Cmk2, a novel serine/threonine kinase in fission yeast

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Abstract **The *cmk2* gene of *Schizosaccharomyces pombe* encodes a 504 amino acid protein kinase with sequence homology with the calmodulin-dependent protein kinase family. The *cmk2*$^+$ gene is not essential for cell viability but overexpression of *cmk2*$^+$ blocks the cell cycle at G2 phase and this inhibition is *cdc2*-dependent. The Cmk2 is a cytoplasmic protein expressed in a cell cycle-dependent manner, peaking at the G1/S boundary. Overexpression of Cmk2 suppresses fission yeast DNA replication checkpoint defects but not DNA damage checkpoint defects, suggesting that the G2 cell cycle arrest mediated by high levels of Cmk2 provides sufficient time to correct DNA replication alterations.**

Key words: Fission yeast; Calmodulin-dependent kinase; Mitosis; Cell growth; Actin; Replication checkpoint

1. Introduction

Calmodulin (CaM) plays an important role in controlling cell proliferation in eukaryotic cells [1]. CaM is essential for viability and cell proliferation in every organism tested [2–4]. Reversible protein phosphorylation plays an important role in the control of cell proliferation. Therefore, it is reasonable to think that the function of CaM in cell cycle control might be mediated at least in part either through regulation of CaM-dependent kinases (CaMKs) or phosphatases. There are several CaMKs [5,6], and at least one CaM-dependent phosphatase (calcineurin) [7,8]. Several evidences suggest that CaMKs play a role in cell cycle regulation at G2/M transition. Lowering CaMKII protein levels in *Aspergillus nidulans* cells slowed the cell cycle in G2 [9]. Additionally, studies in HeLa cells indicate that CaMKII is involved in the onset of mitosis through phosphorylation of Cdc25 phosphatase [10]. Inhibition of CaMKII with KN-93 (a water-soluble inhibitor of CaMKII) or the microinjection of AC3-I (a specific peptide inhibitor of CaMKII) blocks cell cycle in G2. In KN-93-arrested cells, Cdc25 is not phosphorylated and, as a consequence, Cdc2 is not activated [10]. In contrast, overexpression of a constitutively active form of CaMKII in both mouse C127 cells [11] and fission yeast [12] results in G2 arrest. In the case of fission yeast this is due to low Cdc2 kinase activity.

CaMKII also regulates positively G1/S progression in both *A. nidulans* [9] and mammalian cells [1]. CaMKI and CaMKIV have recently been described as members of a CaM-dependent signal transduction cascade. They are activated by CaMK kinase protein, which phosphorylates and enhances the protein kinase activity of CaMKs [5,6]. Recent studies in fission yeast have described a CaMKI homologue, *cmk1*$^+$. The levels of *cmk1*$^+$ mRNA are cell cycle-regulated, being maximal coincident with S phase. Overexpression of a hyperactive form of *cmk1*$^+$ caused cell cycle arrest, and morphological defects, suggesting that Cmk1 may act regulating proliferation in eukaryotic cells [13].

We describe here a fission yeast CaMK homologue, which we named *cmk2*$^+$. *cmk2*$^+$ is a non-essential gene encoding a serine/threonine protein kinase. Overproduction of Cmk2 arrests cells in G2 phase and this G2 arrest is sufficient to bypass DNA replication checkpoint defects but not DNA damage checkpoint defects.

2. Materials and methods

2.1. Fission yeast strains, media and growth conditions

The strains are listed in Table 1. Rich medium was YES, selective medium was EMM2 supplemented with 225 mg/l of the required amino acids [14]. Standard techniques for fission yeast genetics were followed [14]. Fission yeast constructions were transformed by lithium acetate as described [14]. Micrographs of *Schizosaccharomyces pombe* cells were obtained using a Zeiss Axioplan microscope with an MC80 photographic camera incorporated. DAPI staining was as described [14].

2.2. Molecular biology techniques

Standard molecular biology techniques were used. Restriction enzymes were used as recommended by their suppliers (New England Biolabs or MBI Fermentas). DNA fragments were recovered from agarose gels with Clontech's Advantage PCR pure kit, following the manufacturer's instructions.

2.3. DNA manipulation and plasmids construction

The *cmk2*$^+$ gene was PCR-amplified from a *S. pombe* library (Edgar and Norbury, unpublished results) using the primers: cmk2fwd and cmk2rev designed according to the sequence of *cmk2*$^+$ from cosmid C23A1 of fission yeast chromosome I.

Plasmids pREP1-*cmk2*$^+$ and pREP41-*cmk2*$^+$ were constructed as follows: the complete open reading frame (ORF) of *cmk2*$^+$ gene was amplified as described above and the following oligonucleotides: cmk2fwd, containing the *Nde*I restriction site in front of the initiation codon of the *cmk2*$^+$ gene (underlined) CACACACACACATATGTCGATACTAGCGGTA and cmk2rev countering the *Not*I restriction site just after the last codon of the *cmk2*$^+$ gene (underlined) GGGGGGCGGCCGCTATTAACACGTTTAGCAGA. The resulting 1.5 kb PCR product was digested with *Nde*I and *Not*I and ligated into a pREP1 plasmid [15,16] digested with the same enzymes. The resulting plasmids containing the entire *cmk2*$^+$ ORF under the inducible promoters *nmt1* and *nmt41* fused to two hemagglutinin (HA) epitopes and a hexahistidine tag at the COOH-terminal region were transformed to *S. pombe* strains (Table 1) as episomal plasmids. Mutagenesis of *cmk2*$^+$ to create *cmk2KA* was performed by PCR using

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overlapping oligonucleotides at the site of the mutation (K94A). Following subcloning to pREP1 plasmid, the mutation was verified by DNA sequencing.

