Chk1 is a wee1 kinase in the G₂ DNA damage checkpoint inhibiting cdc2 by Y15 phosphorylation

Matthew J.O'Connell^{1,2,3},
Jeanette M.Raleigh¹, Heather M.Verkaide^{1,4}
and Paul Nurse²

¹Trescowthick Research Laboratories, Peter MacCallum Cancer Institute, Locked Bag 1, A'Beckett Street, Melbourne, Victoria 3000, Australia, ²Cell Cycle Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK and ⁴Department of Genetics, University of Melbourne, Parkville, Victoria 3052, Australia

³Corresponding author

The G₂ DNA damage checkpoint ensures maintenance of cell viability by delaying progression into mitosis in cells which have suffered genomic damage. It is controlled by a number of proteins which are hypothesized to transduce signals through cell cycle regulators to delay activation of p34^{cdc2}. Studies in mammalian cells have correlated induction of inhibitory tyrosine 15 (Y15) phosphorylation on p34^{cdc2} with the response to DNA damage. However, genetic studies in fission yeast have suggested that the major Y15 kinase, p107^{wee1}, is not required for the cell cycle delay in response to DNA damage, although it is required for survival after irradiation. Thus, the target of the checkpoint, and hence the mechanism of cell cycle delay, remains unknown. We show here that Y15 phosphorylation is maintained in checkpoint-arrested fission yeast cells. Further, *wee1* is required for cell cycle arrest induced by up-regulation of an essential component of this checkpoint, *chk1*. We observed that p107^{wee1} is hyperphosphorylated in cells delayed by *chk1* overexpression or UV irradiation, and that p56^{chk1} can phosphorylate p107^{wee1} directly *in vitro*. These observations suggest that in response to DNA damage p107^{wee1} is phosphorylated by p56^{chk1} *in vivo*, and this results in maintenance of Y15 phosphorylation and hence G₂ delay. In the absence of *wee1*, other Y15 kinases, such as p66^{mik1}, may partially substitute for p107^{wee1} to induce cell cycle delay, but this *wee1*-independent delay is insufficient to maintain full viability. This study establishes a link between a G₂ DNA damage checkpoint function and a core cell cycle regulator.

Keywords: *chk1*/DNA damage checkpoint/fission yeast/p34^{cdc2}/*wee1*

Introduction

Controls over cell cycle transitions influence the activities of cyclin-dependent protein kinases (cdks). The cdks required for each transition are activated and inactivated sequentially as cells cycle between alternating S-phases and mitoses. There is an interdependency between these cell cycle transitions that must be maintained to ensure

genomic integrity and prevent changes in ploidy; i.e. S-phase onset is dependent on the completion of the previous mitosis, and the onset of mitosis is dependent on the completion of the previous S-phase (Nurse, 1994). Further, as the fidelity of these events is absolutely crucial, a cell will not continue progression through the cell cycle when defects in its division programme are detected. The surveillance mechanisms which monitor the state of the cell, and are believed ultimately to influence cell cycle transitions either directly or indirectly through the timing of cdk activation, are known as checkpoints (Hartwell and Weinert, 1989).

The transition from G₂ into mitosis requires activation of an archetypal cdk, p34^{cdc2} (Nurse, 1990). In the fission yeast *Schizosaccharomyces pombe*, as in all eukaryotes, this requires association of p34^{cdc2} with its regulatory B-type cyclin subunit and phosphorylation on threonine 167 of p34^{cdc2} by a constitutive activity known as cdk-activating kinase (CAK). This potentially active complex is maintained in an inactive state by inhibitory phosphorylation in the active site of p34^{cdc2} on tyrosine 15 (Y15) by *wee1*- and *mik1*-encoded tyrosine kinases throughout the G₂ period. When conditions are appropriate for entry into mitosis, the inhibitory phosphorylation on Y15 is removed by the *cdc25*-encoded Y15 phosphatase, and mitosis is initiated rapidly. This activating modification is the rate-limiting step for entry into mitosis.

