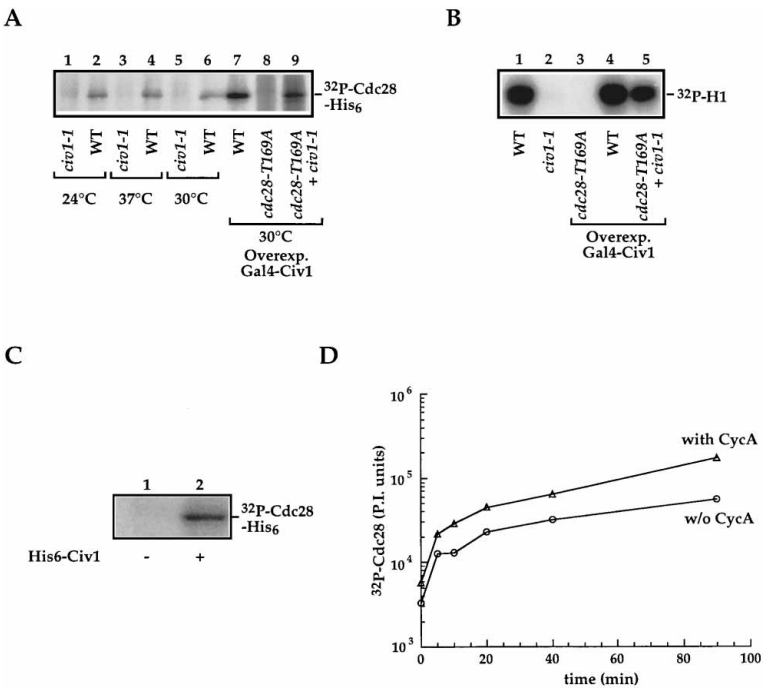

Figure 3. Cdc28–His_6 Phosphorylation and Activation In Vitro Is Dependent on a Wild-Type Civ1 Kinase and Kinetics of Cdc28 Phosphorylation by Civ1

(A) Phosphorylation of Cdc28–His_6. In lanes 1–6, Cdc28–His_6 purified on Ni–NTA–agarose as described in Experimental Procedures from CMY826 *CIV1*$^+$/pDYM1 (lanes 2, 4, and 6) and GF330-1C *civ1-1*/pDYM1 (lanes 1, 3, and 5) was tested for phosphorylation of Cdc28–His_6 by a possibly copurifying CAK at 24°C (lanes 1 and 2), 37°C (lanes 3 and 4), and 30°C (lanes 5 and 6). In lanes 7 and 8, Ni–NTA–agarose-purified fractions of Cdc28–His_6 from YJY88 (Gal4–Civ1, Cdc28–His_6; lane 7) and YJY95 (Gal4–Civ1, Cdc28[T169A]–His_6; lane 8) were tested for phosphorylation of Cdc28–His_6 at 30°C by the copurifying CAK. In lane 9, in vitro complementation: Ni–NTA–agarose-purified Cdc28–His_6 from GF312-17C *civ1-1*/pDYM1 and YJY95 *CIV1*$^+$ (Gal4–Civ1, Cdc28[T169A]–His_6) were mixed in equal amounts and tested for phosphorylation of Cdc28–His_6 at 30°C. We used 100 ng of Cdc28–His_6 or Cdc28(T169A)–His_6 for each test.

(B) Activation of Cdc28–His_6. The ability of Ni–NTA–agarose-purified Cdc28–His_6 to phosphorylate histone H1 after addition of cyclin A was assessed at 30°C as described in Experimental Procedures. Each assay contained 100 ng of Cdc28–His_6 or Cdc28 (T169A)–His_6 purified from yeast and 500 ng of human cyclin A purified from E. coli. Lane 1, Cdc28–His_6 purified from *CIV1*$^+$ (CMY826); lane 2, Cdc28–His_6 purified from *civ1-1* (GF330-1C); lane 3, Cdc28(T169A)–His_6 purified from a Gal4–Civ1 overexpressing strain (YJY95); lane 4, Cdc28–His_6 purified from the Gal4–Civ1 overexpressing strain (YJY88); lane 5 corresponds to lane 9 in (A): Cdc28–His_6 purified from the *civ1-1* strain GF312-17C is activated by Gal4–Civ1 copurified with Cdc28(T169A)–His_6 from strain YJY95.

