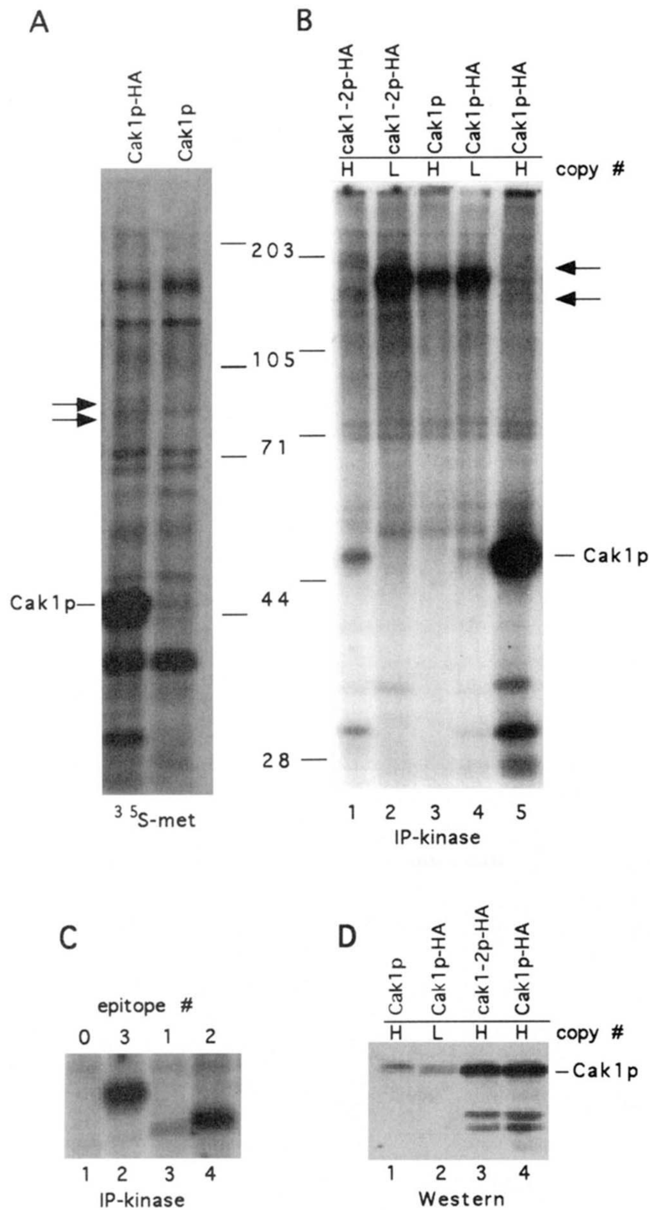

FIGURE 5.—Identification of Cak1p-associated proteins. (A) Extracts were prepared from a strain containing an HA-epitope tagged version of Cak1p expressed from the *GAL1* promoter (Cak1p-HA = SY213) and an isogenic strain in which Cak1p was not HA-tagged (Cak1p = SY50) that had been grown in synthetic minimal medium with galactose as carbon source in the presence of ^{35}S-methionine. Immunoprecipitations were done using the 12CA5 MAb directed against the HA epitope. Immunoprecipitates were subjected to electrophoresis through an 8% polyacrylamide gel, followed by fluorography. Arrows point to high molecular weight bands specific to the immunoprecipitate from the Cak1p-HA strain. Numbers refer to the approximate molecular mass (kD) of standards run on the same gel. (B) Extracts were prepared from the following strains: SY101 (*cak1-2-[HA]$_3$* in YEp24), SY123 (*cak1-2-[HA]$_3$* in YCp50), SY89 (*CAK1* in YEp24), SY86 (*CAK1-[HA]$_3$* in YCp50) and SY106 (*CAK1-[HA]$_3$* in YEp24). Following immunoprecipitation with the 12CA5 MAb, *in vitro* kinase assays were done, and the samples subjected to electrophoresis as in A and autoradiography. Arrows point to high molecular weight phosphoproteins specific to immunoprecipitates from strains expressing Cak1p-HA or cak1-2p-HA from the high copy number YEp24 plasmid. The heavily labeled band of ~170 kD detectable in lanes 2, 3 and 4 is not specific to the HA-tagged Cak1p samples. (C) Extracts were prepared from strains with either 0 (SY89), 3 (SY106), 1 (SY190) or 2 (SY191) tandem copies of the HA epitope at the carboxyl terminus of Cak1p. Samples were analyzed by immunoprecipitation and *in vitro* kinase assay as in B. Only the part of the autoradiograph with the different versions of Cak1p is shown. (D) Western analysis of extracts from strains used in B. The 12CA5 MAb was used to detect Cak1p-HA. A nonspecific protein migrates just above Cak1p-HA, this can be seen in lane 1 and also as the upper band of a doublet in lane 2.

nificant effect on the activity of Pho85p in the acid phosphatase pathway.

