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Specificity of Cdk activation *in vivo* by the two Caks Mcs6 and Csk1 in fission yeast

Damien Hermand[1,2], Thomas Westerling[1], Arno Pihlak[1], Jean-Yves Thuret[3], Tea Vallenius[1], Marianne Tiainen[1], Jean Vandenhaute[2], Guillaume Cottarel[4], Carl Mann[3] and Tomi P.Mäkelä[1,5,6]

[1]Haartman Institute & Biocentrum Helsinki, University of Helsinki, 00014 Helsinki, [5]HUCH Laboratory Diagnostics, 00029 HYKS, Finland, [2]Laboratoire de Génétique Moléculaire (GEMO), University of Namur (FUNDP), 61 Rue de Bruxelles, 5000 Namur, Belgium, [3]Service de Biochimie et Genetique Moléculaire, CEA/Saclay, F-91191 Gif-sur-Yvette Cedex, France and [4]Genome Therapeutics Corp., 100 Beaver Street, Waltham, MA 02154, USA

[6]Corresponding author
e-mail: tomi.makela@helsinki.fi

D.Hermand & T.Westerling and A.Pihlak & J.-Y.Thuret, respectively, contributed equally to this work

Activating phosphorylation of cyclin-dependent kinases (Cdks) is mediated by at least two structurally distinct types of Cdk-activating kinases (Caks): the trimeric Cdk7–cyclin H–Mat1 complex in metazoans and the single-subunit Cak1 in budding yeast. Fission yeast has both Cak types: Mcs6 is a Cdk7 ortholog and Csk1 a single-subunit kinase. Both phosphorylate Cdks *in vitro* and rescue a thermosensitive budding yeast *CAK1* strain. However, this apparent redundancy is not observed in fission yeast *in vivo*. We have identified mutants that exhibit phenotypes attributable to defects in either Mcs6-activating phosphorylation or in Cdc2-activating phosphorylation. Mcs6, human Cdk7 and budding yeast Cak1 were all active as Caks for Cdc2 when expressed in fission yeast. Although Csk1 could activate Mcs6, it was unable to activate Cdc2. Biochemical experiments supported these genetic results: budding yeast Cak1 could bind and phosphorylate Cdc2 from fission yeast lysates, whereas fission yeast Csk1 could not. These results indicate that Mcs6 is the direct activator of Cdc2, and Csk1 only activates Mcs6. This demonstrates *in vivo* specificity in Cdk activation by Caks.

Keywords: Cak1/Cdk-activating kinases/Csk1/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 an activating phosphorylation on a conserved residue in the 'T-loop' of the kinase in order to be fully active (reviewed in Morgan, 1997; Solomon and Kaldis, 1998). The T-loop phosphorylation site is conserved in Cdks from yeast to mammals, and is essential for cell viability in both *Schizosaccharomyces pombe* Cdc2 (T167; Gould *et al*., 1991) and *Saccharomyces cerevisiae* Cdc28 (T169; Lim *et al*., 1996).

The activating phosphorylation on the T-loop of Cdks is mediated by Cdk-activating kinases (Caks; reviewed in Kaldis, 1999). Biochemical purification of a Cak activity for Cdc2 and Cdk2 (Solomon *et al*., 1992) subsequently led to the identification of the previously cloned MO15 serine-threonine kinase (Shuttleworth *et al*., 1990) as the catalytic subunit of the purified Cak (Fesquet *et al*., 1993; Poon *et al*., 1993; Solomon *et al*., 1993). Immunoprecipitation of MO15 revealed stoichiometric binding of 37 and 32 kDa proteins (Tassan *et al*., 1994) identified as 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). As MO15 activity was dependent on the regulatory cyclin subunit, the kinase was renamed Cdk7. The physiological role of the Cdk7–cyclin H–Mat1 complex as a Cak has been addressed by two lines of experimentation. In cycling *Xenopus* egg extracts, immunodepletion of Cdk7 suppressed Cak activity and inhibited entry into M phase (Fesquet *et al*., 1997). The Cak activity was restored by injection of Cdk7 and cyclin H mRNA, demonstrating that a Cdk7 complex is necessary for activation of mitotic Cdk–cyclin complexes (Fesquet *et al*., 1997). In a separate approach, *Drosophila* Cdk7 was found to be necessary for Cak activity of Cdc2–cyclin B and Cdc2–cyclin A *in vivo* using both temperature-sensitive and null alleles of the *Drosophila CDK7* gene (Larochelle *et al*., 1998). These results strongly suggest that the Cdk7–cyclin H–Mat1 complex functions as a Cak *in vivo*, while not excluding the possibility that other Caks exist, as suggested by recent biochemical approaches (Edwards *et al*., 1998; Kaldis and Solomon, 2000).

The trimeric complex of Cdk7–cyclin H–Mat1 is also part of the general transcription factor TFIIH, where the complex phosphorylates the C-terminal domain (CTD) of the large subunit of RNA polymerase II (Pol II) (Feaver *et al*., 1994; Roy *et al*., 1994; Mäkelä *et al*., 1995; Serizawa *et al*., 1995; Shiekhattar *et al*., 1995) and is important for transcription in the early *Drosophila* embryo (Leclerc *et al*., 2000). Cdk7–cyclin H–Mat1 binds the core TFIIH through ERCC2/XPD and can also exist as a free complex (Drapkin *et al*., 1996; Reardon *et al*., 1996). The budding yeast *S.cerevisiae* has a complex closely related to Cdk7–cyclin H–Mat1 that consists of the kinase Kin28, the cyclin Ccl1 and the Mat1 homolog Tfb3/Rig2 (Simon *et al*., 1986; Valay *et al*., 1993; Faye *et al*., 1997; Feaver *et al*., 1997). This complex is also associated with TFIIH and is required for the transcription of most but not all genes (Cismowski *et al*., 1995; Valay *et al*., 1995; Hengartner *et al*., 1998; Lee and Lis, 1998).

In contrast to the Cdk7 complex, the Kin28 complex does not display Cak activity *in vitro* and is not a Cak *in vivo* (Cismowski *et al*., 1995; Valay *et al*., 1995).

