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Fission yeast Csk1 is a CAK-activating kinase (CAKAK)

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Cell cycle progression is dependent on the sequential activity of cyclin-dependent kinases (CDKs). For full activity, CDKs require an activating phosphorylation of a conserved residue (corresponding to Thr160 in human CDK2) carried out by the CDK-activating kinase (CAK). Two distinct CAK kinases have been described: in budding yeast *Saccharomyces cerevisiae*, the Cak1/Civ1 kinase is responsible for CAK activity. In several other species including human, *Xenopus*, *Drosophila* and fission yeast *Schizosaccharomyces pombe*, CAK has been identified as a complex homologous to CDK7–cyclin H (Mcs6–Mcs2 in fission yeast). Here we identify the fission yeast Csk1 kinase as an *in vivo* activating kinase of the Mcs6–Mcs2 CAK defining Csk1 as a CAK-activating kinase (CAKAK).
Keywords: CAK/CAKAK/Csk1/Mcs2/Mcs6

Introduction

Cyclin-dependent kinases (CDKs) are a family of enzymes that initiate and coordinate cell cycle progression. The CDK alone is inactive and requires both association with a regulatory subunit and activating phosphorylation on a conserved residue (Thr160 in human CDK2) located on the 'T-loop' of the kinase to be fully active (for a review, see Morgan, 1997). In inactive, monomeric CDK molecules, the T-loop blocks the catalytic site, preventing substrate binding (De Bondt *et al*., 1993). The binding of the cyclin realigns the PSTAIRE motif helix but also moves the T-loop down towards the C-terminal lobe (Jeffrey *et al*., 1995; Russo *et al*., 1996). The Thr160 phosphorylation contributes to the activation process by stabilizing the T-loop in an active conformation through interactions of the phosphorylated threonine with several cationic residues (Russo *et al*., 1996).

The CDK-activating kinase or CAK was first described as a biochemically purified activity from vertebrate and invertebrate sources (Desai *et al*., 1992; Solomon *et al*., 1992). Subsequently, the $p40^{MO15}$ serine–threonine kinase (Shuttleworth *et al*., 1990) was identified as the catalytic activity in CAK (Fesquet *et al*., 1993; Poon *et al*., 1993; Solomon *et al*., 1993). $p40^{MO15}$ was found to function in complex with cyclin H (Fisher and Morgan, 1994; Mäkelä *et al*., 1994) and assembly factor MAT1 (Devault *et al*., 1995; Fisher *et al*., 1995; Tassan *et al*., 1995) and was renamed CDK7. MAT1 has now also been implicated in substrate recognition (Inamoto *et al*., 1997; Ko *et al*., 1997; Rossignol *et al*., 1997; Yankulov and Bentley, 1997).

The CDK7–cyclin H complex not only has CAK activity but is also part of the TFIIH transcription factor complex where it regulates the activity of RNA polymerase II by phosphorylation of the C-terminal domain (CTD) (Roy *et al*., 1994; Mäkelä *et al*., 1995; Serizawa *et al*., 1995; Shiekhattar *et al*., 1995). Budding yeast *Saccharomyces cerevisiae* has a complex closely related to CDK7–cyclin H–MAT1 and consists of the kinase Kin28, cyclin Ccl1 and Tfb3/Rig2 (Simon *et al*., 1986; Valay *et al*., 1993; Faye *et al*., 1997; Feaver *et al*., 1997). This complex was also identified as part of TFIIH (Feaver *et al*., 1994) and is a CTD kinase important for transcription of most RNA pol II transcripts (Valay *et al*., 1995; Hengartner *et al*., 1998; Lee and Lis, 1998; McNeil *et al*., 1998). However, in contrast to the CDK7 complex, Kin28 is not a CAK (Cismowski *et al*., 1995; Valay *et al*., 1995). Instead, the budding yeast has a distinct single subunit kinase Cak1/Civ1 that activates Cdc28 (and possibly other CDKs) *in vivo*, and is unrelated to CDK7/Kin28 (Espinoza *et al*., 1996; Kaldis *et al*., 1996; Thuret *et al*., 1996).

The appearance of the CDK7–cyclin H–MAT1 in TFIIH and the identification of Cak1/Civ1 raised the question of whether CDK7–cyclin H–MAT1 is an *in vivo* CAK. Two lines of experimentation recently have addressed this question. Using cycling *Xenopus* egg extracts, it was demonstrated that immunodepletion of CDK7 suppressed CAK activity and inhibited entry into M-phase (Fesquet *et al*., 1997). The CAK activity was restored by translation of CDK7 and its associated subunits, demonstrating that a CDK7 complex is necessary for activation of CDK–cyclin complexes. Using both temperature-sensitive and null alleles of the *Drosophila* CDK7 gene, Larochelle *et al.* (1998) showed that CDK7 is necessary for CAK activity of cdc2–cyclin B and cdc2–cyclin A *in vivo* in a multicellular organism.

The homologue of CDK7–cyclin H in fission yeast *Schizosaccharomyces pombe* is Mcs6–Mcs2. This complex is similar to the CDK7 complex in that it has both CTD and CAK activities *in vitro* (Buck *et al*., 1995; Damagnez *et al*., 1995). Both *mcs2* and *mcs6* were isolated originally as potential mitotic inducers in a screen for suppressors of 'mitotic catastrophe', or premature entry into mitosis resulting from elevated, unregulated cdc2 activity (Molz

et al., 1989; Molz and Beach, 1993). Moreover, the alleles isolated during the screen (*mcs6-13* and *mcs2-75*) display allele-specific interactions with *cdc2-3w*, reminiscent of the range of interactions described between *cdc2* and *cdc13* (Booher and Beach, 1987; Molz *et al*., 1989). The similarities of CDK7 and Mcs6 are demonstrated by the ability of CDK7 to replace Mcs6 as well as by the ability of Mcs2 and Mcs6 to associate with CDK7 and cyclin H, respectively (Damagnez *et al*., 1995). In these respects, the mammalian CDK7 and fission yeast Mcs6 appear to be structural and functional homologues, whereas the budding yeast Kin28 kinase is clearly distinct in both its inability to function as a CAK and its inability to replace Mcs6 (Buck *et al*., 1995).

While Mcs6 and CDK7 are CAKs, they are also CDKs, and, indeed similarly to most CDKs, contain a potential activating phosphorylation site in the 'T-loop' (T170 in human CDK7; T176 in *Xenopus* CDK7; S165 in Mcs6). A second potential phosphorylation site on the T-loop is located close by on the N-terminal side (S164 in human CDK7; S170 in *Xenopus* CDK7; T160 in Mcs6). The functional significance of either of these sites has been questioned, as in the presence of MAT1 an unphosphorylated CDK7 can associate with cyclin H to form an active complex (Fisher *et al*., 1995). On the other hand, both sites are phosphorylated *in vivo* (Labbe *et al*., 1994). In addition, in the absence of MAT1, CDK7 requires T170 (or T176 in *Xenopus*) phosphorylation for cyclin H binding (Fisher and Morgan, 1994; Mäkelä *et al*., 1994) and full activity (Labbe *et al*., 1994; Martinez *et al*., 1997; our unpublished results), and indeed biochemical purification of CDK7 complexes from cells has revealed dimeric complexes not containing MAT1 (Devault *et al*., 1995).