The bacterial expression plasmid pGEX-KG allows the expression of GST-fused proteins in *Escherichia coli*. *cmk2*$^+$ and *cmk2KA* were digested with *Nde*I and *Not*I and cloned into the pGEX-KG plasmid.

2.4. *cmk2*$^+$ gene disruption

The *cmk2::ura4*$^+$-deleted mutant was generated by inserting a 1.8 kb fragment encoding the *ura4*$^+$ gene between the *Bgl*II–*Hin*dIII sites of *cmk2*$^+$ from plasmid pVA21 (the plasmid pBluescript that contained the chromosomal *Pst*I–*Xho*I fragment from *cmk2*$^+$).

ura4$^+$ gene was amplified from pURA4 plasmid using VA6 and T7 oligonucleotides, where VA6 is essentially the standard T3 promoter oligonucleotide with an added *Bgl*II site (underlined): CGCCAAAGATCTATTAACCCTCACTAAAG. The amplified fragment was digested with *Bgl*II and *Hin*dIII and ligated to pVA21, creating plasmid pVA24. The fragment *Pst*I–*Bam*HI isolated from the plasmid pVA24 was used to transform wild-type strain. Stable *ura*$^+$ transformants were confirmed by PCR and Southern blotting.

2.5. Chromosomal integration of cmk2-HA6His

To tag genomic *cmk2*$^+$ with two copies of the HA epitope and hexahistidine, plasmid pREP1-*cmk2*$^+$ was digested with *Pst*I and *Sac*I, releasing a ~3 kb fragment that contained the full *nmt1-cmk2*$^+$ expression cassette. This was cloned into pBluescript SK$^-$ (Stratagene) digested with the same enzymes. The resulting plasmid was digested with *Hin*dIII, which released the full *nmt1* promoter and the first 215 amino acids of Cmk2, and ligated to *ura4*$^+$ from pURA4 plasmid digested with *Hin*dIII. The resulting construction was linearised with *Eco*RI and transformed into the appropriate *S. pombe* strains and selected for stable *ura4*$^+$ transformants. Colonies that grew on selective media were screened for HA integration by immunoblotting, with a specific anti-HA epitope antibody.

2.6. Protein extracts and Western blotting

Twenty optical densities (ODs) of exponential growing cultures (OD_{600} = 0.5) were used to prepare boiled protein extracts for Western blotting as described in Hostenbach's web page (http://www.bio.uva.nl/pombe/handbook/). Primary antibodies: 1/1000 of monoclonal antibody anti-HA (12CA5) from ascites fluid or 1/2000 of polyclonal anti-PSTAIR (Upstate Biotechnology) were used and detected with horseradish peroxidase-conjugated antibodies against mouse or rabbit (Bio-Rad). Membranes were visualised by enhanced chemoluminescence (ECL kit, Amersham-Pharmacia).

2.7. Autophosphorylation kinase assay

For in vitro kinase assays, *cmk2*$^+$ and *cmk2KA* fused to GST were expressed in *E. coli* and pellets lysed in NETN buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 plus protease inhibitors). GST-Cmk2 and GST-Cmk2KA were purified by absorption to glutathione-Sepharose beads (Pharmacia).

GST-Cmk2 and GST-Cmk2KA autophosphorylation assays were performed with 350 nM of purified protein in 20 μl kinase buffer (50 mM Tris–HCl pH 8.00, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol) plus 0.2 μl [γ-^{32}P]ATP (10 μCi/μl, Amersham-Pharmacia). Reaction was performed during 30 min at 30°C and stopped with 5 μl 5× sample buffer. The same reaction was also done by adding 350 nM of CaM and 1 mM $CaCl_2$ or 5 mM EGTA. *S. pombe* CaM was expressed in bacteria using the plasmid pET20-*cam1*$^+$ and purified as described in [17].

2.8. Immunofluorescence microscopy

Immunofluorescence microscopy was performed using methanol fixation as described in Hostenbach's web page (http://www.bio.uva.nl/pombe/handbook/). Antibodies to actin (ICN) and rat monoclonal HA (3F10, Boehringer Mannheim) were detected with cyanine 3-conjugated anti-mouse (Jackson Immuno Research), FITC-conjugated goat anti-rat IgG (Sigma Immuno Chemicals).

Cells were examined using a Leica JCS-NT confocal laser-scanning microscope. The cyanine 3 or TRITC and FITC signals were merged with the confocal image analysis software.

2.9. Analysis of hydroxyurea (HU) response

Wild-type cells, *rad1*$^-$ and *rad1*$^-$ cells expressing the different levels of Cmk2 from episomal or integrated plasmid were grown to midlog phase in rich media and HU was added to 12 μM final concentration. From time 0, cell samples were collected every 2 h, washed and counted for survival estimation. 500 cells from each culture were plated on to YES media and incubated for 2 days at 32°C. The septation index was followed under a white light phase contrast microscope. An aliquot of each sample was treated for DAPI staining in order to determine the percentage of cells with the cut phenotype, and an aliquot was treated to estimate the cellular DNA content using a Becton-Dickinson FACScan.

2.10. UV irradiation treatment

Wild-type cells, *rad1*$^-$ and *rad1*$^-$ cells expressing the different levels of Cmk2 from episomal plasmid were grown to midlog phase. 1000 cells of each culture were plated in YES and UV-irradiated at 254 nm with a Stratalinker (Stratagene) at different J/m^2. Survival was assayed as colony number after 4 days of growth on YES plates at 30°C.