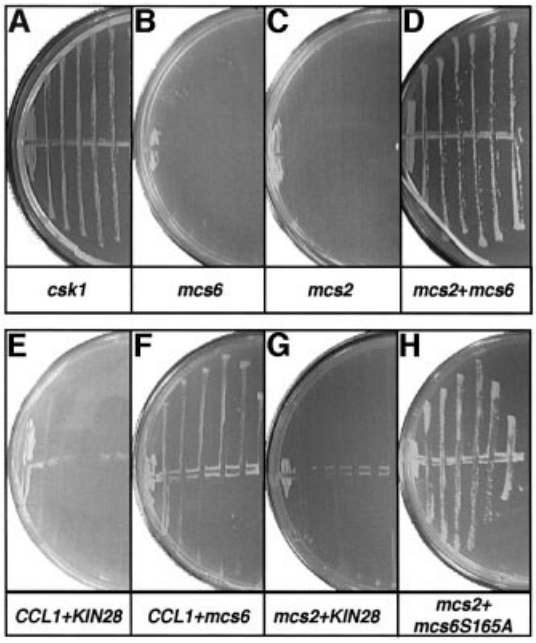


Fig. 1. Complementation of a temperature-sensitive *CAK1* allele by either Csk1 or Mcs6–Mcs2 in budding yeast. The *CAK1* ts strain (*civ1-4*) was transformed by plasmids overexpressing *csk1* (*csk1*-p426), *mcs6* (*mcs6*-p425), *mcs2* (*mcs2*-p424), *KIN28* (*KIN28*-p425), *CCL1* (*CCL1*-p426), *mcs6-S165A* (*mcs6-S165A*-p425), or a combination of these as indicated. Resulting strains were streaked on selective minimal media at 35°C for 4 days and photographed.

Instead, budding yeast *S.cerevisiae* contains a single Cdk-activating kinase, Cak1/Civ1, discovered by biochemical purification of Cak activity (Espinoza *et al*., 1996; Kaldis *et al*., 1996; Thuret *et al*., 1996). Sequence alignments suggest that Cak1 is distantly related to the Cdk family, but biochemical characterization showed that it is active as a monomer. Both genetic and biochemical evidence indicate that Cak1 is the physiological activating kinase of Cdc28 and is important for both G_1–S and G_2–M transitions (Kaldis *et al*., 1996; Thuret *et al*., 1996; Sutton and Freiman, 1997). Cak1 has been demonstrated to be a physiological Cak of more than one Cdk as it also activates Kin28 (Espinoza *et al*., 1998; Kimmelman *et al*., 1999). Thus, it appears that budding yeast has only one Cak.

Cdk activation by Cak thus appears to be mediated by two structurally distinct kinases: a single-subunit kinase in budding yeast and a multi-subunit kinase in metazoans. Interestingly, the fission yeast *S.pombe* is the only known species expressing both Cak types. Mcs6 is the ortholog of Cdk7 and phosphorylates both Cdks and Pol II CTD (Buck *et al*., 1995; Damagnez *et al*., 1995). Mcs6 associates with the cyclin H ortholog Mcs2 (Buck *et al*., 1995; Damagnez *et al*., 1995) and with the Mat1 ortholog Pmh1 (our unpublished results; DDBJ/EMBL/GenBank accession No. AF191500). Both *mcs2* and *mcs6* were originally isolated as potential mitotic inducers in a screen for extragenic suppressors of 'mitotic catastrophe' or premature entry into mitosis resulting from elevated Cdc2 activity (Molz *et al*., 1989). The alleles isolated during the screen (*mcs2-75* and *mcs6-13*) display allele-specific interactions with *cdc2*, reminiscent of the range of interactions described between *cdc2* and *cdc13* (Booher and Beach, 1987; Molz *et al*., 1989). The second fission yeast kinase with Cak activity is the single-subunit Csk1 (Hermand *et al*., 1998; Lee *et al*., 1999). The *csk1* gene was first identified as a multicopy suppressor of the synthetic lethality of *mcs2-75 cdc2-3w cdc25-22* (Molz and Beach, 1993). Subsequently, Csk1 was found to phosphorylate Mcs6 on the T-loop activation site (S165) and activate the Mcs6–Mcs2 complex *in vivo* (Hermand *et al*., 1998). A recent report also implicated Csk1 as a direct activator of Cdc2 (Lee *et al*., 1999), suggesting that Mcs6–Mcs2–Pmh1 and Csk1 function redundantly in Cdc2 activation. In this work, we addressed the *in vivo* functions of the fission yeast Caks Mcs6 and Csk1. Our results indicate that these kinases have distinct non-overlapping functions: Mcs6 acting as the Cdc2-activating kinase and Csk1 as the Mcs6-activating kinase.

Results

Complementation of a temperature-sensitive CAK1 allele by either Csk1 or Mcs6–Mcs2 in budding yeast

The identification of two kinases in fission yeast with Cak activity *in vitro* (Buck *et al*., 1995; Damagnez *et al*., 1995; Hermand *et al*., 1998; Lee *et al*., 1999) prompted us to study whether these kinases could complement a temperature-sensitive (ts) allele of *S.cerevisiae CAK1* (*civ1-4*) (Thuret *et al*., 1996). To this end we initially used an unbiased approach by screening a *S.pombe* cDNA library for clones capable of suppressing the *civ1-4* cell cycle arrest at the restrictive temperature. The only cDNA identified in this screen was *csk1* (Figure 1A). As Mcs6 is a cyclin-dependent kinase, one possibility why it was not identified in this screen is that it requires its cognate cyclin (Mcs2) for activity. To investigate this possibility, plasmids expressing *mcs6* and *mcs2* were transformed into the thermosensitive *CAK1* strain. As shown in Figure 1B–D, overexpression of either *mcs6* or *mcs2* alone does not suppress the *CAK1* thermosensitive phenotype at 35°C, but when both subunits are expressed, the phenotype is fully rescued. This result indicates that the Mcs6–Mcs2 complex has Cak activity *in vivo*.

As might be expected, co-expression of the budding yeast *KIN28* and *CCL1* does not rescue the phenotype (Figure 1E) as the complex lacks Cak activity *in vitro* and is not a Cak *in vivo* (Cismowski *et al*., 1995). The experimental set-up allowed us to investigate whether the heterologous Cdk–cyclin pairs would suppress the thermosensitive *CAK1* strain. The result indicates that Mcs6 and Ccl1 weakly suppress the strain (Figure 1F), whereas Kin28 and Mcs2 do not (Figure 1G).

Phosphorylation of the activation site of Mcs6 was not required for the rescue as a Cak site mutant (Mcs6-S165A) was indistinguishable from Mcs6 (Figure 1H). The functional complementation of Cak1 by Mcs6–Mcs2 demonstrates that a Cak from the Cdk7 family can perform all essential functions of a Cak in heterologous budding yeast cells.