Cak1p autophosphorylates *in vitro* and associates with several high molecular weight proteins: Cak1p can function as a monomer *in vitro* (KALDIS *et al.* 1996), and the bulk of the CAK activity in *S. cerevisiae* elutes from gel filtration columns as a monomer (ESPINOZA *et al.* 1996; KALDIS *et al.* 1996; THURET *et al.* 1996). Nevertheless, it is possible that a fraction of Cak1p associates with regulatory subunits required for its *in vivo* function(s). We therefore used immunoprecipitation to test whether Cak1p associates with other proteins. In the first analysis, we examined Cak1p-associated proteins by immunoprecipitation of Cak1p from cells that had been incubated in the presence of ^{35}S-methionine. In this analysis, Cak1p, tagged at its carboxyl terminus with three tandem copies of the hemagglutinin antigen (HA) epitope (Cak1p-HA), was produced at high levels from the *GAL1* promoter. The results of this analysis are shown in Figure 5A. The heavily labeled band at 49 kD corresponds to HA-tagged Cak1p. Some, but not necessarily all, bands of faster mobility than Cak1p-HA may correspond to degradation products of Cak1p, as similar bands are seen in Western analysis of Cak1p-HA (see Figure 5D). Two higher molecular weight proteins of ~85 and 92 kD are specific to the sample in which the immunoprecipitation was from a strain containing HA-tagged Cak1p. The intensities of these bands are weak, suggesting that only a small amount of Cak1p-HA associates with these proteins. We were unable to detect these co-immunoprecipitating proteins when Cak1p-HA was expressed from its own promoter.

In a separate analysis, we examined the pattern of Cak1p-HA-associated proteins by immunoprecipitation of Cak1p-HA followed by an *in vitro* kinase assay (Figure 5B). Immunoprecipitates from a strain containing *CAK1-HA* on low copy number plasmid YCp50 showed a phosphorylated protein of 49 kD (Figure 5B, lane 4) that was not detected using a strain in which Cak1p was not tagged (Figure 5B, lane 3) and that increased in intensity when *CAK1-HA* was on high copy number plasmid YEp24 (Figure 5B, lane 5). The 49-kD band corresponds to phosphorylated Cak1p-HA as demonstrated by mobility shifts that result when an identical *in vitro* phosphorylation reaction is done using immunoprecipitates from cells in which Cak1p contains only a single

(Figure 5C, lane 3) or two (Figure 5C, lane 4) copies of the HA epitope. This result suggests that Cak1p-HA autophosphorylates *in vitro* or that Cak1p-HA coprecipitates with a protein kinase that phosphorylates it. We repeated the *in vitro* kinase assay using immunoprecipitates from a strain expressing a mutant version of Cak1p-HA (cak1-2p-HA). This protein contains a conservative change in which an invariant lysine in domain II (K31) is changed to arginine. This lysine is critical for the phosphotransfer reaction and mutations in this residue result in a loss of protein kinase activity in a variety of protein kinases (HANKS *et al.* 1988). The K31R mutation in Cak1p-HA severely reduced the amount of phosphorylation of cak1-2p-HA (Figure 5B, compare lane 1 to lane 5). Western analysis showed that the decrease in level of *in vitro* phosphorylation of cak1-2p-HA in this experiment did not result from a decrease in the amount of cak1-2p-HA in cells (Figure 5D, compare lanes 3 and 4). These results suggest that most of the phosphorylation of Cak1p-HA *in vitro* results from autophosphorylation rather than phosphorylation from a coprecipitating protein kinase. Interestingly, strains expressing the cak1-2p protein from either a low or high copy number plasmid as the only version of Cak1p in the cell have growth rates and phenotypes indistinguishable from wild-type strains. The analysis in Figure 5B also reveals several phosphoproteins in addition to Cak1p-HA that are specific to immunoprecipitates from strains containing HA-tagged Cak1p. The most abundant of these migrate faster than Cak1p-HA, and at least some of them may correspond to Cak1p-HA degradation products. A series of phosphoproteins in the molecular mass range of 150–200 kD can be detected in the immunoprecipitate of cak1-2p-HA (Figure 5B, lane 1) as well as in the immunoprecipitate of Cak1p-HA expressed from the high copy number plasmid (Figure 5B, lane 5). These proteins migrate just above and just below a major nonspecific phosphoprotein of variable intensity seen in all the samples and are not detected in the immunoprecipitates of cells in which Cak1p-HA is not tagged (Figure 5B, lane 3) and are clearly different from those detected by *in vivo* labeling of proteins with ^{35}S-methionine (Figure 5A). Whether these proteins are regulatory subunits for Cak1p or *in vivo* substrates of Cak1p or a co-immunoprecipitating protein kinase awaits further characterization.

