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

Damien Hermand¹,², Thomas Westerling¹, Arno Pihlak¹, Jean-Yves Thuret³, Tea Vallenius¹, Marianne Tiainen¹, Jean Vandenhaute², Guillaume Cottarel⁴, Carl Mann³ and Tomi P. Mäkelä¹,⁵,⁶

¹Haartman Institute & Biocentrum Helsinki, University of Helsinki, 00014 Helsinki, ²HUCH Laboratory Diagnostics, 00029 HYKS, Finland, ³Laboratoire de Génétique Moléculaire (GEMO), University of Namur (FUNDP), 61 Rue de Bruxelles, 5000 Namur, Belgium, ⁴Service de Biochimie et Genetique moleculaire, CE/A/Saclay, F-91191 Gif-sur-Yvette Cedex, France and ⁵Genome Therapeutics Corp., 100 Beaver Street, Waltham, MA 02154, USA
⁶Corresponding 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 Cdns in vitro and rescue a thermosensitive budding yeast CAKI 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 Csk1 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).
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 Cdns 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 mc2 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 (*civl-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 *civl-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 *mc2* were transformed into the thermosensitive CAK1 strain. As shown in Figure 1B–D, overexpression of either *mcs6* or *mc2* 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 *Clil1* does not rescue the phenotype (Figure 1E) as the complex lacks Cak activity *in vitro* and is not a Cak in *vivo* (Cisowski 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 mc6-13 csk1Δ phenotype but not the csk1Δ phenotype**

The complementation results described above show that both Mcs6 and Csk1 have Cak activity when
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 sucl 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 G1- 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 G2 activators of Cdc2 rescue this strain, it is likely that it is mainly affected in the G2–M transition. This would be in agreement with previous observations indicating that higher levels of Cdc2 activity are required at the CG2–M transition compared with the G1–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 over-expressed (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
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 Cdk5, 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).
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

*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 Cdns, but not in Kin28. Subsequently, we reproduced this mutation *in vitro* and combined it with the *S165A Csk* 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数据, 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 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 G1 arrest. When fission yeast cells are arrested in G1, Cdc2 is dephosphorylated (Simanis and Nurse, 1986), whereas a G1 arrest in budding yeast cells does not affect Cdc28 phosphorylation (Hadwiger and Reed, 1988). In fission yeast, the Cdc2 dephosphorylation is associated with Rum1 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 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 Cdc2 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+ csk1 Δmcs6-13 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 MATa α1 ura3 leu2 trpl1 by2 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 are on two vectors: pREP3 (Mandrell, 1993) and PAAN (Xu et al., 1990). mcs2-pHA, csk1-pHA, mcs6-pREP3 and mcs-CDK7-pREP3 have been described (Damagnez et al., 1995; Hermand et al., 1998). mcs2-pAH and csk1-pAH results from the exchange of the ura4+ selection marker of pAH by LEU2 from pREP3. pKIN28-pREPS results from transforming a S aficion-HHI (underlined) PCR fragment from the KIN28 cDNA (a kind gift of Michel Simon) using primers 5’-AGGGGCGGTTCCGAGATGAGATATGGAG3’ and 5’-GGGCTATTCACTCAGTACTCAG-ATTATTAGT-3’.

The Csk1 open reading frame (ORF) was amplified from S. cerevisiae genomic DNA using primers 5’-CGGGAAATCAGGTTGAATTGGATCACTG-3’ and 5’-TAGTGAGATATGGAG3’ and cloned as an EcoRI-NorI (underlined) fragment into pGEX-4T-1. The ORF was subsequently transferred into pREP3 in order to express wild-type Csk1 or into pHA (Hermand et al., 1998) and pAH (see above) in order to express HA-Csk1.

In the experiment of Figure 2, cdc2, cdc13, nml1, wee1, cdc25 and succ1 were expressed from genomic inserts cloned into pWH5 (Wright et al., 1989). In the experiment of Figure 5B, a cdc2-PA-HA-pREP3 plasmid (a kind gift from B.Duesmann) was used, and the resulting strain was grown in the presence of thiamine to reduce the toxicity of overexpressed Cdc2-IA.