The kinase responsible for the activating phosphorylation of the CDK7 CAK (CAK-activating kinase or CAKAK) has not been identified *in vivo*. Although both CDK2–cyclin A and cdc2–cyclin B readily catalyse dual phosphorylation of CDK7 T-loop *in vitro* (Fisher *et al*., 1995; Martinez *et al*., 1997), the CDK7 phosphorylation and activation does not correlate well with CDK2- or cdc2-associated kinase activity (Martinez *et al*., 1997), suggesting the existence of other CAKAK(s).

Here the *S.pombe* Csk1 kinase is identified as an *in vivo* activating kinase of the Mcs6–Mcs2 CAK. The *csk1* gene was isolated as a multicopy suppressor of the synthetic lethality of a *mcs2-75 cdc2-3w cdc25-22* triple mutant strain (used to clone *mcs2*; Molz and Beach, 1993). *csk1* displayed a specific genetic interaction with *mcs2*, but unlike *mcs2* is not as essential gene. Here this genetic interaction is extended to reflect a functional *in vivo* interaction of *csk1*, *mcs2* and *mcs6*.

Results

Synthetic lethality of mcs6-13 when combined with csk1::sup3-5

As *csk1* has been isolated as an efficient suppressor of the *mcs2-75* allele (Molz and Beach, 1993), it was interesting to study genetic interactions of *csk1* with *mcs6*, which encodes for the CDK associated with the Mcs2 cyclin. For this purpose, a strain with a *csk1* deletion (*csk1::sup3-5*; Molz and Beach, 1993) and a strain with the *mcs6-13* (Molz *et al*., 1989) allele were used. The *mcs6-13* allele has been isolated as an extragenic suppressor of the mitotic catastrophe phenotype caused by the combination of an activated allele of *cdc2* (*cdc2-3w*) and a recessive temperature-sensitive *wee1* mutation (*wee1-50*; Molz *et al*., 1989).

When these strains were grown on rich medium plates at 30 or 37°C, the *mcs6-13* conferred no phenotype, whereas the *csk1::sup3-5* displayed a weak thermosensitive phenotype (Figure 1A). The same phenotype was observed in a strain with a *URA4+* disruption of *csk1* (data not shown). Combining *mcs6-13* and *csk1::sup3-5* led to a synthetic lethality on rich medium at 37°C (Figure 1A). To analyse the phenotype of the double mutant cells, they were grown in minimal media and stained with calcofluor and 4′,6-diamidino-2-phenylindole (DAPI) 20 h after a temperature shift (Figure 1C). The cells appeared highly elongated, with fragmented chromatin, multiple division septa and aberrant calcofluor staining of cell wall material at the ends of the cells. Overexpression of *csk1* fully rescues the phenotype of the double mutant strain at 37°C (Figure 1D). These results indicate that there is a strong genetic interaction between *csk1* and *mcs6*, as previously reported for *csk1* and *mcs2* (Molz and Beach, 1993). The conditional phenotype of the *mcs6-13 csk1::sup3-5* strain is somewhat reminiscent of those observed with spores harbouring either a *mcs2* or a *mcs6* deletion (Molz and Beach, 1993; Buck *et al*., 1995).

During these studies, it was observed that the *csk1* disruption (both the *csk1::sup3-5* and a *csk1::URA4+* strain) did not grow as well as a wild-type strain on minimal media, although no significant difference was observed on rich media. The poor growth was associated with the aberrant calcofluor staining observed in the synthetic lethal strain, but the other abnormalities of the synthetic lethal strain were not observed, as shown for *csk1::URA4+* in Figure 1F. In addition, when stationary phase wild-type or *csk1::URA4+* cells were diluted into fresh medium, a marked delay in the re-entry of the *csk1::URA4+* strain into the cell cycle was observed (Figure 1G). This could reflect a delay in the re-activation of the inactive and unphosphorylated Cdc2 kinase characteristic of stationary phase (Simanis and Nurse, 1986).

Regulation of Mcs6–Mcs2 kinase activity in vivo by Csk1

The genetic interactions between *csk1*, *mcs6* and *mcs2* suggested that Csk1 may regulate the Mcs6–Mcs2 complex. Indeed, in a previous study, the Mcs2-associated kinase activity was found to decrease 3-fold in a *csk1* deletion strain (*csk1::sup3-5*) when compared with a wild-type strain using myelin basic protein (MBP) as a substrate (Molz and Beach, 1993). In that study, strains where the endogenous *mcs2* was replaced with an allele encoding a hemagglutinin (HA)-tagged Mcs2 (Molz and Beach, 1993) were used. Subsequently, Mcs6 was identified as the Mcs2-associated kinase, new substrates were identified (Buck *et al*., 1995; Damagnez *et al*., 1995) and the specific activity of Mcs6–Mcs2 toward the RNA Pol II CTD was found to be several orders of magnitude higher than toward MBP (data not shown). Therefore, using the strategy described above, the CTD kinase activity of Mcs6–Mcs2 in a wild-type strain (with Mcs2-HA) and in an isogenic strain with a *csk1* disruption were compared