3. Results

3.1. The cmk2 locus encodes an ORF similar to known CaMKs

Examination of the fission yeast genome sequencing project (Sanger Centre) identified an ORF with homology to CaMKs, that we called *cmk2*. The ORF is reported in chromosome 1 cosmid (C23A1, accession number AL021813) and it contains three exons encoding a putative Ser–Thr protein kinase of 504 amino acids and a predicted molecular weight of 57 kDa. A computer-based amino acid sequence homology search for known proteins revealed that the greatest degree of amino acid sequence identity was shared with budding yeast RCK1 and RCK2 (CLK1) kinases (42% and 43% identity respectively), and to CaMKs (40% identity to rat CaMKI and 35% to rat CaMKII) (Fig. 1). RCK1 and RCK2 were first described as suppressors of radiation sensitivity of fission yeast G2 arrest deficient mutants (*rad1*, *rad3*, *rad9*, *rad17* and *chk1*) but not of repair deficient strains (*rad15*, *rad13*,

Table 1
S. pombe strains

Strain	Genotype	Source
RA4	*h-leu1-36 ura4-D18*	Lab stock
VA262	*h-cmk2::ura4 leu1-36 ura4-D18*	This study
RA106	*h-leu1-36 ura4-D18 cdc25-22*	Lab stock
VA126	*h-leu1-36 ura4-D18 cdc25-22 cmk2-HA6His (ura4+)*	This study
	h-leu1-36 ura4-D18 rad1::ura4	J. Millar
MB198	*h-leu1-36 ura4-D18 rad1::ura4 nmt1-cmk2-HA6His*	This study
MB199	*h-leu1-36 ura4-D18 rad1::ura4 nmt41-cmk2-HA6His*	This study
RA78	*h-leu1-36 ura4-D18 cdc2-3w*	Lab stock
	h-leu1-36 ura4-D18 cdc2-3w cdc25::ura4	J. Millar
RA34	*h-leu1-36 cdc2-33 cdcF15A*	Lab stock
	h-leu1-36 ura4-D18 rad3::ura4+	P. Russell

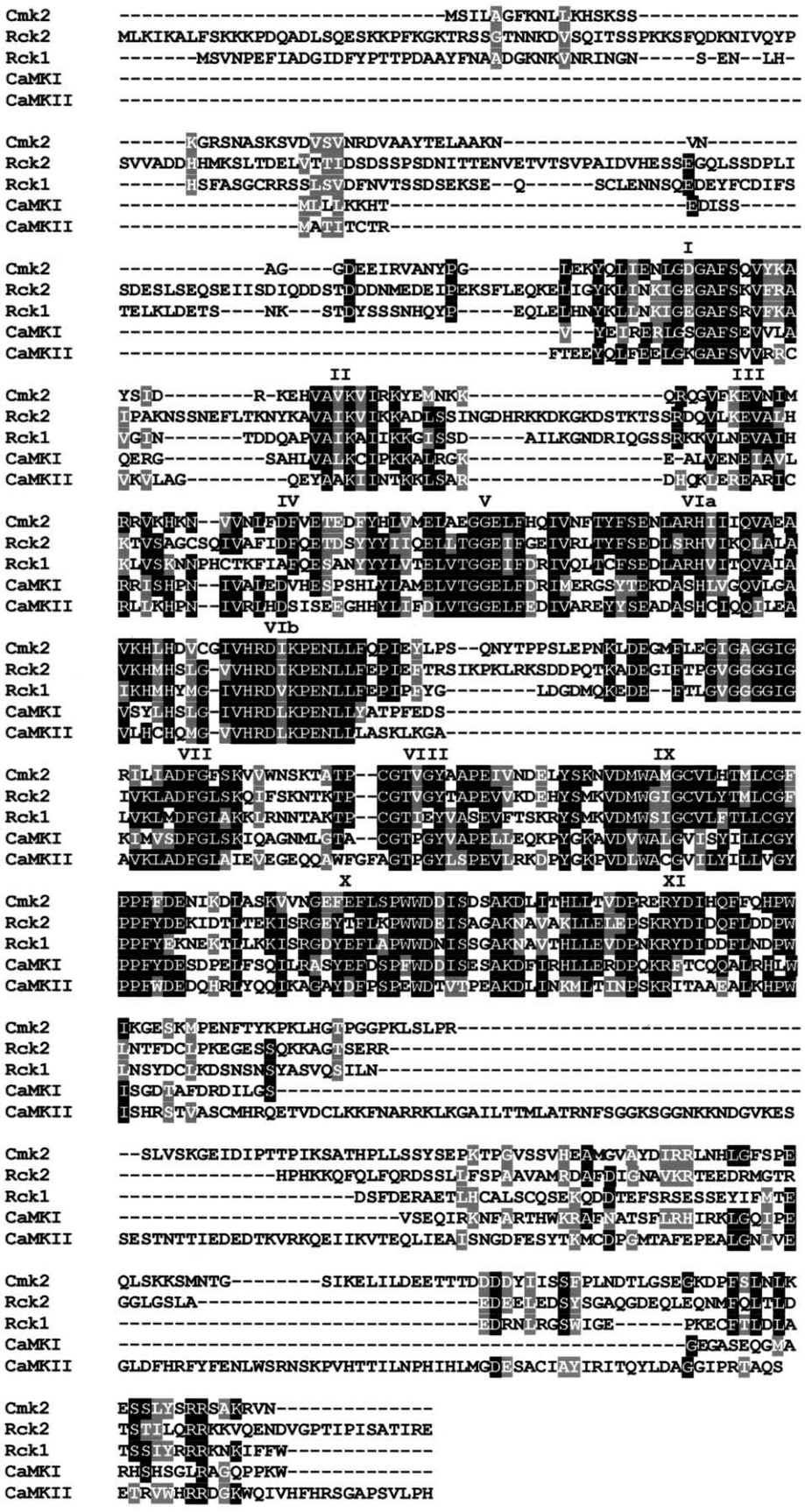

Fig. 1. Amino acid comparison of Cmk2 with RCK1, RCK2, CaMKI and CaMKII. The protein sequences were compared using the ClustalX program. Identical amino acids are shown as white against black, conserved amino acids are shown as white against grey. The 11 consensus domains of the kinases are marked as roman numerals. Dashes indicate single-residue gaps introduced to maximise the alignment.