Activators of Cdc2 suppress the mcs6-13 csk1Δ phenotype but not the csk1Δ phenotype

The complementation results described above show that both Mcs6 and Csk1 have Cak activity when

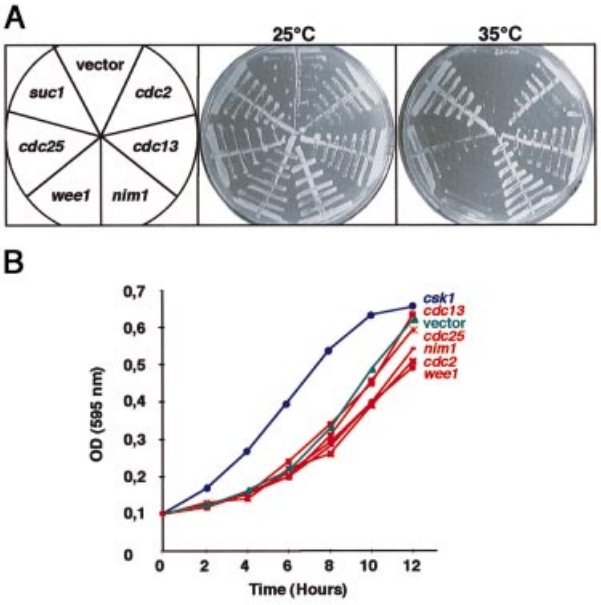


Fig. 2. Activators of Cdc2 suppress the *mcs6-13 csk1Δ* phenotype but not the *csk1Δ* phenotype. (**A**) In order to study the role of the Cdc2–Cdc13 complex in the lethality of the *mcs6-13 csk1Δ* strain, it was transformed with plasmids containing genomic inserts of *cdc2*, *cdc13*, *nim1*, *wee1*, *cdc25*, *suc1* in pWH5 or an empty vector. Subsequently, these strains were streaked as sectors on minimal media and incubated at either 25 or 35°C, as indicated, for 4 days. (**B**) The *csk1Δ* strain was transformed with the same plasmids as in (A). The resulting strains were inoculated to an optical density (OD) of 0.1 from stationary phase cultures in minimal media. Subsequently, ODs were measured at the indicated time points. A representative experiment is shown.

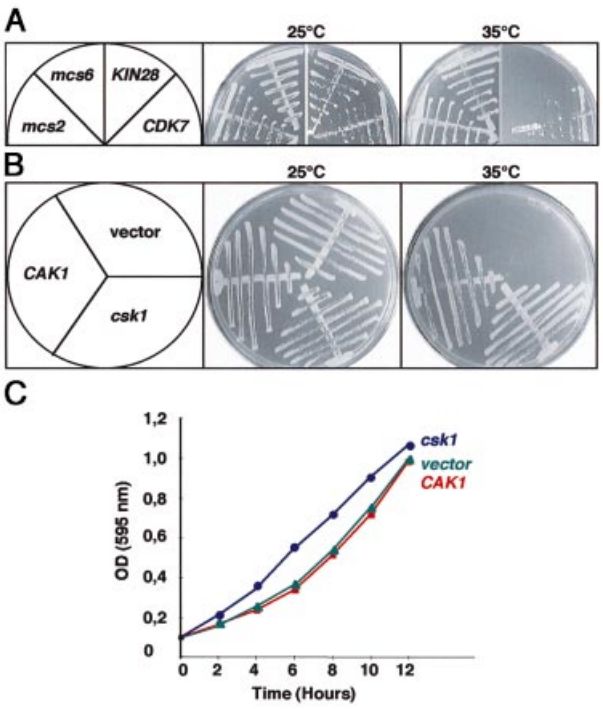


Fig. 3. Cak1 suppresses the *mcs6-13 csk1Δ* but not the *csk1Δ* phenotype. (**A**) The *mcs6-13 csk1Δ* phenotype is suppressed by heterologous Caks. *mcs6-13 csk1Δ* was transformed with plasmids overexpressing *mcs2* (*mcs2*-pAHL), *mcs6* (*mcs6*-pREP3), *KIN28* (*KIN28*-pREP3) or *CDK7* (*CDK7*-pREP3). The resulting strains were streaked on minimal media and incubated at either 25 or 35°C, as indicated, for 4 days. (**B**) As in (A), except that *csk1* (*csk1*-pAHL), CAK1 (*CAK1*-pAHL) or an empty vector was used. (**C**) In order to study whether Cak1 could rescue the phenotype of *csk1Δ*, it was transformed with plasmids overexpressing either *csk1* (*csk1*-pAHL), CAK1 (*CAK1*-pAHL) or an empty vector and assayed for cell cycle re-entry as in Figure 2B.

expressed in budding yeast. This result thus suggests that Mcs6 and Csk1 could be functionally redundant for Cdc2 activation in fission yeast cells, as previously suggested (Lee *et al*., 1999). To address this issue more directly we compared the ability of various Cdc2 regulators to suppress the phenotype of two strains: (i) *csk1::sup3-5*, a simple *csk1* disruption (and referred to as *csk1Δ* subsequently) displaying a delay of entry into exponential growth (Hermand *et al*., 1998); and (ii) *mcs6-13 csk1Δ* (Hermand *et al*., 1998), in which the *csk1* disruption is combined with the *mcs6-13* allele (Molz *et al*., 1989). In combination, these mutations confer a synthetic lethality at 35°C (Hermand *et al*., 1998), whereas the *mcs6-13* mutation alone shows no phenotype (Molz *et al*., 1989).

Transformation of plasmids harboring genomic inserts encoding for *cdc2*, *cdc13*, *nim1*, *cdc25* and *suc1* enabled the growth of *mcs6-13 csk1Δ* at 35°C, while *wee1* did not (Figure 2A). Thus, all of the activators of Cdc2 tested rescued the phenotype, suggesting that impairment of Cdc2 activation is critical for the lethality of *mcs6-13 csk1Δ*, in agreement with Lee *et al*. (1999). We also found that the G_1- and S-phase cyclins Cig1, Cig2 and Puc1 (a kind gift of Sergio Moreno) were unable to suppress the thermosensitive growth of the *mcs6-13 csk1Δ* double mutant (data not shown). As only G_2 activators of Cdc2 rescue this strain, it is likely that it is mainly affected in the G_2–M transition. This would be in agreement with previous observations indicating that higher levels of Cdc2 activity are required at the CG_2–M transition compared with the G_1–S transition (Stern and Nurse, 1996).

Remarkably, none of the Cdc2 regulators that suppressed the *mcs6-13 csk1Δ* phenotype were able to suppress the *csk1Δ* growth delay phenotype (Figure 2B), suggesting that this phenotype is not directly related to Cdc2 activation.