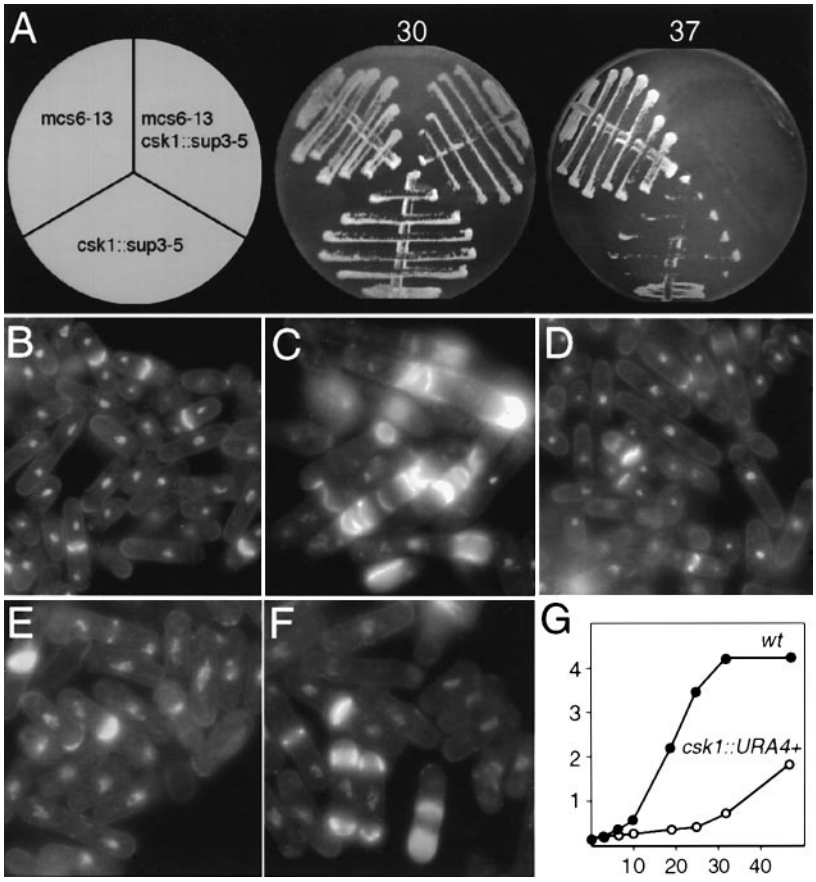

Fig. 1. *csk1::sup3-5* is synthetic lethal with *mcs6-13*. The potential genetic interaction of *csk1* and *mcs6* was tested by crossing *mcs6-13* with a *csk1* disruptant strain *csk1::sup3-5*. In order to analyse the phenotypes, all three mutant strains have been streaked on YES media at either 30 or 37°C (**A**). Separately, cells from the *mcs6-13 csk1::sup3-5* strain (**B** and **C**) or the same strain transformed with *csk1*-pREP3 (**D**) were fixed with glutaraldehyde and stained with DAPI and calcofluor. The cells analysed in (B) were grown at 30°C; the cells in (C) and (D) were analysed 20 h after a shift to 37°C. To analyse further the *csk1* disruption phenotype, a *csk1::URA4+* strain was grown in YES (**E**) or EMM minimal media (**F**), and cells were stained with DAPI and calcofluor. Growth rates of the strain on YES and EMM were measured after inoculation from a culture arrested in stationary phase (**G**).

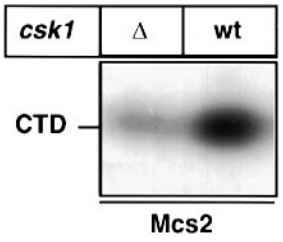

Fig. 2. Mcs2-associated CTD kinase activity is regulated *in vivo* by Csk1. To analyse Mcs2-associated kinase activity, Mcs2 was immunoprecipitated from *S.pombe* cells with an integrated *mcs2*-HA allele (wt) or an isogenic strain with a *csk1* disruption (Δ) using 12CA5 anti-HA monoclonal antibodies. The activity of the kinase toward a triple-CTD peptide was tested in an *in vitro* kinase reaction followed by SDS–PAGE and autoradiography.

(Figure 2). A 3-fold decrease in CTD activity in the *csk1* disruption strain indicates that Csk1 regulates Mcs6–Mcs2 *in vivo*, and is in agreement with previous results with MBP (Molz and Beach, 1993).

Csk1 phosphorylates Mcs6 on the potential CDK activation site S165

The genetic interactions of *csk1* with *mcs6* and *mcs2* and the regulation of Mcs6–Mcs2 activity by Csk1 suggested that it acts upstream of Mcs6–Mcs2. Thus, one possible function of Csk1 could be direct activation of the Mcs6–Mcs2 complex through phosphorylation of Mcs6 S165 (corresponding to the activation site T170 in human CDK7). To investigate this, myc epitope-tagged Mcs6 or Mcs6-S165A proteins were purified from *S.pombe* cells and tested as substrates for Csk1 purified from *S.pombe* cells overexpressing an HA epitope-tagged Csk1 (Figure 3). Following the kinase reaction, the indicated samples were separated on SDS–PAGE, transferred to a nitrocellulose membrane and analysed by autoradiography. A single phosphorylated band was seen in the wild-type Mcs6-containing lane (Figure 3, top panel). This band exactly co-migrated with the Mcs6 protein as demonstrated by anti-myc Western blotting analysis from the same membrane (Figure 3, bottom panel). No phosphorylation of Mcs6-S165A was observed, although the protein levels were comparable. These results indicate that Csk1 phosphorylates Mcs6 on the apparent CDK activation site.

Csk1 phosphorylates CDKs as a single subunit kinase

Csk1 purified from *S.pombe* cells phosphorylated Mcs6 efficiently. To determine whether a recombinant Csk1 is functional, a baculovirus-expressed GST–Csk1 fusion protein was purified and tested for its ability to phos-

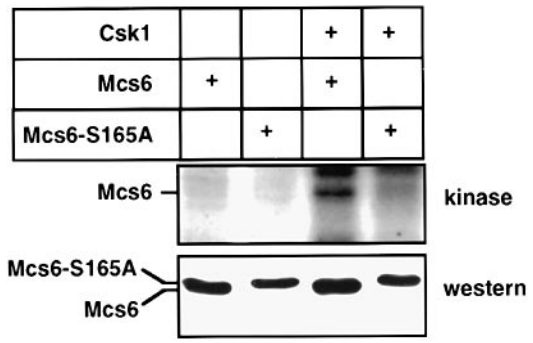

Fig. 3. Csk1 phosphorylates Mcs6 on the potential activation site S165. Top panel: Mcs6 or Mcs6-S165A proteins were tested as substrates for the Csk1 kinase. Mcs6 or Mcs6-S165A were purified from *S.pombe* cells overexpressing the corresponding myc-tagged proteins using 9E10 anti-myc monoclonal antibodies. Csk1 was purified from *S.pombe* cells overexpressing HA-tagged Csk1 using 12CA5 anti-HA monoclonal antibodies. The lanes without Csk1 contained a control purification from cells not expressing HA-tagged Csk1. Following a kinase reaction, products were analysed by SDS–PAGE, transferred to a nitrocellulose membrane and subjected to autoradiography. Bottom panel: the nitrocellulose membrane was used for Western blotting analysis with the 9E10 anti-myc monoclonal antibodies to visualize the myc-tagged Mcs6 or Mcs6-S165A proteins.

phorylate Mcs6 (Figure 4A). In this case, the Mcs6 was purified from insect cells co-infected with *mcs6* and *mcs2* baculoviruses using anti-Mcs6 antiserum, and therefore contains Mcs6 complexed with Mcs2 (see below). As demonstrated in the right lane in Figure 4A, Mcs6 is phosphorylated efficiently by the recombinant GST–Csk1 in these conditions. In parallel experiments, GST–Csk1 was also found to phosphorylate a human CDK7 (complexed with cyclin H; Figure 4B) and GST–CDK2 (Figure 4C), but not an RNA Pol II CTD substrate (Figure 4D). These results indicate that Csk1 phosphorylates Mcs6 and other CDKs as a single subunit kinase. As with Mcs6 (see above), the Csk1-mediated CDK2 phosphorylation was on the T-loop activation site T160 (Figure 4C), and was found to activate a CDK2–cyclin A complex (Figure 4E). A bacterially produced GST–Csk1 also retains CDK-phosphorylating activity (data not shown), indicating that Csk1 does not require kinases or binding partners to be active.