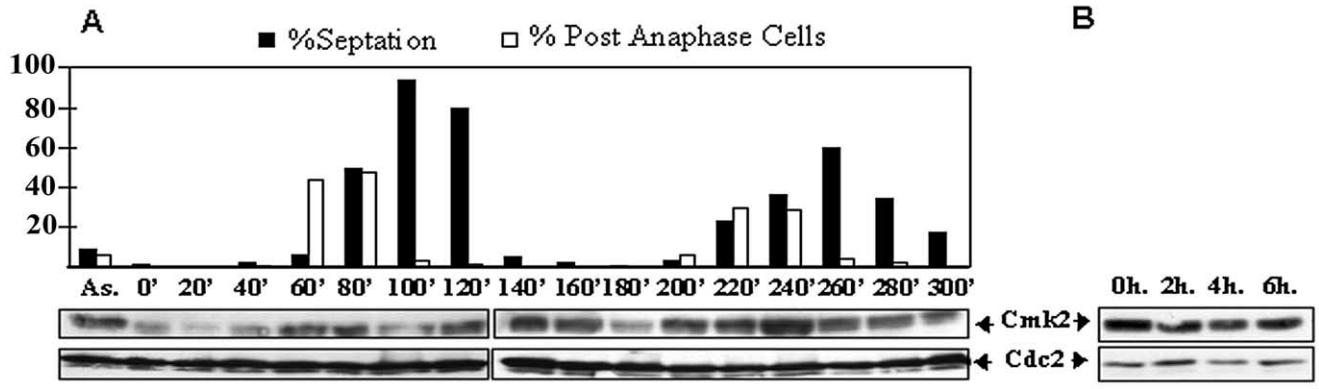

Fig. 2. Cmk2 protein levels oscillate throughout the cell cycle. A: A synchronous culture was generated by block and release of *cdc25-22* strain carrying *cmk2*-HA6His expressed from the endogenous promoter. Cells were blocked at 35°C during 4 h and harvested at the indicated time points after shift to 25°C, extracts were prepared and Cmk2-HA6His detected by Western blotting. Asynchronously growing cells (lane 1) were used as a reference and $p34^{cdc2}$ protein level was assessed on the same blot as a loading control. Synchrony was monitored by septation index (filled boxes) and percentage of cells which have passed anaphase (one cell with two separated nuclei without septum) (empty boxes). B: Cells carrying *cmk2*-HA6His expressed from the endogenous promoter were grown at 25°C to early log phase and shifted to 35°C. After shifting samples were taken at different times to detect Cmk2 and Cdc2 by Western blot as in A.

rad 21 and *rad22*) [18]. They have particular features such as a long Gly rich insert between consensus domains VIb and VII of protein kinases, which is also present in Cmk2. Rck2p/Clk1p was also described as a CaMK-like protein [19] although it did not bind CaM.

Cmk2 also shares homology with CaMKs (CaMKI and CaMKII) from vertebrates, particularly in its catalytic domain and at the C-terminus.

3.2. *cmk2*$^+$ *is not an essential gene*

To examine the role of *cmk2*$^+$ in *S. pombe* cell cycle, a construct was generated in which *cmk2*$^+$ was replaced by the *S. pombe ura4*$^+$ gene (see Section 2). This construct was used to replace the genomic copy of *cmk2*$^+$ in an *ura4-D18* strain. The correct integration of the construct in the resulting strain was confirmed by Southern hybridisation analysis (data not shown). The *cmk2::ura4* strain was viable, and presented no morphological abnormalities.

3.3. *Expression of Cmk2 is cell cycle-regulated*

To examine expression of Cmk2 during the cell cycle, the levels of Cmk2 were assessed by Western blotting in extracts from synchronous cultures of *cdc25-22* cells expressing *cmk2*$^+$-HA6His from its own promoter. The post-anaphase and septation indices were used to gauge synchrony. Cmk2 levels increased at post-anaphase and decreased after septation when cells are entering G2 phase (Fig. 2A), indicating that Cmk2 abundance oscillates during cell cycle progression.

The same result was obtained using *cdc10-129* thermosensitive mutant strain to synchronise the cells (data not shown).

To rule out the temperature effect, wild-type cells with the endogenous *cmk2*$^+$-HA6His were grown at 25°C, shifted to 35°C, and then samples were taken at different time points. Temperature had no effect on Cmk2 protein levels (Fig. 2B).

3.4. *Cmk2 overexpression inhibits entry into mitosis via tyrosine phosphorylation of Cdc2*

In order to determine the effect of Cmk2 in *S. pombe* cells, *cmk2*$^+$ was overexpressed under the control of *nmt1* promoter (pREP1-*cmk2*$^+$) induced by removing thiamine (B1) from the growth medium. Cells overexpressing Cmk2 were unable to grow (Fig. 3A). The lethal effect of Cmk2 overexpression was due to its catalytic activity because the overexpression of the catalytically inactive Cmk2KA (Fig. 4, lane 4), which contains lysine 94 mutated to alanine, did not have a lethal effect and cells did not show any phenotype compared with overexpression of wild-type Cmk2 (Fig. 3A). Fig. 3B shows the protein level of active Cmk2 and inactive Cmk2KA expressed in the cells.

Cell cycle progression was blocked in cells overexpressing Cmk2. Cells were elongated and DAPI staining (Fig. 3C) and FACScan analysis (data not shown) revealed that cells overexpressing Cmk2 have a single nucleus and 2*n* DNA content, indicating a block in G2 phase. The same phenotype was observed when expressing *cmk2*$^+$ from an integrated form of nmt-*cmk2*$^+$ or nmt41-*cmk2*$^+$ (data not shown).