Cak1 suppresses the mcs6-13 csk1Δ but not the csk1Δ phenotype

We then extended the suppressor analysis to kinases reported to have Cak activity in other species. For this purpose, initially two kinases of the Cdk7 family (human Cdk7 and budding yeast Kin28) were tested for their ability to suppress the thermosensitive growth defect of the *mcs6-13 csk1Δ* strain. The results indicate that Cdk7 suppressed the phenotype at 35°C, whereas *KIN28* did not (Figure 3A). In addition, the *mcs2* cyclin-encoding gene was also found to suppress the lethality when overexpressed (Figure 3A), constituting the first direct genetic interaction between *mcs6* and *mcs2*.

The results presented in Figures 2 and 3 suggest that the synthetic lethality of the *mcs6-13 csk1Δ* strain primarily reflects impaired Cdc2 activation by Cak, and not RNA Pol II large subunit CTD phosphorylation. This was further supported by the ability of budding yeast *CAK1* to

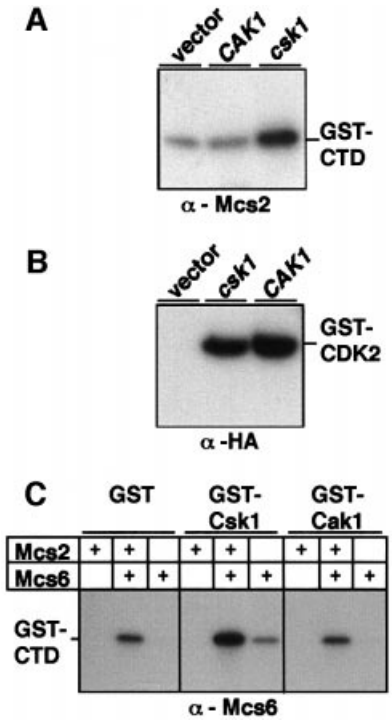


Fig. 4. Cak1 does not activate Mcs6 *in vitro* or *in vivo*. (**A**) In order to analyze the effects of overexpressed Cak1 on Mcs2-associated kinase activity in fission yeast cells, anti-Mcs2 immunoprecipitates made from cells overexpressing HA-Cak1 (*CAK1*) or HA-Csk1 (*csk1*) or a control (vector) were subsequently assayed for kinase activity toward GST–CTD, followed by SDS–PAGE and autoradiography. (**B**) The activity of the overexpressed HA-Cak1 (*CAK1*) or HA-Csk1 (*csk1*) from strains used in (A) was controlled by kinase reactions from anti-HA immunoprecipitates using GST–CDK2 as a substrate. (**C**) Kinase activity of purified Mcs6 or Mcs6–Mcs2 was assessed following a pre-incubation with GST, GST–Csk1 or GST–Cak1 in a cold kinase reaction. The second kinase reaction was performed in the presence of radiolabeled ATP and GST–CTD to assay Mcs6 activity. The basal activity of the Mcs6–Mcs2 complex is shown in lane 2.

suppress *mcs6-13 csk1Δ* at 35°C (Figure 3B). In contrast to the synthetic lethal strain, expression of a *CAK1* plasmid did not suppress the *csk1Δ* growth delay phenotype (Figure 3C), similarly to what was previously observed for the Cdc2 regulators.

Cak1 does not activate Mcs6 unlike Csk1

Based on previous results suggesting that phosphorylation of Mcs6 by Csk1 is required for normal cell cycle entry from stationary phase (Hermand *et al*., 1998), the inability of *CAK1* to suppress the *csk1Δ* phenotype suggested that Cak1 would not activate Mcs6 *in vivo*. Accordingly, Mcs2-associated kinase activity was not increased in Cak1-overexpressing *S.pombe* cells, unlike what has been reported for Csk1-overexpressing cells (Figure 4A; Hermand *et al*., 1998), although the intrinsic kinase activities of Cak1 and Csk1 in the respective strains toward a glutathione *S*-transferase (GST)–CDK2 substrate were comparable (Figure 4B). Furthermore, in contrast to Csk1, Cak1 was unable to activate Mcs6 or Mcs6–Mcs2 *in vitro* (Figure 4C) using baculovirus-expressed GST–Cak1 (or GST–Csk1 as a control) in an activation assay described previously (Hermand *et al*., 1998). These results demonstrate that Cak1 is unable to activate the Mcs6–Mcs2 complex in *S.pombe* cells.

Cdc2 associates with overexpressed Cak1 but not Csk1 in fission yeast cells

Cak1 has been shown not only to phosphorylate, but also to co-purify with Cdc28 from budding yeast lysates (Thuret *et al*., 1996); therefore, we were interested in investigating whether Csk1 would associate with its suggested substrate Cdc2 in an analogous manner. Endogenous Csk1 was not detected co-purifying with fission yeast Cdc2 (data not shown), but this could have been due to a detection problem considering the low levels of endogenous Csk1 (our unpublished data). To control this possibility, GST–Suc1 was used to purify Cdc2 (Cdc2-HA) from *S.pombe* cells also overexpressing either Csk1 or budding yeast Cak1. Subsequent western blotting analysis of the complexes revealed that while overexpressed Cak1 readily associated with Cdc2 in *S.pombe* lysates, Csk1 did not (Figure 5A).

GST–Cak1, but not GST–Csk1, associates with and subsequently phosphorylates S.pombe Cdc2

In a second approach to address whether Csk1 could associate with *S.pombe* Cdc2, recombinant GST–Csk1 or GST–Cak1 was added to fission yeast lysates, and following a 30 min incubation on ice GST–Csk1 or GST–Cak1 and associated proteins were purified and subjected to an *in vitro* kinase reaction with radiolabeled ATP. As no exogenous substrates were added, phosphorylated bands represent potential substrates that had been purified from the fission yeast lysates due to their association with Csk1 or Cak1. This analysis revealed that GST–Cak1 bound and phosphorylated *in vitro* a protein of 34 kDa from a wild-type fission yeast lysate (Figure 5B, lane 5). Moreover, when a fission yeast lysate expressing Cdc2-HA in addition to endogenous Cdc2 was used in the same assays, an additional band of 36 kDa was labeled (Figure 5B, lane 6). Subsequent western blotting analysis revealed that the 34 and 36 kDa bands co-migrated with Cdc2 and Cdc2-HA, respectively (not shown). When GST–Csk1 was used, no labeled bands were detected (Figure 5B, lane 3–4) nor was Cdc2 or Cdc2-HA detected by western blotting (not shown). As Cdc2 is not autophosphorylated (Gould *et al*., 1991; Solomon *et al*., 1992), these results demonstrate that Cak1 can associate with and subsequently phosphorylate fission yeast Cdc2. Furthermore, using a similar approach with baculovirus lysates expressing wild-type *S.pombe* Cdc2 or a Cdc2 (T167A) activation-site mutant (Gould *et al*., 1991), we demonstrate that phosphorylation of Cdc2 by GST–Cak1 was directed at Thr167 (Figure 5C).