(C) Substrate Cdc28–His_6 is in excess with regard to copurifying Civ1. We incubated 100 ng of Cdc28–His_6 purified from the strain CMY826/pDYM1 as in (A), lanes 2, 4, and 6, with [γ-^{32}P]ATP in the absence (lane 1) or the presence (lane 2) of 300 ng of Ni–NTA-purified His_6–Civ1 at 30°C for 15 min. Relative incorporation of ^{32}P onto Cdc28–His_6 in lane 1 was 40-fold less than that in lane 2, and hence cannot be seen in this figure.

(D) Kinetics of Cdc28–His_6 phosphorylation by His_6–Civ1 in conditions of excess substrate. We used 100 ng of Cdc28–His_6 purified from GF330-1C *civ1-1*/pDYM1, in the presence or absence of 200 ng of cyclin A, as substrate in a phosphorylation reaction with 0.6 ng of Ni–NTA-purified His_6–Civ1. His_6–Civ1 was added to the reaction mixes on ice, and aliquots were then distributed into separate tubes on ice. One tube was immediately placed in liquid nitrogen (0 min) and the other tubes were transferred to 30°C for the indicated times before freezing in liquid nitrogen. At the end of the time course, SDS sample buffer was added to the frozen samples, and these were boiled for 3 min. [^{32}P]Cdc28 levels were determined by SDS–PAGE and PhosphorImager analysis. ^{32}P incorporation is shown as relative PhosporImager (P.I.) units.

Cdc28–His_6 in the presence of cyclin A, and this activation was abolished by the Cdc28(T169A) mutation (Figure 5C). Further evidence that this CAK activity is coded for by the *CIV1* gene was obtained by showing that a His_6–Civ1(K31A) mutant, purified as for the wild-type protein, had less than 5% the activity of the wild-type Civ1 in vitro (C. Miled and C. M., unpublished data). This mutation affects a highly conserved lysine residue of the protein kinase family that is involved in binding ATP. Thus, monomeric Civ1–His_6 appears to be both necessary and sufficient for CAK activity in vitro toward both Cdc28–His_6 and GST–Cdk2.

Cdc28 Activity and Phosphorylation Are Inhibited In Vivo in the *civ1-4* Mutant

The preceding results indicated that Civ1 is both necessary and sufficient for in vitro CAK activity. We next sought to determine whether Civ1 is required for CAK activity in vivo. We found that Cdc28 kinase activity and overall phosphorylation of Cdc28 are reduced to 10%–20% of the wild-type levels at 24°C, 30°C, and 39°C in the *civ1-1* mutant (unpublished data). Thus, the *civ1-1* mutation appears to inactivate partially the CAK activity of Civ1 to an equal extent at all temperatures tested. We sought new conditional mutants of Civ1 to see whether temperature-sensitive growth would be correlated with a temperature-sensitive loss of CAK activity in vivo. The *CIV1* gene was polymerase chain reaction (PCR) amplified under mutagenic conditions, and the amplified DNA fragments were integrated at the chromosomal *CIV1* locus as described in Experimental Procedures. We screened for mutants that grew well at 24°C but not at 37°C. We chose the *civ1-4* mutant for further analysis because it arrested cell division within one cell cycle after transfer to 37°C. Furthermore, we found that *civ1-4* is synthetically lethal at 24°C when combined with the *cdc28-1* or *cdc28-4* mutants (see Experimental Procedures). Thus, partial inactivation of Civ1 at the permissive temperature of 24°C is lethal when Cdc28 activity is also reduced by the *cdc28-1* or *cdc28-4* temperature-sensitive mutations. These genetic interactions suggest that the two proteins functionally interact.