Csk1 is a CDK-activating kinase for Mcs6–Mcs2

As shown in Figure 3, Csk1 phosphorylates Mcs6 on the apparent CDK activation site. To investigate whether this phosphorylation indeed activates the Mcs6–Mcs2 complex, insect cells were infected with *mcs6* or *mcs2*, or double-infected with both *mcs6* and *mcs2*. The corresponding proteins were then immunopurified with either anti-Mcs2 or anti-Mcs6 antisera and tested in a Csk1 activation assay (Figure 5A). The assay consisted of an initial kinase reaction where the *mcs2* or *mcs6* immunoprecipitates were incubated with unlabelled ATP and either GST–Csk1 or GST. Subsequently, GST–Csk1 or GST were removed with three washes, and the remaining Mcs2 or Mcs6 complexes were tested for either GST–CTD or GST–CDK2 activity. In anti-Mcs2 complexes, activity was only detected in the Mcs6–Mcs2 co-infection, and this activity was significantly increased by Csk1 activation (Figure 5A, top panel). Similarly, anti-Mcs6 complexes had activity in the Mcs6–Mcs2 co-infection, and an

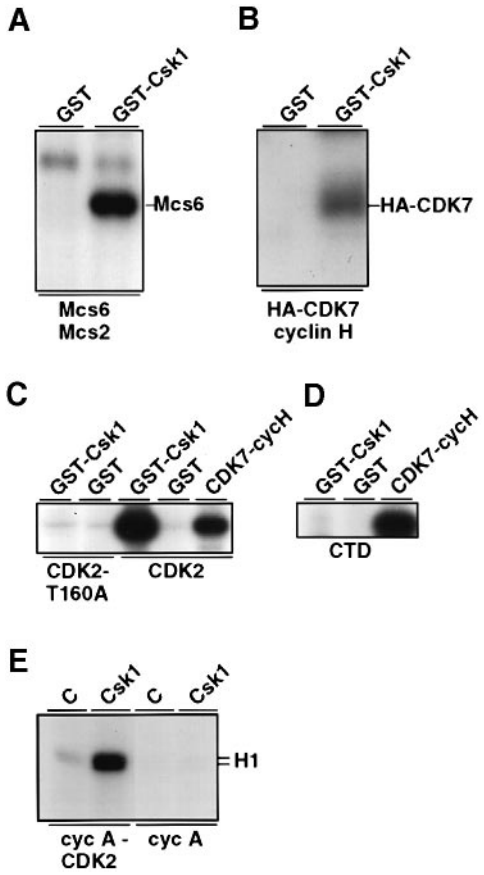

Fig. 4. Csk1 phosphorylates CDKs as a single subunit enzyme. Baculovirus-expressed recombinant GST–Csk1 (see Materials and methods) was assayed in a kinase reaction with: baculovirus Mcs6–Mcs2 purified with an anti-Mcs6 antiserum (**A**); baculovirus HA-tagged CDK7–cyclin H purified with anti-HA monoclonal antibodies (**B**); GST–CTD (**C**); and GST–CDK2 or the GST–CDK2 T160A mutant produced and purified from *Escherichia coli* (**D**). In order to show activation of the CDK2–cyclin A complex by Csk1, immunopurified CDK2 from rabbit reticulocyte lysate was associated with recombinant cyclin A, and phosphorylated by Csk1 produced in rabbit reticulocyte lysate (or control lysate). Subsequently, CDK2–cyclin A complexes were re-purified and assayed for histone H1 activity (**E**).

increase in the activity of Mcs6–Mcs2 toward GST–CTD after Csk1 pre-activation was observed (figure 5A, middle panel). Quantitation of the Csk1-induced increase in both anti-Mcs2 and anti-Mcs6 complexes indicated a 3-fold activation (data not shown). The activity of the Mcs6–Mcs2 complex toward GST–CDK2 was consistently weaker than toward GST–CTD. However, pre-activation with Csk1 significantly increased the GST–CDK2 activity of the Mcs6–Mcs2 complex (Figure 5A, bottom panel; in lanes with GST–Csk1 a low background phosphorylation of GST–CDK2 was observed). The slightly higher activity of the Mcs6–Mcs2 complex in anti-Mcs6 purifications at least partially reflects the lower affinity of the Mcs2 polyclonal antiserum toward the Mcs6–Mcs2 complex, as verified by Western blotting analysis (data not shown).

Csk1 phosphorylates and activates cyclin-free Mcs6

In addition to phosphorylating and activating Mcs6 complexed to Mcs2, Mcs6 purified from the *mcs6* single infections was active toward both GST–CTD and GST–CDK2,

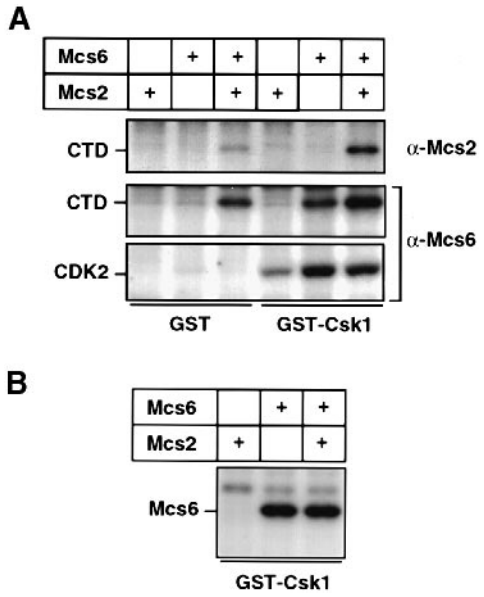

Fig. 5. Csk1 phosphorylates and activates both Mcs6 and the Mcs6–Mcs2 complex *in vitro*. In order to test the ability of GST–Csk1 to phosphorylate and activate Mcs6 and the Mcs6–Mcs2 complex, all three proteins were produced and purified from baculovirus-infected cells (see Materials and methods). (**A**) Immunoprecipitated Mcs6, Mcs2 or Mcs6–Mcs2 complex were activated by GST–Csk1 in the presence of ATP for 6 min and, after washing out the GST–Csk1 (or GST), activities were tested for either GST–CTD (top and middle panel) or GST–CDK2 (bottom panel) after immunoprecipitations using either anti-Mcs2 antibodies (upper panel) or anti-Mcs6 antibodies (middle and bottom panel). (**B**) To test Csk1-mediated phosphorylation of Mcs6 alone or in complex with Mcs2, kinase reactions using the indicated complexes were performed as indicated.