The inability of Csk1 to associate with and subsequently phosphorylate *S.pombe* Cdc2 does not reflect a general inability of this kinase to bind to Cdks, as GST–Csk1 readily associated with and subsequently phosphorylated the closely related human Cdc2 (Figure 5C, lane 3) expressed under identical conditions to the *S.pombe* wild-type and mutant Cdc2. On the other hand, the ability of the single-subunit kinases Cak1 and Csk1 to form stable complexes appears to be substrate specific, as no association of Csk1 with Mcs6 has been detected (data not shown).

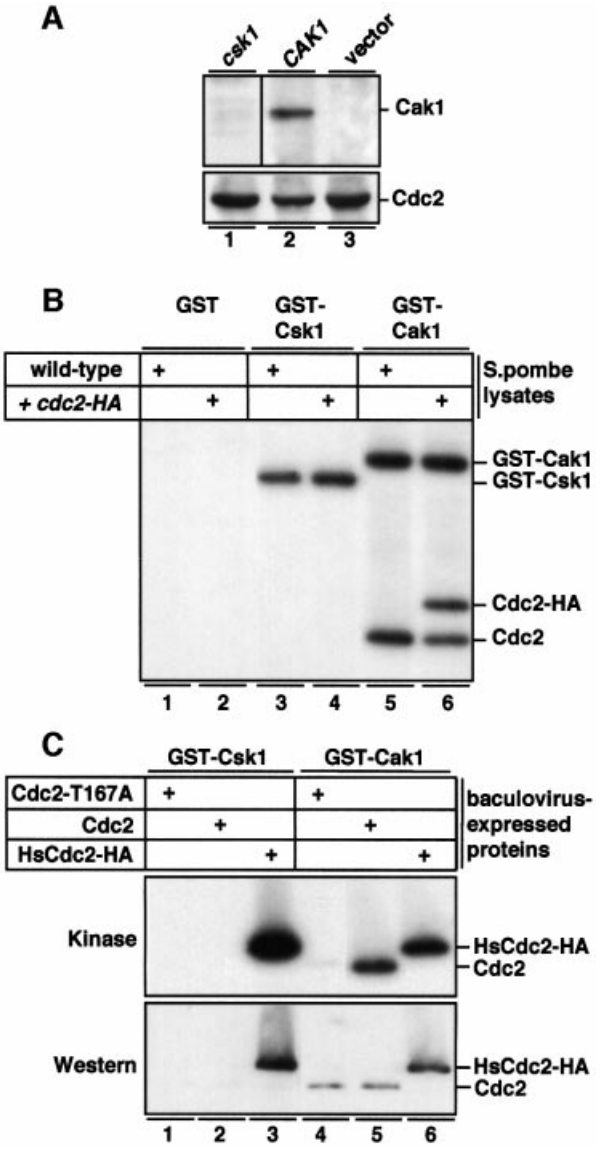


Fig. 5. Association and phosphorylation of *S.pombe* Cdc2 by Cak1, but not Csk1. (**A**) GST–Suc1 was used to purify Cdc2 from *S.pombe* cells also overexpressing either Csk1 (*csk1*-pAAUN) or Cak1 (*CAK1*-pAHA). Precipitated complexes were analyzed by western blotting using either anti-Csk1 (lane 1) or anti-HA (lanes 2–3) antibodies (upper panel). Subsequently, the blot was stripped and probed using anti-PSTAIRE antibodies (lower panel). (**B**) Lysates of wild-type *S.pombe* cells (lanes 1, 3 and 5) or cells moderately overexpressing (see Materials and methods) *S.pombe* Cdc2-HA (lanes 2, 4 and 6) were incubated for 90 min with GST (lanes 1 and 2), GST–Csk1 (lanes 3 and 4) or GST–Cak1 (lanes 5 and 6). Following glutathione–Sepharose purification, a kinase reaction was performed in the presence of [γ-^{32}P]ATP without additional substrates, followed by SDS–PAGE and autoradiography. Phosphorylated proteins are indicated on the right. (**C**) Insect cell lysates producing *S.pombe* Cdc2 (Cdc2), a T167A Cdc2 mutant (Cdc2-T167A) or human HA-tagged Cdc2 (HsCdc2-HA) were incubated with GST–Csk1 or GST–Cak1 as indicated, and subsequently GST proteins were purified and subjected to a kinase reaction (Kinase) as in (B). The kinase gel was subsequently transferred to nitrocellulose and probed with anti-PSTAIRE antibodies.

A mcs6-S165A mutation mimics the csk1 disruption phenotype in fission yeast

The genetic and biochemical results presented above suggest that Csk1 and Mcs6 are not redundant Caks of

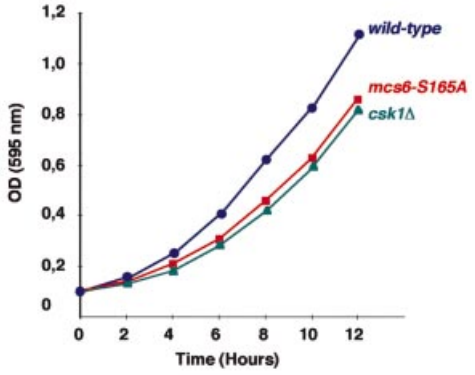


Fig. 6. A *mcs6-S165A* mutation mimics the *csk1* disruption phenotype in fission yeast. After integration of the *mcs6-S165A* allele (see Materials and methods), growth of either wild-type or *csk1Δ* or *mcs6-S165A* was compared as in Figure 2.

S.pombe Cdc2, but instead the data support a linear activation cascade: *csk1* → *mcs6* → *cdc2*. In this model, Csk1 phosphorylation of the Mcs6 T-loop Ser165 is not essential (due to an alternative activation mechanism), whereas Mcs6 phosphorylation of Cdc2 on Thr167 is essential.