Flow cytometric analysis showed that 70% of *civ1-4* mutant cells arrested division at 37°C with a 1N DNA content and 30% with a 2N DNA content (Figure 6B). Microscopic analysis revealed that 75% of the *civ1-4*

reactions, human cyclin A purified from E. coli, or Civ1 purified from yeast, was added to the reaction mixtures as specified in the figure legends. Human cyclin A was prepared in E. coli as described previously (Pagano et al., 1992) except that partial purification was limited to ammonium sulfate precipitation (30% of saturation) of the soluble, abundant cyclin A. In tests for Cdc28–His_6 activation in vitro, the reaction buffer contained in addition 2 μg of histone H1 (Boehringer Mannheim). Phosphorylation by Civ1 of 500 ng of GST–Cdk2, purified from E. coli as described previously (Poon et al., 1993), was assayed using the same reaction conditions as for Cdc28.

Histone H1 Kinase Activity of Cdc28–HA Immunoprecipitated from *civ1-4* and Wild-Type Cells

We collected 25 ml of cells growing in YPD (GF312-17C wild type), CMY979 (*civ1-4 CDC28–HA*), and CMY951 (wild-type *CDC28–HA*) at 24°C or 37°C at an OD (600 nm) of 0.5. Cell extract preparation, Cdc28–HA immunoprecipitation, and immune complex kinase assays were carried out as described previously for Slt2–HA (Zarzov et al., 1996) except that histone H1 was used as the protein substrate.

In Vivo [^{33}P]Phosphate Labeling of Cdc28 in *civ1-4* and Wild-Type Cells

GF312-17C (wild type)/pDYM1, CMY979 (*civ1-4 CDC28–HA*), and CMY951 (wild-type *CDC28–HA*) cells were grown in YPD low phosphate medium at 24°C or 37°C to an OD (600 nm) of 0.5. We then centrifuged and resuspended 25 OD units of cells in 1 ml of YPD low phosphate medium. We then added 1 mCi of [^{33}P]orthophosphate (Amersham BF 1003), and cells were labeled for 1 hr. Cells were then centrifuged and resuspended in 0.3 ml of cold extraction buffer (25 mM Tris–HCl [pH 7.5], 5% glycerol, 10 mM EGTA, 1 mM dithiothreitol, 1 mM NaN_3) containing protease inhibitors (2 mg/ml each of leupeptin, aprotinin, chymostatin, pepstatin, and 1 mM PMSF) and phosphatase inhibitors (50 mM β-glycerophosphate, 15 mM p-NO2-phenylphosphate, 10 mM Na-orthovanadate). An equal volume of glass beads was added, and cells were broken by vortexing six times for 30 s with 15 s of cooling on ice between each vortexing. We then added 0.5 ml of IP buffer (50 mM Tris–HCl [pH 8.0], 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.3% sodium desoxycholate, 0.2% sodium dodecyl sulfate) containing protease and phosphatase inhibitors as above, and clarified the extract by centrifugation for 15 min at 4°C. Cdc28–HA was immunoprecipitated by adding 3 μl of polyclonal anti-Cdc28 antiserum and incubating for 1 hr at 4°C, followed by the addition of 25 μl of protein A–Sepharose beads and a further incubation of 1 hr at 4°C. The antigen-bound beads were then washed three times with IP buffer, transferred to a new Eppendorf tube, and then washed twice again with IP buffer. The washed beads were then resuspended in SDS–PAGE sample buffer and heated at 80°C for 4 min.

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Civ1 (CAK In Vivo), a Novel Cdk-Activating Kinase

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Summary

Cyclin-dependent protein kinases (Cdks) play key roles in regulating cell division and gene expression. Most Cdks require binding of a cyclin and phosphorylation by a Cdk-activating kinase (CAK) to be active. We report the identification of Civ1 (CAK in vivo), a novel CAK activity in S. cerevisiae. Civ1 is most similar in sequence to the Cdks, but unlike them is active as a monomer and may thus be the founding member of a novel family of kinases. Civ1 binds tightly to and phosphorylates Cdc28, thereby allowing its subsequent activation by the binding of a cyclin. The *CIV1* gene is essential for yeast cell viability, and Cdc28 phosphorylation and activity are conditionally inhibited in a *civ1-4* temperature-sensitive mutant. Civ1 is the only CAK for which there are genetic data indicating that its activity is physiologically relevant in vivo.