***In vitro* Cak1p activity does not fluctuate during the cell cycle:** It is not known whether phosphorylation of Cdc28p at threonine 169 is regulated during the cell cycle. It has recently been shown that a mutant Cdc28 protein in which threonine 169 has been changed to glutamic acid (which may mimic constitutive phosphorylation) can bind to Clb2p, but not to Cln2p, and is unable to allow progression through G_1 (LIM *et al.* 1996). It has therefore been suggested that dephosphorylation of Cdc28p at threonine 169 may be required for association with G_1 cyclins prior to progression through Start (LIM *et al.* 1996). If so, then Cak1 protein levels and/or activity might decrease in early G_1, when Cdc28p must be dephosphorylated and increase in late G_1 when Cln1,2p/Cdc28p complexes are formed and must be activated. Cell cycle changes in Cak1 protein and activity have previously been examined in cells that were synchronized by α-factor arrest/release (ESPINOZA *et al.* 1996). The levels of Cak1 protein and activity did not change significantly during the course of this experiment. However, in this analysis, cells were only followed from late G_1 into M phase, and any differences in Cak1p levels or activity in late M or in early G_1 would have been missed. We therefore took two additional approaches to test for cell cycle-specific changes in Cak1p activity. In the first, we synchronized *cdc15* cells containing Cak1p-HA by an arrest/release regime similar to that described above. Samples were taken as the cells progressed synchronously out of M phase and through 1.5 cell cycles. We measured the levels of Cak1p in the cells by Western analysis and the levels of Cak1p activity by immunoprecipitation followed by an *in vitro* kinase assay with Cdk2 as substrate. We have previously shown that vertebrate Cdk2, as well as Cdc28p, is phosphorylated by Cak1p *in vitro*, and that the level of phosphorylation of Cdk2 *in vitro* reflects the amount of active Cak1p protein kinase (KALDIS *et al.* 1996). In this analysis, we were unable to detect any significant differences in the levels of Cak1 protein or in Cak1p protein kinase activity at different points during the cell cycle (Figure 6A). Because this analysis might have missed a decrease in Cak1p activity in early G_1, we repeated the analysis using cells that had been fractionated by centrifugal elutriation. This procedure, which separates cells on the basis of their volume, provides an enriched source of small, early G_1 cells. None of the fractions of unbudded cells, which ranged in mean cell volume from 17 fl to 28 fl (Figure 6B, fractions 1–8) showed significantly different levels of Cak1p protein kinase activity than budded cells (Figure 6B, fractions 10–11) or an asynchronous population of cells (Figure 6B, cycling).

DISCUSSION

Cak1p is required at multiple points in the cell cycle: Our analyses with two different *cak1* mutants suggest that the activation of Cdc28p for progression from G_2 to M is most sensitive to loss of CAK function in *S. cerevisiae*. However, we also present data which implicate Cak1p in activation of Cdc28p for the G_1 to S transition. Genetic interactions between *cak1* mutations and mutations in genes encoding the G_1 cyclins, α-factor sensitivity of *cak1* mutants, and the fact that 30% of *cak1-22* cells arrest as unbudded cells support a requirement for Cak1p in activation of Cdc28p for the G_1 to S transition. Further support comes from the finding that a temperature-sensitive allele of *CAK1* has recently been obtained