To examine whether overexpression of Cmk2 inhibits entry into mitosis through Cdc2 kinase, Cmk2 was overexpressed in a *cdc2-33 cdc2Y15F* strain. *cdc2-33 cdc2Y15F* strain contains two alleles of *cdc2*$^+$, a thermosensitive *cdc2-33* allele and *cdc2Y15F*, which is defective in tyrosine 15 phosphorylation. In this strain G2/M transition was delayed at permissive temperature by overexpressing Wee1 kinase, which phosphorylates tyrosine 15 of the *cdc2-33* allele (Fig. 3D, bottom right panel) but at restrictive temperature cells entered mitosis at a reduced size (wee phenotype) (Fig. 3D, bottom left panel). Cmk2 overexpression delayed mitosis of *cdc2-33 cdc2Y15F* at 25°C (Fig. 3D, middle right panel) but not at 32°C (Fig. 3D, middle left panel). This experiment demonstrated that the Cmk2-dependent mitotic arrest acts through the inhibition of $p34^{cdc2}$ mitotic kinase.

3.5. *In vitro Cmk2 kinase activity*

We expressed Cmk2 and Cmk2KA proteins in *E. coli* as GST-Cmk2 and GST-Cmk2KA fusion proteins to assess its kinase activity by autophosphorylation assays. Purified GST-Cmk2 and the catalytically inactive GST-Cmk2KA were incubated in the presence of [γ-^{32}P]ATP. As shown in Fig. 4, only wild-type Cmk2 was autophosphorylated. The autophosphorylation assay was also done in the presence of various concentrations of Ca^{2+}/CaM but no significant difference in its activity was observed (data not shown).