To test the first part of the model rigorously, we analyzed the effect of replacing *mcs6* with a mutant encoding a protein in which the T-loop activation site Ser165 was mutated to Ala, thus blocking Csk1 from activating Mcs6. Comparison of the phenotype of this *mcs6::mcs6-S165A* strain with that of the *csk1* disruption strain (*csk1Δ*) should reveal to what extent the observed delay of entry into exponential growth reflects an abolished activation of Mcs6 in *csk1Δ* cells. The results indicate that the *mcs6-S165A* strain displayed a delay of entry into exponential growth very similar to that observed in the *csk1Δ* strain (Figure 6, compare *mcs6-S165A* and *csk1Δ*). These data strongly suggest that the *csk1Δ* phenotype is solely due to the absence of T-loop phosphorylation on Mcs6 and are consistent with the observation that overexpression of Mcs6 can rescue the *csk1Δ* phenotype (Hermand *et al.*, 1998).

mcs6-SALR, combining mcs6-S165A and mcs6-13 mutations, arrests at 35°C

The similarity of the *mcs6-S165A* and *csk1Δ* phenotypes suggested that it should be possible to generate an *mcs6* allele in *csk1* wild-type background that would mimic the synthetic lethality observed in the *mcs6-13 csk1Δ* strain at 35°C. To this end, we sequenced the *mcs6-13* allele, and identified a single T → G mutation (nucleotide 832 in DDBJ/EMBL/GenBank accession No. L47353), which results in the replacement of Mcs6 Leu238 with arginine. It is interesting to note that this residue is conserved in Cdk7 and several other Cdks, but not in Kin28. Subsequently, we reproduced this mutation *in vitro* and combined it with the S165A Cak site mutation to generate *mcs6-S165A-L238R* (referred to as *mcs6-SALR* from now on; see Figure 7A for schematic). Following the replacement of the genomic *mcs6* with *mcs6-SALR*, the phenotype of this strain was compared with that of *mcs6-13 csk1Δ*. As shown in Figure 7B, the *mcs6-SALR* strain is unable to grow at 35°C, just like *mcs6-13 csk1Δ* (Figure 7B). Importantly, in contrast to the *mcs6-13 csk1Δ* strain, the

mcs6-SALR strain is not rescued by overexpressed *csk1* (Figure 7C), as predicted by the presence of wild-type *csk1* in this strain. In all other respects, the *mcs6-SALR* strain was indistinguishable from *mcs6-13 csk1Δ*, as shown by suppression of the thermosensitivity by *CAK1* (Figure 7C) as well as by all the same Cdc2 regulators and Caks described previously for *mcs6-13 csk1Δ* and summarized in Figure 7D. These results demonstrate impaired Cdc2 activation in the *mcs6-SALR* mutant strain at 35°C—a defect that the wild-type Csk1 or even overexpressed Csk1 does not suppress.

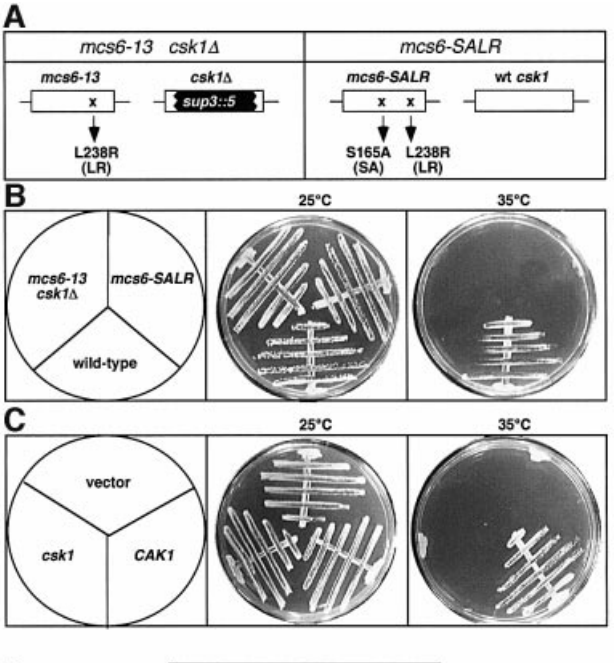
Discussion

Here we studied the role of Mcs6 and Csk1 in activation of Cdc2 in fission yeast. Previous studies have implicated both kinases in the activation of Cdc2 (Molz *et al*., 1989; Molz and Beach, 1993; Hermand *et al*., 1998; Lee *et al*., 1999). *mcs6* and *mcs2* encoding for its cyclin partner display strong genetic interactions with *cdc2* (Booher and Beach, 1987; Molz *et al*., 1989).

Csk1 has been implicated in Cdc2 regulation more indirectly by analysis of the *mcs6-13 csk1Δ* strain, which is synthetically lethal at 35°C (Hermand *et al*., 1998). Arrested cells from this strain display reduced Cdc2-associated H1 kinase activity not seen in either the *mcs6-13* or the *csk1Δ* single-mutant strains (Lee *et al*., 1999). These data, together with the fact that the *mcs6-13 csk1Δ* strain is mutated in two genes encoding proteins with Cak activity *in vitro*, strongly suggested that the phenotype is due to a Cdc2 activation defect. Results presented here demonstrate this to be the case as both multiple Cdc2 regulators (Figure 2), as well as two heterologous Cdk-activating kinases *CDK7* and *CAK1* (Figure 3), rescued the thermosensitivity of the strain at 35°C.

The inability of Cdc2 regulators (Figure 2) or *CAK1* (Figure 3) to rescue the *csk1Δ* phenotype suggests that this phenotype is not a Cdc2 activation defect. These data together with the observations that the *csk1Δ* phenotype is rescued by *mcs6* (Hermand *et al*., 1998) and that the *mcs6-S165A* strain displays a phenotype indistinguishable from *csk1Δ* (Figure 6) indicate that T-loop phosphorylation of Mcs6 is not absolutely required for Cdc2 activation. Therefore, the phenotype of *csk1Δ* and *mcs6-S165A* strains is likely to reflect a transcription defect relating to the as yet uncharacterized TFIIH-associated function of Mcs6 together with Mcs2 and Pmh1.

The suppressor analyses together with the biochemical data showing that Cak1 is unable to activate Mcs6 and that Csk1 is unable to associate with and subsequently phosphorylate fission yeast Cdc2 argued for a linear activation cascade *csk1* → *mcs6* → *cdc2*. Previous reports (Lee *et al*., 1999) on this subject were based on analysis of the double-mutant *mcs6-13 csk1Δ* strain, which does not allow a clear distinction between the roles of the two Caks. Encouraged by the phenotype of the *mcs6-S165A* strain, we therefore attempted to resolve this issue by generating an *mcs6* allele that combined the *mcs6-13* mutation with the T-loop activation site mutant (S165A). The resulting *mcs6-SALR* strain was phenotypically indistinguishable from *mcs6-13 csk1Δ*, and therefore allowed the analysis of



D

	mcs6-13 csk1Δ	mcs6-SALR
csk1	+	-
CAK1	+	+
mcs6	+	+
mcs2	+	+
CDK7	+	+
KIN28	-	-
cdc2	+	+
cdc13	+	+
nim1	+	+
cdc25	+	+
wee1	-	-
suc1	+	+