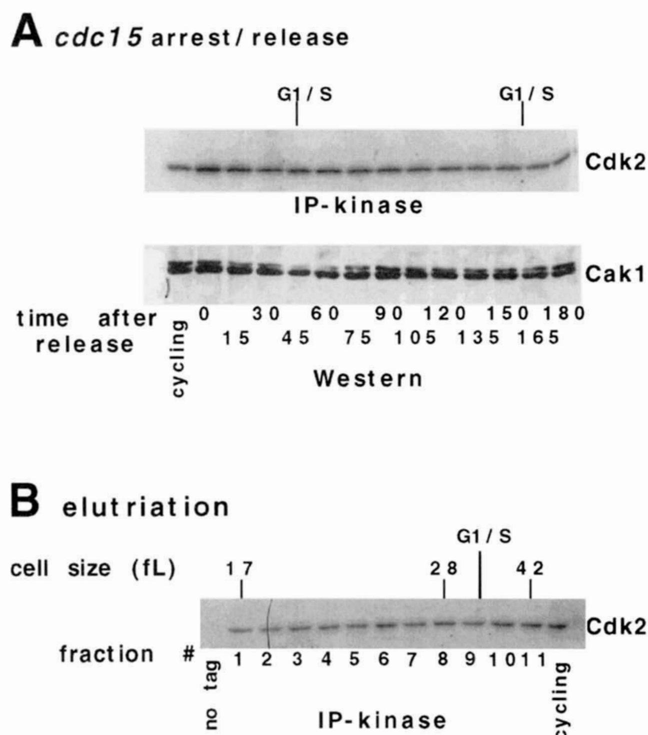

FIGURE 6.—Cell cycle analysis of Cak1p activity. (A) A *cdc15* strain with an HA-tagged version of Cak1p (SY326) was arrested in M by growth for 3.3 hr at 37°. Following release from the *cdc15* block by shift to 24°, samples were taken at the indicated times (min). Extracts were prepared and analyzed for *in vitro* kinase activity of immunoprecipitated Cak1p with Cdk2 as exogenous substrate and for Cak1p-HA protein levels by Western using the 12CA5 MAb. The upper band in the Western is not specific (see Figure 5D). The G_1 to S transitions (50% cells have new buds) occurred at 45 and 155 min. (B) A strain expressing Cak1p-HA (SY86) was grown in YP-ethanol and subjected to centrifugal elutriation. Cell extracts were prepared from fractions and analyzed by immunoprecipitation of Cak1p-HA followed by *in vitro* kinase assay with Cdk2 as substrate as in A. The average cell volume of each fraction was determined using a Coulter channelyzer. Cells in fractions 1–8 were largely unbudded, ~50% of the cells in fraction 9 were budded. The no tag sample was prepared from an exponential culture of strain SY80 (*CAK1* in YCp50).

(*civ1–4*) in which 70% of cells arrest in G_1 at the nonpermissive temperature (THURET *et al.* 1996). The differences between effects of our *cak1* mutations on cell cycle progression with those obtained with the *civ1–4* mutation may result from the nature of the specific alleles isolated or from differences in the strain backgrounds used.

FACS analyses of synchronous *cak1-1* mutants reproducibly show a broad S phase peak (Figure 2B) and a slight delay in the accumulation during S phase of *CLB3* RNA (Figure 2C). These results suggest that *cak1* mutants may be defective in S phase progression as well. The terminal phenotypes of cells containing deletion of *CAK1* are heterogeneous, as reported in this study and by others (ESPINOZA *et al.* 1996; THURET *et al.* 1996), but the majority of cells are either singly or multiply budded, often with a filamentous phenotype similar to that reported for strains in which the B-type cyclins (Clb1-6p), but not the G_1 cyclins, are inactivated (SCHWOB *et al.* 1994). The *in vitro* activity of both Cln2p/Cdc28p (DESHAIES and KIRSCHNER 1995) and Clb2p/Cdc28p (KALDIS *et al.* 1996) requires phosphorylation of threonine 169. We suspect that the *in vivo* activity of Cdc28p throughout the cell cycle also requires this phosphorylation and that Cak1p provides the only CAK activity required for the *in vivo* function of Cdc28p.