During G₂, there are checkpoints which monitor completion of S-phase and prevent mitosis in its absence, and which also monitor the integrity of the genome and act to prevent the onset of mitosis when damage is detected. Genetic studies in fission yeast have identified a number of checkpoint proteins which are required for either, or in most cases both, of these checkpoints (Humphrey and Enoch, 1995). These proteins are non-essential, and so act to alter cell cycle transitions only under conditions of stress. In some cases, the predicted amino acid sequences have given some clue as to their function, but in general we have little idea as to how these proteins function to elicit cell cycle arrest. Genetic studies so far have not been particularly illuminating, as the non-essential nature of these genes has led in most part to the isolation of null alleles, which then fail to show interactions with each other. Similarly, it has also been difficult to build direct links between the checkpoint proteins and the core cell cycle regulators in response to G₂ checkpoint-mediated signals. In the case of the DNA replication checkpoint, inhibitory Y15 phosphorylation has been shown to be the end-point of this checkpoint, but what lies directly upstream is unknown (Enoch and Nurse, 1990). With the G₂ DNA damage checkpoint, the involvement of p34^{cdc2} Y15 phosphorylation is less clear. Cells lacking the major Y15 kinase encoded by *wee1*, p107^{wee1}, delay cell cycle progression following DNA damage and yet are still

hypersensitive to DNA-damaging agents (Barbet and Carr, 1993). Therefore, this delay may not represent a true checkpoint in that cell viability is not maintained. This is compounded further by the observation that *wee1*⁻ mutants are synthetically lethal with G₂ checkpoint mutants, indicating that these cells require a constitutively active checkpoint to maintain viability (Al-Khodairy *et al.*, 1994). Although there have been no reports as to the effects of evoking the G₂ DNA damage checkpoint on Y15 phosphorylation in fission yeast, in mammalian cells it has been shown to correlate with cell cycle arrest following DNA damage in G₂ (Kharbanda *et al.*, 1994a). However, the kinase responsible for this is unknown, although possible candidates are the *wee1* kinase and the *src*-related kinase p53/p56^{lyn} (Kharbanda *et al.*, 1994b). Furthermore, in the G₁ DNA damage checkpoint, it has been shown through overexpression of a mutant *cdk4* allele (*Cdk4*^{F17}) that cell cycle progression may in part be delayed through tyrosine phosphorylation of *cdk4* (Tereda *et al.*, 1995), in addition to the well established mechanism acting through *cdk2* inhibition by binding of the inhibitory protein p21^{CIP1/WAF1} (Dulic *et al.*, 1994; Macleod *et al.*, 1995).

Given the confusion surrounding these issues, we chose to address the role of p34^{cdc2} Y15 phosphorylation in the fission yeast G₂ DNA damage checkpoint. Our studies described here show that Y15 remains phosphorylated after cells sustain DNA damage in G₂ even though they have passed beyond the point at which Y15 normally would be dephosphorylated. This suggests that evoking the checkpoint leads to a maintenance of G₂ through maintaining p34^{cdc2} in its Y15-phosphorylated state. To confirm that maintenance of Y15 phosphorylation is linked to activation of the checkpoint, we have used the gain-of-function phenotype caused by overexpression of one component of the G₂ DNA damage checkpoint, *chk1*. This gene encodes a protein kinase, p56^{chk1}, which appears to function downstream of the other identified checkpoint functions. We show here that p56^{chk1} elicits a cell cycle arrest in G₂ that acts through p107^{wee1}-dependent Y15 phosphorylation of p34^{cdc2}. Further, we show that p107^{wee1} is phosphorylated in cells arrested by *chk1* overexpression or UV irradiation. Finally, we show that p56^{chk1} can phosphorylate p107^{wee1} directly *in vitro*, thus establishing a potentially direct link between the signal transduction cascade which makes up the G₂ DNA damage checkpoint and a core cell cycle regulator.

Results

DNA damage-induced cell cycle arrest results in maintenance of p34^{cdc2} Y15 phosphorylation

In fission yeast, it has been established that activation of p34^{cdc2} through dephosphorylation of Y15 is the rate-limiting step for entry into mitosis (Millar *et al.*, 1991). During the cell cycle, there is a checkpoint which prevents the onset of mitosis until the completion of S-phase. This checkpoint has been shown, at least in part, to act through inhibitory Y15 phosphorylation of p34^{cdc2} (Enoch and Nurse, 1990). Many proteins involved in this checkpoint have been identified by mutational studies, and most of these are also required for the checkpoint which prevents mitosis from occurring when the cell has suffered DNA