Fig. 7. The *mcs6-SALR* mutant, combining the *mcs6-13* and *S165A* mutations, is thermosensitive. (**A**) Schematic comparison of *mcs6-13 csk1Δ* and *mcs6-SALR*. (**B**) The *mcs6-13 csk1Δ* and *mcs6-SALR* mutants have an identical thermosensitive phenotype. The *mcs6-13 csk1Δ*, *mcs6-SALR* and wild-type control strains were streaked on minimal media and incubated at either 25 or 35°C, as indicated, for 4 days. (**C**) The *mcs6-SALR* strain is not rescued by *csk1* overexpression. *mcs6-SALR* strains overexpressing either *csk1* (*csk1*-pAHL), *CAK1* (*CAK1*-pAHL) or an empty vector were streaked on minimal media and incubated at either 25 or 35°C for 4 days. (**D**) Comparison of suppressors of the *mcs6-13 csk1Δ* and the *mcs6–SALR* fission yeast strains by the indicated Cdc2 regulators and Caks.

the role of Mcs6 in Cdc2 activation. The results demonstrated that Csk1 is not directly involved in Cdc2 activation. Unlike in the report of Lee *et al*. (1999), in our experimental set-up we did not detect Csk1 phosphorylating fission yeast Cdc2, although we do find that Csk1 can phosphorylate human Cdc2. Our biochemical results are thus in agreement with the genetic results indicating an inability of Csk1 to function as a Cak for Cdc2 *in vivo*. The functions of Csk1 and Mcs6 are thus distinct and non-redundant.

The linear activation cascade *csk1* → *mcs6* → *cdc2* also explains how Cdc2 activity is unchanged in the *csk1Δ* strain (Hermand *et al*., 1998; Lee *et al*., 1999) and the fact that both *mcs6* and *mcs2* were isolated as loss-of-function mutants that rescued a hyperactive Cdc2 (Molz *et al*., 1989) although the strain was wild type with respect to *csk1*.

When compared with other species, our results on *in vivo* activation of Cdc2 indicate that fission yeast in this respect

is similar to *Xenopus* (Fesquet *et al*., 1997) and *Drosophila* (Larochelle *et al*., 1998), where Cdk7 has been implicated as the Cak of Cdc2. The role of Cdk7 in Cdc2 activation in *Drosophila* was questioned by results demonstrating that expression of a dominant-negative Cdk7 mutant during early division cycles did not inhibit Cdc2 phosphorylation (Leclerc *et al*., 2000). However, this could be due to stable maternal Cdk7 complexes in the early embryonic cycles.

The differences in the Cdc2- and Cdc28-activating kinases in fission yeast and budding yeast, respectively, may also at least partly explain interesting differences noted in the activating phosphorylation of Cdc2 and Cdc28 during a G_1 arrest. When fission yeast cells are arrested in G_1, Cdc2 is dephosphorylated (Simanis and Nurse, 1986), whereas a G_1 arrest in budding yeast cells does not affect Cdc28 phosphorylation (Hadwiger and Reed, 1988). In fission yeast, the Cdc2 dephosphorylation is associated with Rum 1 binding and cyclin B (Cdc13) degradation (Stern and Nurse, 1998), leading to a monomeric kinase, which is not expected to be a substrate of the Cdk7-type Mcs6 kinase (Kaldis *et al*., 1998). In budding yeast, the G_1 arrest is associated with disassembly of the Cdc28 complex (Wittenberg and Reed, 1988), but as Cak1 favors monomers (Kaldis *et al*., 1998), Cdc28 would continue to be phosphorylated. Interestingly, the mammalian counterpart Cdc2 becomes dephosphorylated upon shifting from exponential growth to quiescence (Lee *et al*., 1988), which may reflect the inability of Cdk7 to phosphorylate a monomeric Cdc2.

Materials and methods

Yeast strains and techniques

The *S.pombe* strains used in this study were: *h*$^+$ *ade6-210 ura4D18 leu1-32 his3-D1* (Burke and Gould, 1994), *h*$^+$ *mcs2::mcs2F leu1-32 ura4D18 ade6* (Molz and Beach, 1993); *h*$^-$ *csk1Δ mcs6-13 leu1-32 ade6-704* and *h*$^+$ *csk1::ura4*$^+$ *mcs2::mcs2F leu1-32 ura4D18 ade6* (Hermand *et al*., 1998). *Schizosaccharomyces pombe* was transformed using lithium acetate as described previously (Moreno *et al*., 1991).

The *S.cerevisiae* strains used were GF2351 *MAT*α *civ1-4 ura3 leu2 trp1 lys2 ade2 ade3* (Thuret *et al*., 1996). A high-efficiency transformation method was used (Gietz and Woods, 1998) in order to screen for *S.pombe* cDNAs capable of rescuing the GF2351 strain. The *S.pombe* cDNA library was kindly provided by Dr Michelle Minet and Dr Francois Lacroute.

Fission yeast expression vectors

All expression constructs used in *S.pombe* are based on two vectors: pREP3 (Maundrell, 1993) and pAAUN (Xu *et al*., 1990). *mcs2*-pAHA, csk1-pAHA, *myc-mcs6*-pREP3 and *myc-CDK7*-pREP3 have been described (Damagnez *et al*., 1995; Hermand *et al*., 1998). *mcs2*-pAHL and *csk1*-pAHL result from the exchange of the *ura4*$^+$ selection marker of pAHA by *LEU2* from pREP3. *KIN28*-pREP3 results from transferring a *Sal*I–*Bam*HI (underlined) PCR fragment from the *KIN28* cDNA (a kind gift of Michel Simon) using primers 5′-AGCGGCCGCGTCGAC-ATGAAAGTGAATATGGAG3′ and 5′-GCGGGATCCTCAGTTACG-TATTTTTATTG-3′.

The *CAK1* open reading frame (ORF) was amplified from *S.cerevisiae* genomic DNA using primers 5′-CGGAATTCACCATGAAACTGG-ATAGTATAGAC-3′ and 5′-TATGCGGCCGCTTATGGCTTTTCT-AATTCT-3′, and cloned as an *EcoR*I–*Not*I (underlined) fragment into pGEX-4T. The ORF was subsequently transferred into pREP3 in order to express wild-type Cak1p, or into pAHA (Hermand *et al*., 1998) and pAHL (see above) in order to express HA-Cak1.

In the experiment of Figure 2, *cdc2*, *cdc13*, *nim1*, *wee1*, *cdc25* and *suc1* were expressed from genomic inserts cloned in pWH5 (Wright *et al*., 1986). In the experiment in Figure 5B, a *cdc2-HA-pREP3* plasmid (a kind gift from B.Ducommun) was used, and the resulting strain was grown in the presence of thiamine to reduce the toxicity of overexpressed Cdc2-HA.