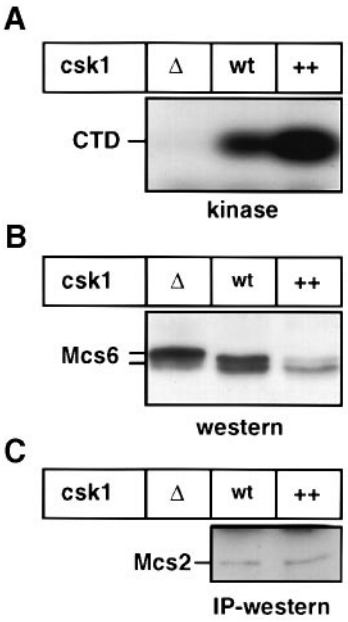

Fig. 6. Mcs6 activity and protein levels are directly controlled by Csk1 *in vivo*. Cell lysates were prepared from three strains all overexpressing a myc-Mcs6 fusion from the strong *nmt-1* promoter, and an integrated HA-mcs2, but with a different *csk1* status: disruption (Δ), wild-type (wt) or overexpression from the ADH promoter (++). (**A**) The indicated lysates were immunoprecipitated using anti-myc antibodies and tested for kinase activity towards a CTD peptide. (**B**) The lysates were immunoblotted for myc-Mcs6 using the same antibodies. (**C**) The lysates were immunoprecipitated using anti-myc antibodies and tested for the presence of Mcs6-associated HA-Mcs2 using anti HA antibodies.

indicating that Mcs6 was active without Mcs2. Interestingly, this activity was completely dependent on prior Csk1 activation (Figure 5A). Quantitation indicated that the activity of Csk1-activated Mcs6 alone was 3-fold weaker than that of a Csk1-activated Mcs6–Mcs2 complex on GST–CTD, whereas on GST–CDK2 Mcs6 alone was as active as the Mcs6–Mcs2 complex. This result also implied that the phosphorylation of Mcs6 by Csk1 is not dependent on Mcs2 binding. Indeed, when Csk1 was used to phosphorylate either Mcs6 or Mcs6–Mcs2, no difference in the efficiency of phosphorylation was observed (Figure 5B), indicating that Csk1 phosphorylates Mcs6 regardless of Mcs2 binding.

The cyclin-free Mcs6 was strongly dependent on Csk1 activation *in vitro* (see above). To analyse the effect of Csk1 on cyclin-free Mcs6 in *S.pombe* cells, a myc-tagged Mcs6 was overexpressed using the strong *nmt1* promoter in *S.pombe* cells with a *csk1* disruption (Δ), wild-type *csk1* (wt) or overexpressed *csk1* (++; Figure 6). Subsequently, anti-myc immunoprecipitates from these strains were analysed for CTD kinase activity (Figure 6A). In the cells with wild-type *csk1*, the CTD kinase activity was clearly higher than that seen previously for endogenous Mcs2 (see Figure 2), and thus shorter exposures are shown. The stronger activity is likely to reflect cyclin-free Mcs6 activity. Moreover, the regulation of the myc-Mcs6 activity by Csk1 is very pronounced; the activity in the disruptant is >30-fold decreased compared with wild-type, and the activity in the overexpression strain is 10-fold increased as determined by quantitation of incorporated radioactivity.

Western blotting analysis of myc-Mcs6 from total lysates from the strain wild-type for *csk1* (Figure 6B) indicates that myc-Mcs6 is a doublet as previously reported (Damagnez *et al*., 1995), although the separation of the two bands is somewhat dependent on gel running conditions (see, for example, Figure 3). A similar pattern is seen with several other CDKs, e.g. CDK7 (Martinez *et al*., 1997), where the faster migrating band represents CDK7 phosphorylated in the T-loop activation site. Accordingly, in Mcs6, the faster migrating band represents S165-phosphorylated Mcs6 based on the migration of the Mcs6-S165A mutant (Figures 3 and 7). In the *csk1* disruptant, only the unphosphorylated form is detectable. On the contrary, myc-Mcs6 in the Csk1-overexpressing lysate is mostly phosphorylated. These data indicate that Csk1 is the major if not only kinase phosphorylating Mcs6 on S165 *in vivo*. Another interesting observation from this experiment is that the level of the overexpressed Mcs6 in the lysates is inversely correlated with Csk1 levels, suggesting that the phosphorylated overexpressed Mcs6 is targeted for degradation. Despite the lower levels of Mcs6 in the Csk1-overexpressing strain, the amounts of Mcs2 associated were similar (Figure 6C), indicating that only a subset of Mcs6 is associated with Mcs2. This is not surprising, as the Mcs6 is overexpressed from the strong *nmt1* promoter, and thus the endogenous levels of Mcs2 are likely to be limiting.

Phosphorylation on the conserved CAK site of Mcs6 is not required to form a functional Mcs6–Mcs2 complex in vivo

Schizosaccharomyces pombe cells with a *csk1* disruption grow poorly on minimal media (Figure 7A). Mcs6 over-

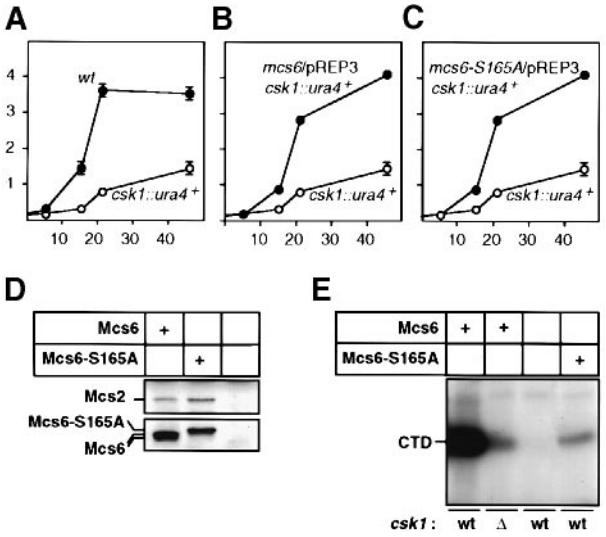

Fig. 7. Mcs6-S165A can associate with Mcs2 *in vivo* to form a functional complex. To test the ability of the Mcs6-S165A mutant to complement the growth retardation of a *csk1::ura4+* mutant (**A**), growth rates of *csk1* disruptant strains transformed with either myc epitope-tagged *mcs6*-pREP3 (**B**) or the *mcs6* S165A-pREP3 (**C**) were measured. (**D**) Anti-myc immunoprecipitates from strains with the *mcs6*-pREP3 or *mcs6* S165A-pREP3 plasmids and an HA-tagged integrated *mcs2* were analysed for the presence of *mcs2* with an anti-HA Western blot (upper panel). The levels of immunoprecipitated myc-Mcs6 or myc-Mcs6-S165A were then controlled using an anti-Myc Western blot (bottom panel). (**E**) The CTD kinase activity associated with myc-Mcs6-S165A in the strain described above or associated with myc-Mcs6 in a *Csk1::ura4+* strain with myc-epitope tagged *mcs6*-pREP3 was measured in an *in vitro* kinase as described in Figure 2.