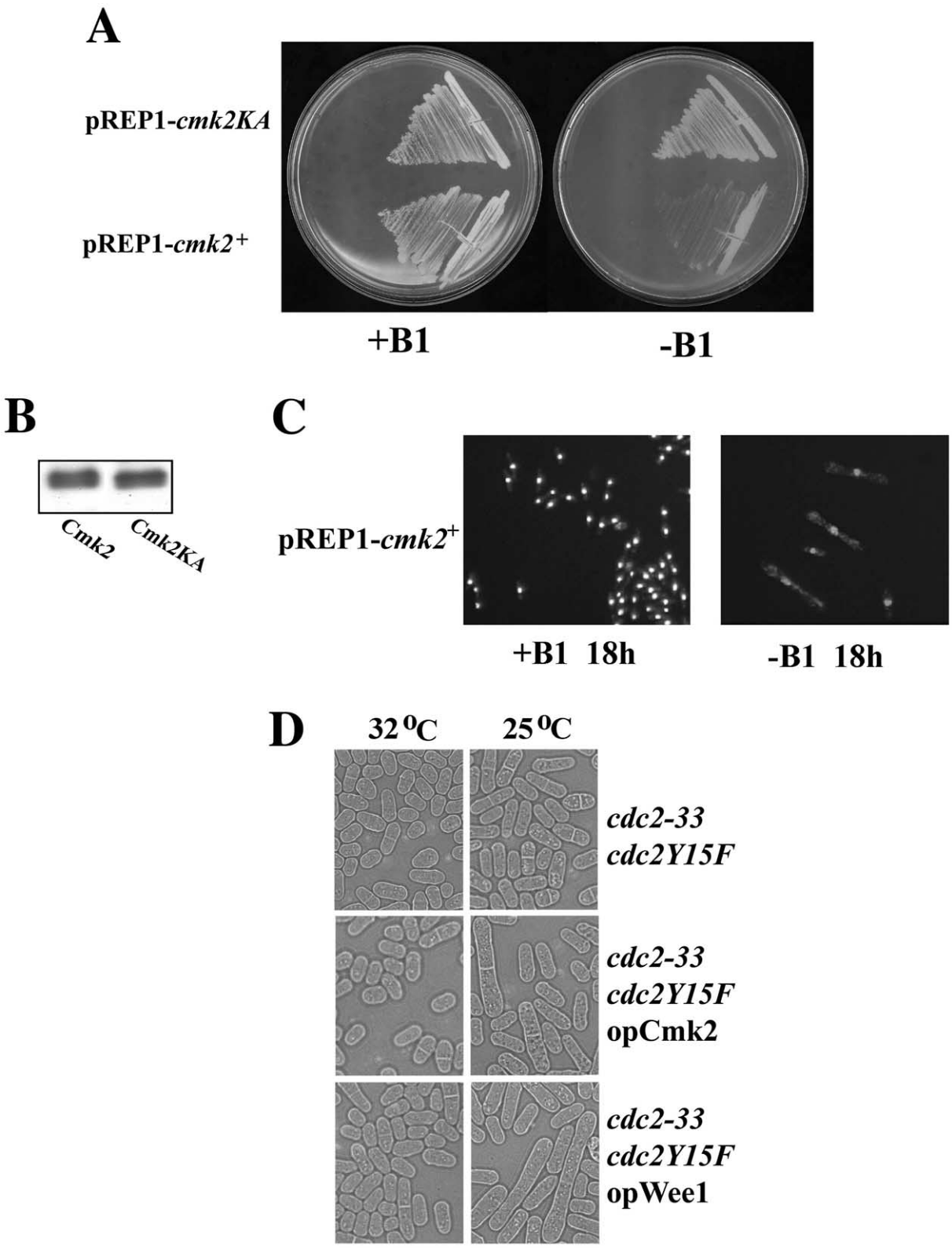

Fig. 3. Overexpression of *cmk2*$^+$ provokes G2 arrest. Wild-type cells expressing *cmk2*$^+$ or *cmk2KA* under the control of a strong *nmt1* promoter (pREP1-*cmk2*$^+$ and pREP1-*cmk2KA*), repressed (+B1) or induced (−B1). B: Western blot of cells overexpressing Cmk2 from pREP1-*cmk2*$^+$ and Cmk2KA from pREP1-*cmk2KA* using anti-HA antibody. C: Cells overexpressing Cmk2 were fixed in ethanol and stained with 4,6-diamino-2-phenylindole. The cells used were the same as in A. D: *cdc2-33 cdc2Y15F* grown at permissive (25°C) and semi-restrictive (32°C) temperature. Cmk2 and Wee1 kinases expressed from pREP1-*cmk2*$^+$ and pREP1-*wee1*$^+$ in *cdc2-33 cdc2Y15F* at permissive (25°C) and semi-restrictive (32°C) temperature in the absence of thiamine. Wee1 kinase was used as control of inhibitory kinase activity in *cdc2-33 cdc2Y15F* strain.

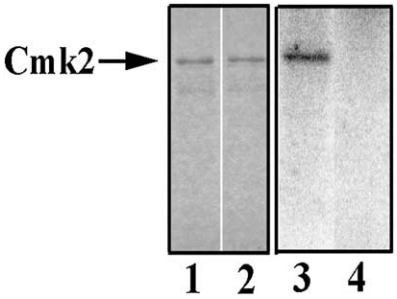

Fig. 4. Kinase activity of Cmk2. GST-Cmk2 (lanes 1 and 3) and GST-Cmk2KA (lanes 2 and 4) were purified from *E. coli* and assayed for autophosphorylation (lanes 3 and 4). Lanes 1 and 2 show Coomassie blue staining of purified proteins.

3.6. *Cmk2 overproduction suppresses DNA replication but not DNA damage checkpoint defects*

As shown in Fig. 1 *cmk2*$^+$ has a high homology with budding yeast *RCK1* and *RCK2* genes. Because RCK1 and RCK2 overexpression suppress cell cycle S/M checkpoint mutants in *S. pombe* [18], we examined whether *cmk2*$^+$ suppresses the checkpoint deficiency in *S. pombe rad1*-deleted mutant. Wild-type cells exposed to 12 mM HU cease to divide due to a checkpoint arrest [20]. Upon removal of the drug, wild-type cells reinitiate mitosis and division. The *rad1*-deleted strain is deficient in this arrest and cells continue to attempt going through mitosis and division when exposed to HU, which compromises cell viability (Fig. 5A). Overexpression of *cmk2*$^+$ in *rad1* mutant restores cell viability to wild-type level after HU treatment (Fig. 5A), indicating that Cmk2 can suppress sensitivity to DNA replication defects of *rad1* mutant. Overexpression of the ribosomal protein S7 in *rad1* mutant cells was used as a control to rule out the possibility that any overexpressed protein will rescue the viability of *rad1* mutant cells (Fig. 5A).

In *rad1* mutant cells the septum bisects the single nucleus resulting in aneuploid or anucleate cells; this resembles the morphology of cut mutants [21]. To analyse the effect of overexpressing *cmk2*$^+$ in *rad1*-deleted cells during exposure to HU we measured the percentage of cells that showed cut phenotype. As described, the *rad1* mutant showed a high percentage of cut phenotype cells (Fig. 5B). In contrast, expression of Cmk2 in these cells suppressed the cut phenotype (Fig. 5B).

To examine whether the ability of Cmk2 to suppress checkpoint defects was restricted to the *rad1* mutant or it was valid