Fig. 1. DNA damage-induced cell cycle delay is associated with maintenance of p34^{cdc2} Y15 phosphorylation. Early G₂ cells (972h⁻) were collected by centrifugal elutriation. Half were irradiated with 100 J/m² UV-C, the other half were left as unirradiated control. (A) Septation index (open symbols) and cell number increases (solid symbols) show that cell cycle progression in irradiated cells (circles) is delayed by ~40 min compared with unirradiated controls (squares). (B) Western blot analysis shows that Y15 phosphorylation of p34^{cdc2} is maintained during the period of the delay. At the indicated times, extracts were prepared (Moreno *et al.*, 1991) from boiled cells in HB buffer supplemented with 0.1% SDS, electrophoresed on a 10% SDS-PAGE gel and blotted to nitrocellulose. Y15 phosphorylation was detected with a rabbit polyclonal phospho-specific antibody to p34^{cdc2} (New England Biolabs) and ECL detection reagents (Amersham). The blot was stripped and reprobed with a rabbit polyclonal antibody directed against the PSTAIRE region of p34^{cdc2} (a kind gift from Dr H.Nishitani) as a loading control.

damage (Humphrey and Enoch, 1995). Despite this, no effect of DNA damage-induced arrest in G₂ on Y15 phosphorylation has yet been demonstrated, and so we sought to establish whether evoking this checkpoint influenced the phosphorylation of p34^{cdc2} on Y15.

In order to investigate this, we prepared small fission yeast cells synchronized in early G₂ by centrifugal elutriation. These cells were split into two populations: one was irradiated with 100 J/m² UV-C to evoke the DNA damage checkpoint, and the other was left unirradiated as a control. These cells were then re-inoculated into media, and samples were taken over a timecourse to assay for cell cycle progression, and for p34^{cdc2} Y15 phosphorylation (Figure 1). In the irradiated cells, we observed a delay in cell cycle progression and dephosphorylation of Y15 compared with unirradiated controls. Dephosphorylation of Y15 was evident after 60 min in the control cells, but not until 120 min in the irradiated cells. By the end of the timecourse (180 min), Y15 phosphorylation re-appeared in the unirradiated controls as they had passed START of the subsequent cell cycle. A similar delay was also observed for activation of p34^{cdc2} kinase activity (data not shown). These data show that in fission yeast, as in mammalian cells, p34^{cdc2} is maintained in its Y15-phosphorylated state during a G₂ cell cycle delay in response to DNA damage. Although there was not an increase in

Y15 phosphorylation, this was already at high levels as the cells used for the experiment had been synchronized in G₂. However, this G₂ level of Y15 phosphorylation is maintained past the point at which p34^{cdc2} normally would be activated by Y15 dephosphorylation due to the cells passing both the size and completion of S-phase requirements for mitosis. This finding suggests that the maintenance of G₂ as a consequence of the DNA damage checkpoint control in fission yeast may involve inhibition of p34^{cdc2} by Y15 phosphorylation.

Chk1 promotes cell cycle arrest associated with inhibition of p34^{cdc2} by Y15 phosphorylation

One problem in studying the G₂ DNA damage checkpoint in fission yeast has been that the majority of identified mutations represent null alleles, and so it has been difficult to establish genetic interactions between the checkpoint functions themselves, or with core cell cycle regulators. One exception to this is *chk1*, which encodes a protein kinase, p56^{chk1}, that is essential for the G₂ DNA damage checkpoint. There is a gain-of-function caused by overexpression of *chk1* which can, by itself, elicit a cell cycle arrest in G₂ in both wild-type and checkpoint mutant cells (Walworth *et al.*, 1993; Al-Khodairy *et al.*, 1994; Ford *et al.*, 1994). In keeping with this, moderate increases in the level of *chk1* can rescue the radiation sensitivity caused by lack of some checkpoint functions. Furthermore, p56^{chk1} is phosphorylated in response to DNA damage, but this response is blocked in cells carrying mutations in the other checkpoint genes (Walworth and Bernards, 1996). It would appear, therefore, that p56^{chk1} acts near the end of the G₂ DNA damage checkpoint signal transduction pathway, downstream of the other identified components, and possibly close to p34^{cdc2}.