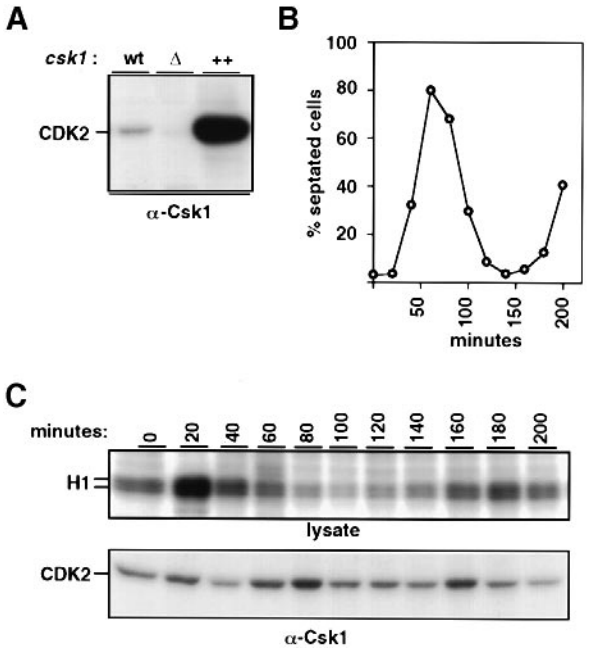

Fig. 8. Csk1 activity is constant throughout the cell cycle. (**A**) Csk1 polyclonal antiserum was tested by immunoprecipitation from lysates of three strains with different *csk1* status [wild-type (wt), disruption (Δ) and overexpression (++)] and subsequent kinase assay on GST–CDK2. (**B**) *cdc25-22* cells were synchronized by a temperature block-release and aliquots were taken every 20 min for the preparation of lysates. The percentage of septated cells is shown. (**C**) The lysates described above were used to monitor histone H1 activity directly (upper panel) or assayed for GST–CDK2 activity following Csk1 immunoprecipitations (bottom panel).

expression in this strain rescued this growth retardation phenotype completely (Figure 7B). This suggests that Mcs6 phosphorylation on S165 is not required when Mcs6 is overexpressed. Accordingly, the Mcs6-S165A mutant was indistinguishable from Mcs6 in rescuing the *csk1* disruption phenotype (Figure 7C), and indeed the S165A mutant of Mcs6 also rescued the synthetic lethality of the *mcs6-13 csk1::sup3-5* strain (data not shown).

The rescue by Mcs6-S165A presumably resulted from an active Mcs6-S165A–Mcs2 complex. To investigate the relative activities of Mcs6 and Mcs6-S165A, these proteins were expressed in *S.pombe* cells with a wild-type Csk1 to enable Mcs6 to become phosphorylated. As shown in Figure 7D, Mcs2 associated with both wild-type and mutant Mcs6 in a similar fashion. The kinase activity of the overexpressed Mcs6-S165A was low but detectable (Figure 7E), and indeed comparable with the kinase activity of overexpressed Mcs6 from the Csk1 disruptant (Figure 7E; a 30 h exposure is shown compared with the 3 h exposure in Figure 6A).

In conclusion, the characteristics of Mcs6-S165A *in vivo* are in agreement with several biochemical observations made with the apparent functional homologue CDK7 (Devault *et al*., 1995; Fisher *et al*., 1995; Martinez *et al*., 1997). Taken together, these results suggest that with either Mcs6 or CDK7, phosphorylation on the conserved CAK site is not required for cyclin binding or for activity. In CDK7, the alternative activation mechanism involves the assembly factor MAT1, and an analogous mechanism may exist in *S.pombe*.

Csk1 kinase activity is constant throughout the cell cycle

The total levels of Mcs2 and the activity of the Mcs2-associated kinase (presumably Mcs6) are constant throughout the cell cycle (Molz and Beach, 1993), which may not be surprising if this complex is involved in both CDK activation and RNA polymerase II CTD phosphorylation. To study Csk1 activity during the cell cycle, we analysed the kinase activity associated with a specific (Figure 8A) polyclonal Csk1 antiserum from lysates of synchronized (Figure 8B) *cdc25-22* cells (Figure 8C). As a control for the synchrony, histone H1 kinase activity from full lysates was performed from parallel samples (Figure 8C), demonstrating a first peak at 20 min following the temperature shift, indicating that the cells were entering mitosis from the arrest in G_2. No significant differences in the activity of Csk1 was observed at any point during the first cell cycle and up to the second mitosis (at 180 min). As neither Mcs6–Mcs2 (Molz and Beach, 1993) nor Csk1 activities are cell cycle regulated, the CAK phosphorylation on cdc2 may be a required, but not a regulated modification. In this light, it is quite surprising to identify an elaborate cascade of kinases fulfilling this function, and it may be that this pathway could be used in a regulatory fashion in, for example, response to stress or nutritional and environmental conditions.

Discussion

The *S.pombe csk1* gene was identified originally as a multicopy suppressor of a mutant allele of the *mcs2* cyclin,

and was suggested to encode the catalytic subunit of Mcs2 (Molz and Beach, 1993). Later, Mcs6 (originally termed Mop1/Crk1) was identified as the CDK partner of Mcs2, implying another function for Csk1. In this study, the functional basis for the observed genetic interaction of *csk1* with *mcs2* was investigated.

Initially, the genetic interaction of *csk1* with *mcs2* was extended to include *mcs6*. A synthetic lethal interaction between *csk1* disruption and *mcs6-13* was identified, with a terminal phenotype reminiscent of that seen with either *mcs6* or *mcs2* disruptions. The terminal phenotype includes highly elongated cells with multiple septa and fragmented nuclei. However, the multiple septa phenotype only appears after 18 h although the growth arrest following the temperature shift is very rapid and is accompanied by fragmented nuclei. Interestingly, the synthetic lethality was rescued not only by Csk1 or Mcs6 overexpression, but also by overexpression of the *S.cerevisiae* Cak1/Civ1 (T.Westerling, A.Pihlak, D.Hermand, J.-Y.Thuret, C.Mann and T.P.Mäkelä, in preparation). In contrast, the growth retardation phenotype in the *csk1* disruptant strain was rescued by Mcs6 overexpression, but not by Cak1/Civ1 overexpression (T.Westerling, A.Pihlak, D.Hermand, J.-Y.Thuret, C.Mann and T.P.Mäkelä, in preparation). This suggests that the *csk1* disruptant phenotype is due mainly to inefficient activation of Mcs6, indicating that phosphorylation of Mcs6 on the CAK site is not essential, but clearly beneficial for Mcs6, and that Csk1 is likely to be the major kinase phosphorylating Mcs6 *in vivo*.