Introduction

Cyclin-dependent protein kinases (Cdks) were first identified as key regulators of the eukaryotic cell division cycle (Nurse, 1990; Murray and Hunt, 1993). Cdc28 of Saccharomyces cerevisiae and Cdc2 of Schizosaccharomyces pombe are the founding members of this family and are required at both the G1/S and G2/M transitions of the cell cycle (Forsburg and Nurse, 1991). In higher eukaryotes, the multiple functions of the yeast Cdc2/Cdc28 kinase are carried out by several related Cdks (Dorée and Galas, 1994; Pines, 1994; Nigg, 1995). More recently discovered members of the Cdk family have also been implicated in regulating gene expression and DNA repair (Poon and Hunter, 1995b). Cdks require for their activation, in addition to the binding of a cyclin molecule, their phosphorylation on a conserved threonine in a region called the T-loop (Morgan, 1995). The mechanism of this activation is not yet clear. Based on the crystal structure of Cdk2 bound to a fragment of cyclin A, phosphorylation of Cdk2 Thr-160 is postulated to cause the T-loop to become tethered to the carboxy-terminal lobe of the kinase in a conformation similar to that found in the corresponding region of the catalytically active cyclic AMP–dependent protein kinase (PKA) (Jeffrey et al., 1995). Purified PKA can activate itself by autophosphorylation on a threonine of its T-loop (Steinberg et al., 1993). In contrast, activation by phosphorylation of the Cdks is carried out by a distinct kinase activity that has been baptized CAK (Cdk-activating kinase) (Solomon et al., 1992; Solomon, 1994).

A CAK capable of phosphorylating and activating Cdc2 and Cdk2 has been isolated from Xenopus, starfish, and mammalian cells (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993; Darbon et al., 1994; Fisher and Morgan, 1994; Labbé et al., 1994; Wu et al., 1994). The catalytic subunit of this kinase was identified as p40-MO15 and later renamed cdk7 because it was found to be associated with a cyclin-like activating subunit called cyclin H (Fisher and Morgan, 1994; Mäkelä et al., 1994) as well as an assembly factor called MAT1 (Devault et al., 1995; Fisher et al., 1995; Tassan et al., 1995; Yee et al., 1995). Remarkably, cdk7–cyclin H was also found to be a component of the transcription factor complex called TFIIH (Roy et al., 1994; Serizawa et al., 1995; Shiekhattar et al., 1995). TFIIH is required for the basal transcription of genes by RNA polymerase II, and the cdk7–cyclin H kinase can phosphorylate the repeated sequences of the carboxy-terminal domain (CTD) of the large subunit of this polymerase. This unexpected activity of CAK could be an indication that cdk7–cyclin H coordinately regulates gene expression and cell cycle progression. However, it cannot be ruled out that the biochemical specificity of cdk7–cyclin H is more promiscuous in vitro than was initially anticipated and that its true biological function in vivo is more limited.

Given the high degree of functional conservation in eukaryotic cell cycle regulators, the identification of cdk7–cyclin H homologs in more genetically tractable organisms should allow a test of the in vivo functions of this kinase. Apparent homologs of cdk7–cyclin H have indeed been identified in the budding and fission yeasts. The Mop1(Crk1)–Mcs2 kinase of S. pombe is very similar in sequence to cdk7–cyclin H and has been shown in vitro to have both CAK and CTD kinase activity (Buck et al., 1995; Damagnez et al., 1995). The study of mutant forms of this kinase will be decisive in deciding whether it participates in Cdc2 phosphorylation and activation or TFIIH activity (or both) in vivo in the fission yeast. In S. cerevisiae, Kin28–Ccl1 is the apparent homolog of cdk7–cyclin H in terms of sequence similarity and by its presence in budding yeast TFIIH (Simon et al., 1986; Valay et al., 1993; Feaver et al., 1994; Cismowski et al., 1995; Valay et al., 1995). Kin28 immunoprecipitated from whole-cell extracts shows CTD kinase activity, but unlike Mop1(Crk1) has no CAK activity (Cismowski et al., 1995). Interestingly, *kin28-ts* mutants (Cismowski et al., 1995; Valay et al., 1995) and *ccl1-ts* mutants (Valay et al., 1996) are clearly defective at the restrictive temperature in the transcription of class II genes, and the large subunit of RNA polymerase II is hypophosphorylated in

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