Does Cak1p have other functions in the cell in addition to activation of Cdc28p? *S. cerevisiae* has several protein kinases, including Kin28p and Pho85p, which are homologous to Cdc28p, depend on cyclins for activity and have serine or threonine residues in the site analogous to threonine 169 of Cdc28p. These protein kinases, like Cdc28p, may require phosphorylation at these sites for activation. We saw no effect of *cak1* mutations on the levels of RNAs whose steady-state levels depend on Kin28p function. It is possible that the *cak1-22* mutation specifically affects the ability of Cak1p to activate Cdc28p, but not its ability to activate Kin28p; however, another mutant allele of *CAK1*, *cak1-1*, also showed no defects in general transcription. However, if the turnover rate of phosphorylated Kin28p is slower than that of phosphorylated Cdc28p, effects of *cak1* mutations on Kin28p function would not be observed in our analysis. We also cannot rule out the possibility that while phosphorylation of Kin28p by Cak1p might be required for the transcription of some genes, unphosphorylated Kin28p is active for transcription of the genes measured in this analysis. *CAK1* on a high copy number plasmid was found to suppress the temperature sensitivity of a *kin28* mutant, and *cak1* mutations are lethal when combined with a *kin28* mutation (VALAY *et al.* 1995). While this could result if Cak1p is required for Kin28p function, other explanations are possible. For example, while *kin28* mutants are defective in transcription of many genes, they may be most sensitive to reduction in transcription of genes whose RNAs are very unstable. These would include the *CLN* and *CLB* RNAs. High levels of Cak1p could therefore suppress *kin28* mutants by providing more phosphorylated Cdc28p to combine with the few Cln or Clb molecules made in *kin28* cells.

We did see reduced transcription of *CLB2* in our analyses of the *cak1-1* and the *cak1-22* strains. The effect on *CLB2* transcription probably results from the fact that active Clb1,2p/Cdc28p is required for the maximal expression of *CLB2* (AMON *et al.* 1993). The difference between *CLB2* RNA levels in cycling *cak1-1* and wild-type cells (Figure 2C and D) appears greater than that between arrested *cak1-22* and wild-type cells (Figure 4). Part, if not all, of the discrepancy results from the fact that *CLB2* RNA levels in the *cak1-1* strain are compared

to synchronous, wild-type cells at G_2/M, which are expressing *CLB2* RNA to maximal levels; while the *CLB2* RNA levels for the *cak1-22* cells are compared to levels in asynchronous wild-type cells, which are considerably lower than peak levels. However, it is also possible that the *cak1-1* allele, although not a conditional mutation, is more defective in activation Cdc28p (and therefore indirectly in *CLB2* transcription) than the temperature-sensitive *cak1-22* allele. This scenario would be possible if, for example, the temperature sensitivity of the *cak1-22* mutation results in part from defects in a Cak1p function not related to its phosphorylation of Cdc28p.

We detected no major effects of the *cak1-22* allele on the activity of the Pho80p/Pho85p cyclin/Cdk complex. Unless the *cak1-22* mutation is specific for the Cak1p-Cdc28p interaction, as postulated above, this results suggests that the Pho80p/Pho85p complex does not require activation by Cak1p. Pho85p also associates with additional cyclins, including Pcl1p (Hcs26p) (ESPINOZA *et al.* 1994) and Pcl2p (OrfDp) (MEASDAY *et al.* 1995) and the function of these cyclin/CDK complexes is for cell cycle control. It is therefore possible that these complexes, but not the Pho80p/Pho85p complex, require activation by Cak1p; effects of *cak1* mutations on the function of these complexes for cell cycle control would be obscured by the effects of *cak1* mutations on the function of the Cdc28p protein kinase.

In addition to its role in activation of Cdc28p for cell cycle progression, Cak1p also has an essential role in spore formation. *CAK1* was isolated as a dosage suppressor of a temperature-sensitive *smk1* strain (WAGNER *et al.* 1997). *SMK1* encodes a MAP kinase in yeast required for spore wall formation (KRISAK *et al.* 1994). Further, temperature-sensitive *cak1* mutants fail to form spores at the nonpermissive temperature (WAGNER *et al.* 1997). Whether the role of Cak1p in spore formation is via Cdc28p or via another substrate remains to be elucidated.