We wished to determine whether the cell cycle arrest caused by *chk1* overexpression was equivalent to that involving activation of p56^{chk1} by upstream components of the checkpoint following DNA damage, and whether this would result in our observed maintenance of Y15 phosphorylation. To this end, we overexpressed *chk1* from the *nmt1* promoter in wild-type cells. Between 10 and 12 h growth in the absence of thiamine, which is required for derepression of the promoter (Maundrell, 1993), we observed a cessation of cell division and loss of p34^{cdc2} kinase activity, which was confirmed to be concomitant with induction of p56^{chk1} by Western blotting (Figure 2). Unlike the more transient effects of evoking the G₂ DNA damage checkpoint by UV irradiation, continued overexpression of *chk1* led to cells becoming highly elongated, as they were maintained in a G₂ arrest with inactive p34^{cdc2}. Consistent with this, strains arrested by *chk1* overexpression accumulated significantly higher levels of Y15-phosphorylated p34^{cdc2} than did unarrested controls (Figure 2). Thus *chk1* overexpression has a similar effect to evoking the G₂ DNA damage checkpoint: cells are arrested in G₂ because p34^{cdc2} is maintained inactive by Y15 phosphorylation, and continuation of *chk1* overexpression is, therefore, analogous to a constitutively acting checkpoint arrest.

Chk1 overexpression elicits a cell cycle arrest through wee1

wee1 encodes the major p34^{cdc2} Y15 kinase in fission yeast, p107^{wee1} (Russell and Nurse 1987b; Lundgren *et al.*,

1991). We therefore asked whether *chk1* mediates its arrest on cell cycle progression via p107^{wee1}. If this were the case, then a *wee1*Δ mutant should be defective in its response to *chk1* overexpression. We tested this hypothesis by overexpressing *chk1* in a strain (*wee1*Δ) lacking the p107^{wee1} kinase. Under the same conditions as the wild-type controls, *wee1*Δ strains overexpressing *chk1* continued to divide at the same rate (Figure 3), and maintained the cell size and cell cycle distribution of vector-only controls (Figure 3), i.e. in the absence of *wee1*, cells are completely non-responsive to overexpression of *chk1*. These data show that the *chk1*-mediated cell cycle arrest acts through *wee1*. If p56^{chk1} were alternatively acting by inhibiting the p80^{cdc25} phosphatase, then we would have observed a suppression of the 'wee' phenotype in these cells, which was clearly not the case. This is in keeping with the biochemical studies showing p80^{cdc25} levels to be low in G₂ and activated on entry into mitosis (Moreno *et al.*, 1990; Dunphy, 1994).

The p107^{wee1} protein kinase phosphorylates p34^{cdc2} on Y15, and levels of this accumulate during a *wee1*-dependent cell cycle arrest induced by *chk1* overexpression. We wished, therefore, to confirm that under these conditions p107^{wee1} and p56^{chk1} were acting through this mechanism. To this end, we compared the effects of *chk1* overexpression in strains carrying alleles of *cdc2* which are altered in their sensitivity to regulators of Y15 phosphorylation, and confer a *wee* phenotype due to a shortening of the G₂ period. As predicted, *cdc2-1w* mutants, which have a reduced sensitivity to *wee1* (Russell and Nurse, 1987b), were less sensitive to *chk1* overexpression than *cdc2-3w* mutants, which have a reduced requirement for the *cdc25*-encoded phosphatase (Nurse, 1975) (Figure 3). Furthermore, the *chk1*-induced arrest was suppressed by co-overexpression of the *cdc25* phosphatase, and was not dependent on the presence of the *nim1/cdr1* kinase (Russell and Nurse, 1987a), a negative regulator of p107^{wee1} (data not shown). These data confirm that *chk1* overexpression arrests cells due to maintaining *wee1*-mediated Y15 phosphorylation of p34^{cdc2}.

p107^{wee1} is hyperphosphorylated in cells arrested by DNA damage or chk1 overexpression