Although *csk1* is not an essential gene, it is clearly implicated in the control of cell growth. A *csk1* disruptant is sensitive to medium composition, and the strain is defective in re-growth from stationary phase and presents a growth retardation. However, the only clear phenotype of the *csk1* disruptant with aberrant calcofluor staining of cell wall material at the ends of the cells is difficult to interpret with current knowledge, and could be either cell cycle or transcription related.

Mcs2-associated kinase activity was decreased 3-fold in a *csk1* disruptant strain both toward the originally used substrate MBP (Molz and Beach, 1993) and the RNA polymerase II CTD substrate (this study), establishing a functional link between Csk1 and the Mcs6–Mcs2 complex. When using recombinant proteins, Csk1 was found to activate the Mcs6–Mcs2 complex toward both RNA Pol II CTD and GST–CDK2. These results identify the fission yeast Csk1 kinase as an *in vivo* activating kinase of the Mcs6–Mcs2 CAK, defining Csk1 as a CAKAK. Whether similar kinases exist in other species remains to be studied, but it is interesting to note that Csk1 phosphorylated and activated a CDK7–cyclin H complex in conditions where no activation by GST–CDK2–cyclin A was observed (Figure 4, and data not shown).

The Mcs6 homologue CDK7 is phosphorylated on two sites in the T-loop. Phosphorylation of T170 (T176 in *Xenopus*) enhances association (Fisher and Morgan, 1994; Mäkelä *et al*., 1994) and activity (Labbe *et al*., 1994) of CDK7–cyclin H, and this site corresponds to the conserved activation site of CDKs (CAK site). The role of S164 (S170 in *Xenopus*) phosphorylation is less clear (Poon *et al*., 1994; Fisher *et al*., 1995; Martinez *et al*., 1997; see also below). Both of these sites can be phosphorylated by CDK2–cyclin A and cdc2–cyclin B *in vitro* (Fisher *et al*., 1995; Martinez *et al*., 1997), and indeed, based on the ability of these kinases to activate CDK7–cyclin H, they were defined as CAKAKs (Fisher *et al*., 1995). In this regard, *in vivo* CAKAK Csk1 was distinct in that it was found to phosphorylate Mcs6 only on the conserved CAK site (S165), suggesting that if the site corresponding to CDK7 S164 (T160 in Mcs6) is phosphorylated *in vivo*, it is mediated by a distinct kinase (see also below).

Phosphorylation of the CAKAK site of Mcs6 *in vivo* is not essential, as Mcs6-S165A rescued the synthetic lethality of *mcs6-13 csk1::sup3-5*. The mammalian CDK7–cyclin H complex can be activated *in vitro* either by phosphorylation on the CAKAK site or by associating with MAT1. The contributions of these alternative (but not exclusive) activation mechanisms *in vivo* is not clear. It is interesting to speculate that the rescue by Mcs6-S165A is based on a MAT1 homologue in *S.pombe* cells. A MAT1 homologue in *S.pombe* could also be important in determining the substrate specificity of the Mcs6 kinase, as has been identified in other systems (Inamoto *et al*., 1997; Ko *et al*., 1997; Rossignol *et al*., 1997; Yankulov and Bentley, 1997).

In contrast to the general dogma of a CDK being dependent for catalytic activity on its cognate cyclin subunit, a baculovirus-expressed Mcs6 kinase can be phosphorylated and activated directly by Csk1. Both the activity and tight regulation by CAKAK site phosphorylation of monomeric Mcs6 is similar to activities described for *in vitro* translated *Xenopus* CDK7 (Martinez *et al*., 1997). More importantly, when Mcs6 is overexpressed from the strong *nmt-1* promoter in fission yeast cells, the kinase activity associated with the overexpressed Mcs6 is significantly higher and more tightly under Csk1 regulation than activity associated with endogenous Mcs6–Mcs2 complexes (Figures 2 and 6). This suggests that the Mcs6 can act as a monomer *in vivo*, although as of yet there is no evidence for a physiological role for such a kinase.

The ability of Csk1 to phosphorylate Mcs6 in the absence of the Mcs2 cyclin is reminiscent of what is seen with another single subunit CAK: the budding yeast Cak1/Civ1 kinase, which phosphorylates CDC28 before cyclin binding (Espinoza *et al*., 1996; Kaldis *et al*., 1996, 1998; Thuret *et al*., 1996). In this regard, it is interesting to point out that although Cak1/Civ1 and Csk1 are only distantly related in primary structure, they share several features not common in serine–threonine kinases. Moreover, Csk1 is able to complement a temperature-sensitive mutant of Cak1/Civ1 in budding yeast, indicating that in these conditions, Csk1 is in fact an *in vivo* CAK of CDC28 (T.Westerling, A.Pihlak, D.Hermand, J.-Y.Thuret, C.Mann and T.P.Mäkelä, in preparation). Taken together with the capacity of Csk1 to activate other CDKs (Figure 4), it is tempting to speculate that perhaps Csk1 could also function as a CAK for cdc2. These questions will be interesting to study when reagents become available. However, the current results unambiguously indicate that fission yeast has at least two CAKs: Csk1 that activates Mcs6, and another CAK (the only candidate being Mcs6–Mcs2) responsible for the essential cdc2 activation, and present in the viable Csk1 disruptant strain.

Materials and methods

Yeast strains and techniques

The *S.pombe* strains used in this study were: h^{+N} *ade6-216 leu1-32 ura4D18*, h^{+} *mcs2::mcs2F leu1-32 ura4D18 ade6*, h^{+} *csk1::sup3-5*

leu1-32 ade6-704 (Molz and Beach, 1993*), h⁻ mcs6-13 leu1-32 ura4D18 ade6* (Molz *et al*., 1989) and *h⁻ cdc25-22 leu1-32 ura4-D18 ade6-216, h⁻ csk1::sup3-5 mcs6-13 leu1-32 ade6-704, h⁺ csk1::ura4+ mcs2::mcs2F leu1-32 ura4D18 ade6*. The *S.pombe* were transformed using the lithium acetate high efficiency transformation protocol (Moreno *et al*., 1991). Genetic crosses, synchronization using the *cdc25-22* block–release protocol and analysis were performed according to Moreno *et al*. (1991).

Cloning of csk1

The *csk1* open reading frame (ORF) was amplified from a *S.pombe* cDNA library (a kind gift from Drs Michelle Minet and Francois Lacroute) using the CF1 (5′-GGG-GAA-TTC-AAT-TTA-ATG-AAA-TCA-GTC-3′) and CR1 (5′-TCT-CTC-TCG-AGT-TAT-GCA-TAT-TGT-GAA-AGC-C-3′) primers. The *Eco*RI–*Xho*I-digested PCR product was introduced into the pGEX-4T bacterial expression vector (Pharmacia) and confirmed by sequencing.