Budding yeast expression vectors

mcs6, *mcs2* and *csk1* ORFs were cloned under the control of a methionine-repressible promoter in p425, p424 and p426 (Mumberg *et al*., 1994), respectively. *mcs6-S165A* (Hermand *et al*., 1998) was transferred as a *Cla*I–*Pst*I fragment into *mcs6*-p425.

KIN28-p425 was made by transfer of the *KIN28* cDNA from pREP3 (see above) to p425. The insert for *CCL1*-p426 was made by PCR from *S.cerevisiae* genomic DNA using oligonucleotides 5′-ACG-CGTCGACGAATCCACCATGACGGATATTCAACTAAATGG-3′ and 5′-GCGGGATCCCTCGAGCGGTAACAGAGCTGTTTCATG-3′, and cloned as *Sal*I–*Xho*I (underlined) into p426. All plasmids were transformed in GF2351 (Thuret *et al*., 1996) and streaked out on media lacking methionine and the respective selection nutrients.

Construction and integration of mcs6 mutants

Construction of the *myc-mcs6-S165A*-pREP3 plasmid was as described previously (Hermand *et al*., 1998). The S165AL238R mutant of *mcs6* was made by replacing the insert in *myc-mcs6*-pREP3 with a fragment generated by a two-step PCR approach using the following primers: MopS165A1, 5′-AGCGGCCGCGTCGACACCATGGAACAG-3′; MopL238R2, 5′-GGGACGCTGTTGCATACTTTTAATG-3′; MopL238R3, 5′-AAAAGTATGCAACAGCGTCCC-3′; MopS165A4, 5′-TGCGGCC-GCGGATCCTTAAACAAATT-3′. The resulting product was verified by direct sequencing.

In order to create the S165E mutant of *mcs6*, the same strategy was used except that MopS165E2 (5′-TGGTGCTCCAT**GTG**GCTTG-3′) and MopS165E3 (5′-AAGCCACATG**GAG**CACCAGG-3′) were used. All constructs were verified by sequencing the resulting plasmids. Subsequently, *Sal*I–*Bam*HI inserts containing the SA, SE or SALR mutants were transferred to the pRS306 plasmid (Sikorski and Hieter, 1989) with the *kan*R cassette of pFA6a-kanMX (Bahler *et al*., 1998).

An *Eco*RV fragment of the pSK-*mcs6* G, containing a *Hin*dIII *mcs6* genomic insert, was then replaced by *Eco*RV inserts harboring the mutants and the *kan*R cassette. The *Xho*I–*Xba*I linear fragments were used to transform the strain: *h*$^+$ *ade6-216 ura4D18 leu1-32 his3-D1*. Transformants were plated on YE media for 12 h and replicated on YE media supplemented with G418 in order to select for the presence of integrated *mcs6* mutants. Integration at the correct locus was confirmed by Southern blotting.

Antibodies and immunoprecipitations from S.pombe cells

The rabbit polyclonal Csk1, Mcs6 and Mcs2 antisera have been described (Hermand *et al*., 1998). Monoclonal HA antibody (Boehringer Mannheim) or Anti-FLAG M5 antibody (Eastman Kodak Company) were used as per the manufacturer's instructions. 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 (PBS), 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, 5 mM NaF]. Following disruption, lysates were adjusted to 0.5% NP-40 for immunoprecipitation. 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 dithiothreitol (DTT)].

Recombinant protein expression and purification

For protein expression, GST–Cak1 (a kind gift from Philipp Kaldis and Mark Solomon; Kaldis *et al*., 1998), GST–Csk1 (Hermand *et al*., 1998), Mcs6 (Hermand *et al*., 1998) and Mcs2 (Hermand *et al*., 1998) baculoviruses were propagated in Hi5 insect cells (Invitrogen) for 48 h. The GST–Csk1, GST–Cak1 and GST proteins were purified using glutathione–Sepharose and eluted with glutathione. Ten nanograms of purified protein were used per assay. Mcs6, Mcs6–Mcs2 and Mcs6–Mcs2-Pmh1 complexes were purified with Mcs6 antiserum and used bound to protein A–Sepharose. Protein amounts were estimated by silver staining and 100 ng of purified proteins were used for kinase assays. Bacterially expressed GST–CDK2-D155N and GST–CTD proteins were purified as described (Damagnez *et al*., 1995; Hermand *et al*., 1998).

Affinity purification with GST–suc1, GST–Cak1 and GST–Csk1

Extracts from fission cells were prepared from 100 ml cultures as above, washed once in PBS, and disrupted in 1× pellet volume of lysis buffer

[buffer A (50 mM Tris pH 7.5, 50 mM KCl, 5 mM EDTA, 10 mM β-glycerophosphate, 2 mM PMSF, aprotinin, leupeptin) supplemented with 0.5 M NaCl, 5 mM DTT and 1% Igepal CA-630] and subsequently diluted with 2× volume buffer A.

Baculovirus-infected (48 h) Hi5 cells were washed in PBS, treated 10 min on ice in buffer A with 5 mM DTT and disrupted with a 23G needle. The lysates were diluted with an equal volume of buffer A with 300 mM NaCl and 0.2% Igepal CA-630.

To purify Cdc2 from *S.pombe* lysates, 1 µl of GST–Suc1 agarose (Upstate Biotechnology) was incubated with 200 µg of lysate on ice for 30 min. Alternatively, yeast or insect cell lysates (1 mg in 400 µl) were supplemented with 2–4 µg of purified GST, GST–Csk1 or GST–Cak1 and incubated on ice for 30 min followed by purification with glutathione–Sepharose as above. Beads were washed four times with washing buffer (50 mM Tris pH 7.5, 50 mM KCl, 0.1% Igepal CA-630) and used in kinase assay (see below).

Kinase assays

Kinase assays on immunoprecipitates from *S.pombe* cells were performed essentially as described (Hermand *et al*., 1998). Briefly, reactions were performed in 30 µl of kinase buffer supplemented with 10 µCi of [γ-^{32}P]ATP and indicated substrates (4 µg of GST–CDK2-D155N, 4 µg of GST–CTD) for 30 min at 30°C. When using baculovirus proteins, 10 ng of GST–Csk1, GST–Cak1, or 100 ng of Mcs6 (and complexed proteins as indicated) in immunoprecipitates were used as kinases. In the Cdk-activation experiments, GST–Csk1 or GST–Cak1 was incubated with Mcs6, Mcs2 or Mcs6–Mcs2 bound to beads for 6 min at 30°C in kinase buffer supplemented with 1 mM ATP. Subsequently, the beads were washed three times with kinase buffer before a kinase reaction with [γ-^{32}P]ATP with GST–CTD substrate. Phosphorylated substrates were analyzed by 10% SDS–PAGE followed by autoradiography.

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