Budding yeast expression vectors

mcs6, mcs2 and csk1 ORFs were cloned under the control of a methionine-repressible promoter in p425, p424 and p426 (Munberg et al., 1994), respectively. mcs6/S165A (Hermand et al., 1998) was transferred as a Cak-Prep fragment into mcs6-p425.

KIN28-p425 was made by transfer of the KIN28 cDNA from pREP3 (see above) to p425. The insert for CLL1-p426 was made by PCR from S. cerevisiae genomic DNA using oligonucleotides 5’-AGGCTCGAGGATCCATGAGATATGGAG-3’ and 5’-GGGCTATTCACTCAGTACTCAG-3’, and cloned as SalI-XhoI (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 mcs6/S165A pREP3 plasmid was as described previously (Hermand et al., 1998). The S165A/CL238R mutant of mcs6 was made by replacing the insert in mvc6-238-rep3 with a fragment generated by a two-step PCR approach using the following primers: Mops/S165A, 5’-AGGGGCGGTTCCATGAGATATGGAG-3’ and Mops/L238R, 5’-GGGCTATTCACTCAGTACTCAG-3’. Mops/L238R, 5’-AGGGGCGGTTCCATGAGATATGGAG-3’ and Mops/S165E3, 5’-AGGGGCGGTTCCATGAGATATGGAG-3’ were used. All constructs were verified by sequencing the resulting plasmids. Subsequently, SalI-BamHI inserts containing the SA, SE or SALK mutants were transferred to the pRS306 plasmid (Sikorski and Hieter, 1989) with the kanR cassette of pFAGu-kanMX (Baker et al., 1998).

An EcoRV fragment of the pSK-mcs6 G, containing a HindIII mcs6 genomic insert, was then replaced by EcoRV inserts harboring the mutants and the kanR cassette. The XhoI–XhoI 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) was 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 MgCl2, 2.5 mM MnCl2, 1 mM dithiothreitol (DTT)].

Recombinant protein expression and purification

For protein expression, GST–Csk1 (a kind gift from Philipp Kaldis and Mark Solomon; Kaldis et al., 1998) or GST–Csk1 (Hermand et al., 1998), Mcs6 (Hermand et al., 1998) and Mcs2 (Hermand et al., 1998) baculoviruses were propagated in H5 insect cells (Invitrogen) for 48 h. The GST–Csk1, GST–Csk1 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–pM1 complexes were purified with Mcs6 antisera 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–Csk1 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
Kinase assays
Kinase assays on immunoprecipitates from S. pombe cells were performed essentially as described (Hermund et al., 1998). Briefly, reactions were performed in 30 µl of kinase buffer supplemented with 10 µCi of [γ-32P]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-32P]ATP with GST–CTD substrate. Phosphorylated substrates were analyzed by 10% SDS–PAGE followed by autoradiography.

Acknowledgements
We thank Philipp Kaldis, Mark Solomon, Paul Nurse, Sergio Moreno, Gerard Faye, Michel Simon, Michelle Minet, Francois Lacroute, Eberhard Schneider, Bernard Ducommun, David Beach, Peter Wagner and Nina Korsisara for providing reagents. We are grateful to Beata Grallert and Eric Boye for fruitful discussions and help with fission yeast genetics. This study was supported by grants from Academy of Finland, University of Helsinki, Finnish Cancer Organization, Finnish Cancer Institute and Sigrid Juselius Foundation. D.H. is a FNRS Postdoctoral Researcher; A.P. is a graduate student of the Helsinki Graduate School in Biotechnology and Molecular Biology; T.W. and T.V. are graduate students of Helsinki Biomedical Graduate School.

References


Fesquet,D. et al. (1993) The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. EMBO J., 12, 3111–3121.


*Received November 2000; revised and accepted November 20, 2000*