Schizosaccharomyces pombe expression vectors

The *csk1* ORF described above was introduced into the *S.pombe* expression vectors pAHA [a modified pAAUN (Xu *et al*., 1990) with an N-terminal HA epitope tag] and into pREP3 (Maundrell, 1993). *myc-mop1*-pREP-3 was described previously (Damagnez *et al*., 1995). The S165A mutant of *mcs6* (or *mop1*) was made by replacing the insert in *myc-mop1*-pREP-3-by a two-step PCR approach using pREP-3-*myc-mop1* as the template. In the first step, the 5′ PCR fragment was made with primers MopS165A1, 5′-AGC-GGC-CGC-GTC-GAC-ACC-ATG-GAA-CAG-3′, and MopS165A2, 5′-C-CTG-ATG-AGC-CAT-ATG-GCT-TGG-G-3′, where Ser165 was mutated to alanine with the introduction of a silent *Nde*I restriction site (underlined). The 3′ PCR fragment was made with primers MopS165A3, 5′-C-CCA-AGC-CAT-ATG-GCT-CAT-CAG-G-3′, where Ser165 was mutated to alanine with the introduction of a *Nde*I restriction site (underlined), and MopS165A4, 5′-TGC-GGC-CGC-GGA-TCC-TTA-AAC-AAA-TT-3′. The second PCR was made with MopS165A1 and MopS165A4 using the described PCR fragments as template. Sequencing checked the full-length sequence of the mutant.

Disruption of csk1

A 300 bp internal *Hin*dIII fragment from *csk1* was replaced by the *URA4+* cassette from pAAUN (Xu *et al*., 1990). Subsequently, the 2.5 kb *Eco*RI–*Xho*I insert containing the *URA4+* marker was used to transform the *S.pombe* strain *h⁺ mcs2::mcs2F leu1-32 ura4D18 ade6*. The correct integration was confirmed by PCR followed by a Southern blot.

Csk1, Mcs6 and Mcs2 antibodies

C-terminal 15 amino acid peptides from Csk1 (IESFPKVSARLSQYA) or Msc6 (QRQNNFPMRANIKFV) were synthesized, linked to keyhole limpet hemocyanin (KLH), and used for immunization of rabbits. The resulting polyclonal antisera were tested for specificity using the recombinant baculoviruses and *S.pombe* strains overexpressing the corresponding proteins with epitope tags by Western blots and immunoprecipitation. The Mcs2 rabbit polyclonal antiserum was generated against a full-length Mcs2 protein, and was a kind gift from Drs Eberhard Schneider and Peter Wagner (unpublished).

Generation of baculoviruses

The GST–Csk1-expressing baculovirus was made by inserting the *csk1* ORF as a *Eco*RI–*Not*I fragment from pGEX-4T-*csk1* into pAcGHLT-A (Pharmingen) transfer vector, which was introduced into Sf9 insect cells together with BaculoGold (Pharmingen) baculovirus DNA as recommended by the manufacturer. An empty pAcGHLT was used to make a control GST-expressing virus. The *mcs2* and *mcs6* viruses were made by inserting the ORF of the respective genes into the pVL1392 (Pharmingen) transfer vector following the protocol above. The cyclin H virus was generated by inserting a *Bam*HI–*Xba*I fragment containing the ORF of human cyclin H into *Bgl*II–*Xba*I of the pEV55 transfer vector. The HA epitope-tagged CDK7 virus (Fisher and Morgan, 1994) was a kind gift from Robert Fisher and David Morgan.

Protein expression, purifications and immunoprecipitations

For protein expression, the baculoviruses were propagated in Hi5 insect cells (Invitrogen) for 48 h. The GST–Csk1 and GST proteins were purified using glutathione–Sepharose and eluted with glutathione. About 10 ng of purified protein was used per assay. Mcs6, Mcs2 and Mcs6–Mcs2 complexes were purified with specific antisera as indicated and used still bound to protein A–Sepharose. The HA-CDK7–cyclin H complex was immunopurified with HA 12CA5 mouse monoclonal antibody (Boehringer Mannheim, Germany).

Bacterially expressed GST–CDK2-D155N, GST–CDK2-T160A and GST–CTD (a kind gift from David Chao and Richard Young) proteins were purified using glutathione–Sepharose beads (Damagnez *et al*., 1995). The CTD peptide was a triple repeat of the RNA polymerase II CTD motif linked to biotin (YSPTSPSYSPTSPSYSPTSPS-biotin).

Production of Csk1 in rabbit reticulocyte lysate using T7 RNA polymerase was performed according to the manufacturer's instructions (Promega, TNT kit).

For immunoprecipitations from *S.pombe* cells, yeast strains (10 ml) were grown overnight in selective media. Subsequently, cell pellets were washed once in phosphate-buffered saline, and disrupted in lysis buffer [150 mM NaCl, 50 mM HEPES pH 7.5, 5 mM EDTA, 1% NP-40 with 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM β-glycerophosphate and 5 mM NaF]. Following disruption, lysates were adjusted to 0.5% NP-40 for immunoprecipitation with the HA antibody (Boehringer Mannheim), the myc 9E10 mouse monoclonal antibody (Babco Inc., Berkeley, CA) or other antisera. Immunoprecipitates on protein A–Sepharose (Sigma) beads were washed four times with lysis buffer containing 0.1% NP-40, and once with kinase buffer [20 mM Tris pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 1 mM dithiotreitol (DTT)].

Kinase assays and Western blotting

For standard kinase reactions, 10 ng of GST–Csk1, 100 ng of Mcs6–Mcs2, 100 ng of HA-CDK7 or immunoprecipitates as indicated were used as kinases. Kinase reactions were performed in 30 µl of kinase buffer supplemented with 10 µCi of [γ-^{32}P]ATP and the indicated substrates (2 µg of CTD peptide, 4 µg of GST–CDK2-D155N, 4 µg of GST–CDK2-D155N-T160A, 4 µg of GST–CTD) for 30 min at 30°C.

For the initial kinase reaction of the CAK assay presented in Figure 4E, 500 ng of cyclin A [a kind gift of Jean-Yves Thuret and Carl Mann (Thuret *et al*., 1996)] and 5 µl of a reticulocyte lysate programmed with *csk1* (or unprogrammed as control) were incubated with CDK2 immunopurified (M2, Santa Cruz Biotechnology) from rabbit reticulocyte lysate in kinase buffer supplemented with 1 mM cold ATP for 10 min at 30°C. The CDK2 still bound on beads was purified by three washes followed by a kinase reaction with 10 µCi of [γ-^{32}P]ATP and 4 µg of histone H1 as a substrate.

In the CDK activation experiments in Figure 5A, GST–Csk1 was incubated with Mcs6, Mcs2 or Mcs6–Mcs2 bound to beads for 6 min at 30°C in kinase buffer supplemented with 100 µM ATP. Subsequently, the beads were washed three times with kinase buffer before a kinase reaction with [γ-^{32}P]ATP with GST–CTD or GST–CDK2 substrates. Phosphorylated substrates were analysed by 10% SDS–PAGE followed by autoradiography. Western blotting analysis was according to standard procedures.

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