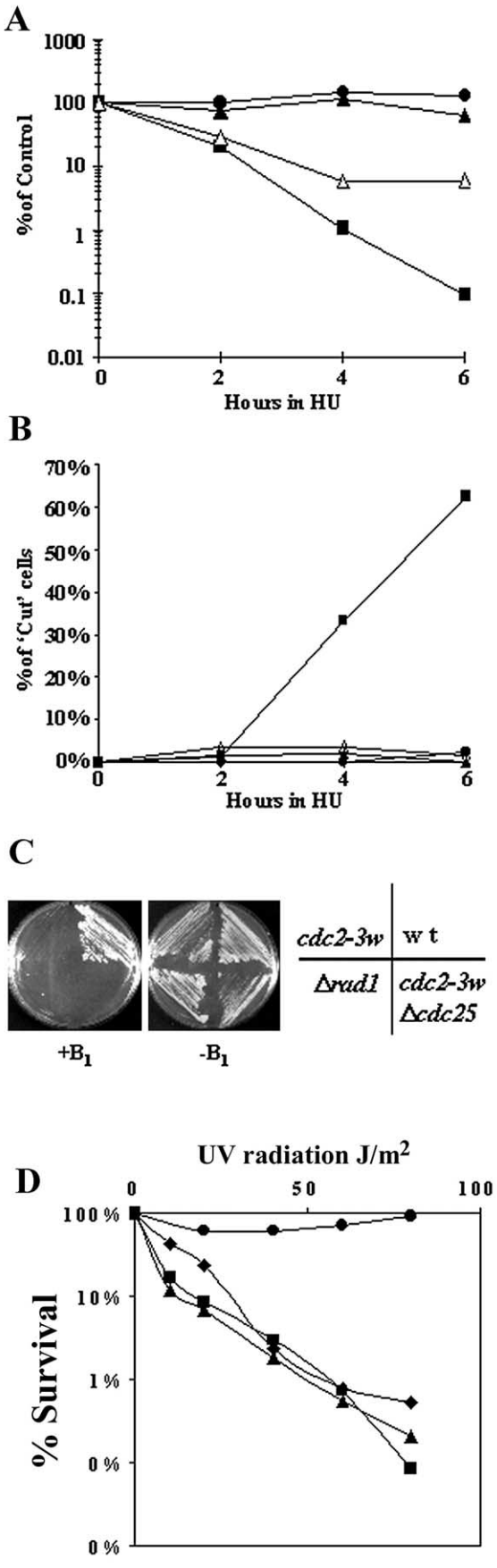

Fig. 5. cmk2$^+$ overexpression suppresses HU but not UV DNA damage sensitivity of checkpoint deficient mutants. A: Percentage of surviving cells after HU treatment of wild-type (filled circles), Δ*rad1* (filled squares) and Δ*rad1* overexpressing Cmk2 (filled triangles) or ribosomal protein S7 (open triangles) as a control of overexpression. B: Percentage of cells with defects in chromosome segregation ('cut' phenotype) during HU treatment. Cells from wild-type (filled circles), Δ*rad1* (filled squares), Δ*rad1* overexpressing *cmk2*$^+$ from strong *nmt1* promoter (open triangles) or weaker *nmt41* promoter (filled triangles). C: Cells from *cdc2-3w*, *cdc2-3w* Δ*cdc25*, wild-type and Δ*rad1* strains overexpressing Cmk2 ($-B_1$, right) or not ($+B_1$, left) were streaked on plates containing 5 mM HU. Photographs were taken after 2 days of growing at 32°C. D: Percentage of UV survival of wild-type cells (filled circles), Δ*rad1* (filled squares), Δ*rad1* overexpressing *cmk2*$^+$ from strong *nmt1* promoter (filled diamonds) or weaker *nmt41* promoter (filled triangles) strains.

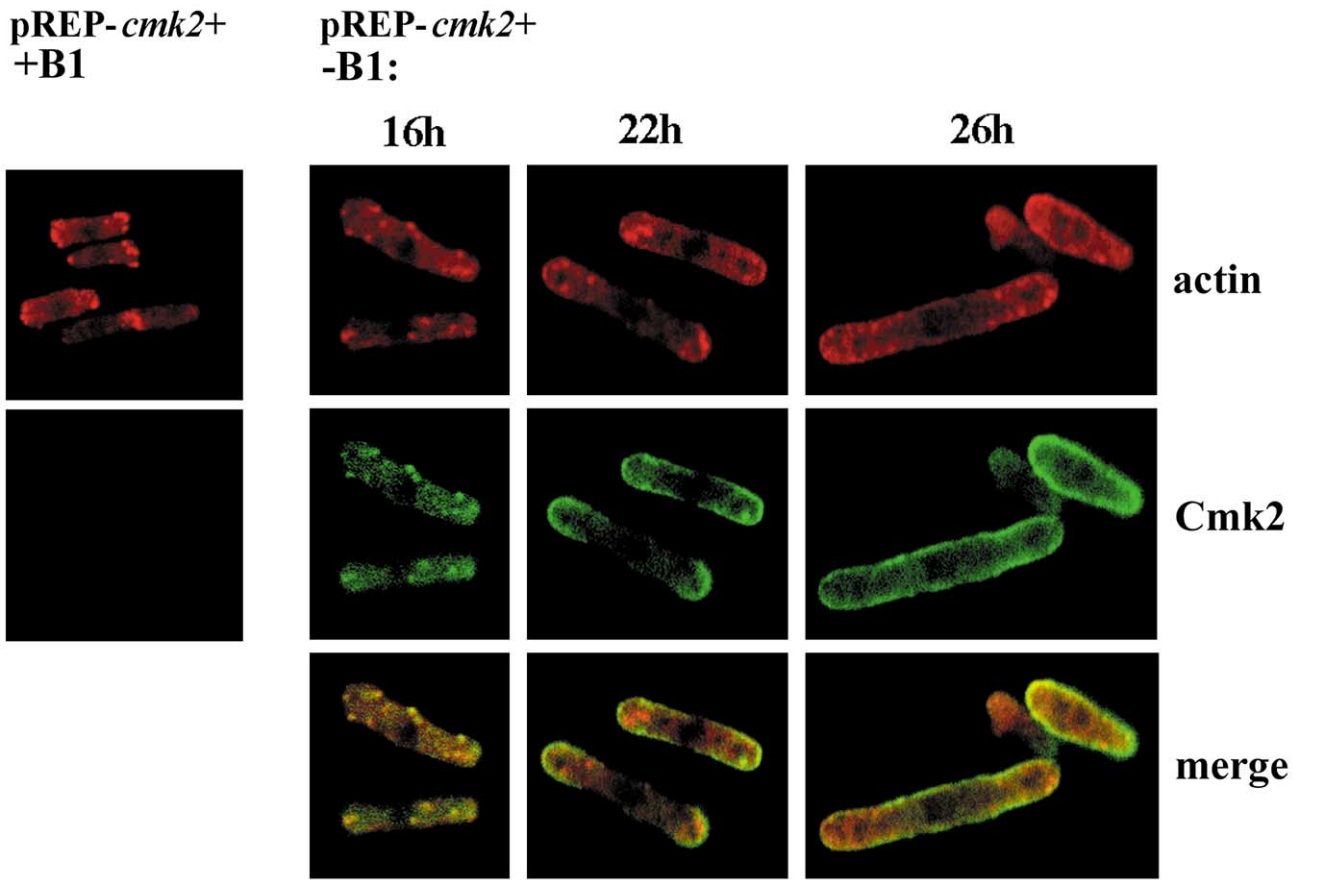

Fig. 6. Intracellular localisation of Cmk2. Overexpression of *cmk2*$^+$ from the *nmt41* promoter was repressed (+B1) or induced (−B1) at the indicated time points. Indirect confocal immunofluorescence was carried out using anti-actin antibodies and anti-HA antibodies to detect actin (red) and Cmk2 (green) proteins. Merging of the red anti-actin and green anti-HA (bottom panels).

for other DNA replication checkpoint mutants, we analysed other mutants defective in the S/M checkpoint as: *cdc2-3w* and *cdc2-3w Δcdc25*. These mutants fail to form colonies in the presence of HU [22]. In contrast, wild-type cells initially undergo cell cycle arrest in the presence of HU, and then resume cell cycle with a longer S phase, forming slow growing colonies of elongated cells. As shown in Fig. 5C, Cmk2 expressed in *cdc2-3w* and *cdc2-3w Δcdc25* mutants suppressed the sensitivity to HU as in *rad1* mutant.