Cak1p-associated proteins: Cak1p is active when expressed from *Escherichia coli* (KALDIS *et al.* 1996), and the bulk of Cak1p activity fractionates as a monomer in yeast extracts (ESPINOZA *et al.* 1996; KALDIS *et al.* 1996; THURET *et al.* 1996). However, Cak1p may require regulatory subunits for its *in vivo* function, either for modulation of its activity, association with specific substrates or localization. We found that Cak1p associates with several high molecular weight proteins in immunoprecipitation experiments from cells metabolically labeled with ^{35}S-methionine. Furthermore, additional high molecular weight proteins are visible as phosphorylated proteins in *in vitro* kinase reactions of Cak1p immunoprecipitates. The phosphoproteins may be substrates of Cak1p or substrates of a Cak1p-associated protein kinase. The fact that the intensity of these phosphoproteins does not decrease when the *in vitro* kinase assay is done with a catalytically defective (but not inactive) Cak1p suggests either that these proteins associate more tightly with the mutant cak1-2 protein or that the proteins are phosphorylated by a co-immunoprecipitating kinase. Cdc28p has been shown to co-immunoprecipitate with Cak1p under certain conditions (THURET *et al.* 1996) and could therefore be responsible for the phosphorylation of these proteins. Our Cak1p immunopreciptiates do not have protein kinase activity toward histone H_1 (data not shown), suggesting that there is not much active Cdc28p present. However, sufficient activity may be present to phosphorylate proteins within the same complex. The Cak1p immunoprecipitates also show that Cak1p autophosphorylates *in vitro,* and we have also found that Cak1p is phosphorylated *in vivo* (data not shown). Whether the *in vivo* phosphorylation results from autophosphorylation is under investigation.

Cak1p and the Sit4p protein phosphatase: The genetic screen that identified Cak1p was designed in part to discover functions for the Sit4p protein phosphatase in addition to its role in the activation of the G_1 cyclins. Why *cak1-1,* which causes severe defects in progression from G_2 into M, results in lethality in the absence of Sit4p, which is required for progression from G_1 to S, is not obvious. Sit4p is required for the normal accumulation of RNA for the G_1 cyclins, Cln1p and Cln2p. We have presented in this paper evidence that Cak1p is required to activate the Cln/Cdc28p protein kinase as well as the Clb/Cdc28p protein kinase. In the *cak1-1 pGAL:SIT4* strain grown in glucose, there may not be enough active Cln/Cdc28p for cells to progress through the cell cycle. What argues against this model is that expression of *CLN2* from a *SIT4*-independent promoter is not able to suppress the lethality of the *cak1-1 pGAL:SIT4* strain. A second possibility is that Sit4p has additional important functions in the G_2 to M transition but that the *sit4* mutations we have studied mainly affect the G_1 to S role. Consistent with this possibility is the finding that under certain experimental conditions *sit4* mutants can be induced to undergo S phase and bud emergence. These mutants, however, appear to be blocked in cell cycle progression in G_2 or M (DOSEFF and ARNDT 1995). Perhaps Sit4p, like Cak1p, functions in a pathway at G_2 necessary for the formation of active Clb/Cdc28p protein kinase.

Cak1p activity during the cell cycle: We and others (ESPINOZA *et al.* 1996) have shown that Cak1p protein levels and *in vitro* protein kinase activity do not fluctuate during the cell cycle. The phosphorylation of Cdc28p at threonine 169 may be constitutive, with newly synthesized Cdc28 protein immediately phosphorylated by Cak1p. However, indirect evidence raises the possibility that the phosphorylation state of Cdc28p at threonine 169 is under cell cycle control (LIM *et al.* 1996). If so, this could result from cell cycle regulation of the association of Cak1p with Cdc28p, perhaps due to changes in protein localization. Alternatively, there may be cell cycle fluctuations in Cak1p activity *in vivo,* even though

we did not detect any *in vitro*. For example, Cak1p might associate during part of the cell cycle with inhibitors that were lost during immunoprecipitation and therefore did not affect *in vitro* activity. Finally, regulation of threonine 169 phosphorylation could result from changes in the activity of a protein phosphatase that dephosphorylates threonine 169. The *in vitro* activity of the vertebrate CAK, Cdk7/MO15, also shows no cell cycle-dependent changes. This constitutive *in vitro* CAK activity is the only major property that Cak1p of budding yeast has in common with the vertebrate CAK. The two protein kinases are very different in protein sequence, in their requirement for subunits for *in vitro* activity and in their involvement in general transcription. Whether a vertebrate CAK which more closely resembles Cak1p exists is currently under investigation.

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