In contrast, overexpression of Cmk2 could not suppress *rad1* sensitivity to DNA damage caused by UV light (Fig. 5D). The same results obtained in *rad1* were observed in *rad3* mutant (data not shown).

3.7. Subcellular localisation of Cmk2

To shed additional light on the potential physiological function of Cmk2, we determined in which subcellular compartment Cmk2 resides. We first used the *cmk2*$^+$-HA6His strain to localise Cmk2 but endogenous Cmk2 expression was too low to be detected. In a second experiment using the strain that overproduces *cmk2*$^+$ under the control of a moderate version of the *nmt1* promoter (*nmt41*) the anti-HA signal was exclusively cytoplasmic, but also showed accumulation of Cmk2 at the cortical sites of the cells (Fig. 6) similar to the actin patches. The increase in protein expression showed Cmk2 more concentrated at the polar ends (Fig. 6, middle panel) and, at longer expression, it was localised to or near the plasma membrane along the cell cortex (Fig. 6, middle right panel). Immunostaining of actin protein from the same samples showed a delocalised pattern throughout Cmk2 expression (Fig. 6, top panels). Actin patches were dispersed outside the polar zones. The delocalisation of actin patches was coincident with the localisation of Cmk2 to the poles. Although actin and Cmk2 co-migrated during the Cmk2 expression they did not co-localise (Fig. 6, bottom panels). These results suggest that Cmk2 movement to the cortical sites of the cell delocalises actin from the growing tips.

4. Discussion

We report the cloning and characterisation of a fission yeast gene, *cmk2*$^+$, which encodes a serine/threonine kinase similar to the CaMK family. It has high homology with mammalian CaMKs but the highest homology is shared with Rck1p and Rck2p kinases from *Saccharomyces cerevisiae* [23]. *RCK1* and *RCK2* were isolated as suppressors of radiation sensitivity in *S. pombe* checkpoint mutants and they also have high homology with CaMKs. Like RCK1 and RCK2, Cmk2 has a high density of serine doublets which, according to protein structure prediction [24], are placed in flexible hydrophilic potential phosphorylation sites. In contrast to RCK1 and RCK2, Cmk2 has its catalytic domain near the N-terminus, like the CaMKs [25].

Cmk2 contributes to the regulation of G2/M transition.

cmk2$^+$ is not essential for cell viability but overexpression of Cmk2 blocks cells in G2 phase suggesting that Cmk2 has an inhibitory role in G2/M transition. This G2 phase block requires Cdc2 inhibition, as seen in *cdc2Y15A cdc2-33* strain, where Cmk2 overexpression had no effect.

Cmk2 overproduction rescues DNA replication checkpoint defects, but not sensitivity to DNA damage of checkpoint mutants. Although Cmk2 rescues HU sensitivity of different checkpoint mutants, *cmk2*-deleted cells had a normal checkpoint response. Checkpoint rescue could be a consequence of the general negative regulation of cell cycle by Cmk2. Overexpression of Cmk2 provokes G2 phase block, which is sufficient to protect checkpoint mutant cells from DNA replication insults (HU), but not from UV DNA damage. In addition, the rescue of HU sensitivity by overexpression of Cmk2 is Cdc2-dependent because Cmk2 does not rescue the HU sensitivity of *cdc2Y15A cdc2-33* strain (data not shown). Thus, Cmk2 blocks the cell cycle in a way that is independent of the S/M checkpoint response, but related to cell cycle machinery.

Our results also indicate that Cmk2 protein levels oscillate during the cell cycle. Levels increase at post-anaphase, when *S. pombe* cells are finishing mitosis and start G1/S, and decrease when cells are entering G2 phase. In *S. pombe* cells several cellular processes are co-ordinated within the time of Cmk2 increase: duplication of DNA and cell growth that involves the actin cytoskeleton. After DNA duplication, cells in G2 phase enter mitosis only when they have grown to the appropriate size. During cell growth actin patches accumulate at the sites of cell growth and it has been shown to associate directly to the plasma membrane [26]. Cmk2 protein is located near the plasma membrane along the cell cortex and the growth sites of the cell. However, our data are based on the overexpression of Cmk2 protein, and therefore they may not reflect the native Cmk2 localisation. Nevertheless, some observations from our study suggest the hypothesis that Cmk2 is linked to cell morphology and cortical components distribution. First, transient movement of Cmk2 from the growth zones to the cell cortex correlates with gradual displacement of actin patches from these sites. Second, overproduction of Cmk2 protein leads to lysis (personal unpublished observations) associated with Cmk2 kinase activity because overproduction of the inactive Cmk2KA kinase does not cause lysis. Third, cells overexpressing Cmk2 show tap pool ends morphology (personal unpublished observations) resembling the phenotype associated to actin components disruption [27].

The fact that *cmk2*-deleted cells have normal morphology makes us think that Cmk2 kinase might be involved in responding to specific environmental perturbation rather than regulating general cell morphology.

Recently, we have identified *cmk2*$^+$ as essential for oxidative stress response [28]. *cmk2*-deleted cells are sensitive to oxidative stress. Cmk2 is phosphorylated in vivo and in vitro by the stress-activated mitogen-activated protein kinase Sty1/Spc1 during oxidative stress. Ongoing studies are focus in analysing the possible role of Cmk2 regulating cell morphology in response to oxidative stress.

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