Given the requirement for p107^{wee1} for *chk1*-mediated cell cycle arrest, we decided to assay p107^{wee1} for modifications consistent with a direct interaction between p107^{wee1} and p56^{chk1}. Previous studies have shown that p107^{wee1} is present at concentrations too low to be detected by conventional antibodies (Aligue *et al.*, 1994). Detection of p107^{wee1} cannot be aided by overexpression of the protein as this induces a strong cell cycle arrest. In order to alleviate this problem, we constructed strains expressing a fully functional *wee1* from its endogenous promoter, which was engineered to allow detection and isolation of p107^{wee1}: a triplicated influenza virus haemagglutinin (HA) tag, which has been shown to be effective in detecting low abundance proteins (Tyers *et al.*, 1993), was added to allow immunodetection of the protein with the 12CA5 monoclonal antibody. Six consecutive histidines were also added to allow isolation of the protein on Ni-agarose under conditions which would preserve any post-translational modifications (Figure 4). That the tagged form of *wee1* was functional was confirmed by complementation of

Fig. 2. *chk1* overexpression elicits a G₂ cell cycle arrest. (A) *chk1* was expressed from the *nmt1* promoter in pREP1 as described (Maundrell, 1993) in wild-type cells, and cells carrying an empty vector were used as a control. Induction of *chk1* expression, assayed by Western blotting using polyclonal sera raised in rabbits against bacterially expressed p56^{chk1}, was evident between 10 and 12 h growth in the absence of thiamine at 30°C and is correlated with an arrest of cell cycle progression. Up to this point, the cultures grow with the same kinetics. (B) Extracts were prepared from the *chk1*-overexpressing cells in (A), and assayed for p34^{cdc2} activity from p56^{cdc13} immunoprecipitates using histone H1 as a substrate, and quantified with a Molecular Dynamics PhosphorImager. p34^{cdc2} activity was inhibited with the same kinetics as p56^{chk1} induction. (C) Western blot analysis of extracts prepared from the 16 h timepoints under the conditions described in the legend to Figure 1 showed arrested cells have increased levels of p34^{cdc2} Y15 phosphorylation. (D) FACS analysis and DAPI staining of ethanol-fixed cells from the 16 h timepoints in (A) show that the cells were arrested in G₂. Bar, 10 μm. Elongation of the cells results in a slight increase in the signal for DNA content.

mutations in *wee1* (*wee1-50* and *wee1Δ*) and the ability of this construct to induce G₂ arrest when overexpressed from the *nmt1* promoter (data not shown).

Using Western blotting, we showed that p107^{wee1} migrated predominantly as a single band with a smearing of higher molecular weight species. However, when *chk1* was overexpressed, p107^{wee1} now migrated as a doublet, and the amount of the higher molecular weight band was dependent on the level of *chk1* expression (Figure 4). In cells containing a single integrated copy of *nmt1::chk1*, the higher molecular weight form of p107^{wee1} was dependent on the derepression of the promoter. A similar distribution of p107^{wee1} species was seen in cells arrested by *chk1* overexpression from a multi-copy plasmid. Even under repressing conditions, the *nmt1* promoter directs substantial levels of gene expression (Forsburg, 1993). Therefore, cells carrying multi-copy *nmt1::chk1* have increased levels of p56^{chk1} (data not shown). In these strains, a substantial proportion of p107^{wee1} was retarded in mobility, but this was to a lesser extent than when the promoter was derepressed and was insufficient to result in cell cycle arrest. Thus, there is a *chk1* dose-dependent modification on p107^{wee1} which is associated with cell cycle arrest and maintenance of p34^{cdc2} Y15 phosphoryl-

ation. In no case did we observe a mobility shift for all the p107^{wee1}, even though cells were first boiled and extracted under denaturing conditions in the presence of phosphatase inhibitors (Materials and methods). This observation suggests that a proportion of this protein may be inaccessible to p56^{chk1} *in vivo*, and may reside in a separate cellular compartment.

Overexpression of *chk1* has a similar effect on p34^{cdc2} Y15 phosphorylation as did DNA damage. We therefore assayed the effect of DNA damage on p107^{wee1}, and observed that it was similarly retarded in mobility when prepared from irradiated cells, although to a lesser extent (Figure 4). This may be due to the more transient effects of radiation-induced DNA damage (Figure 1), as compared with the sustained cell cycle arrest elicited by continued *chk1* overexpression (Figure 2). The latter would allow a more pronounced accumulation of the modified form of p107^{wee1} and Y15-phosphorylated p34^{cdc2}. The mobility shift was not, however, a consequence of G₂ arrest in these cells, as it was not observed in elutriated G₂ cells, or in cells arrested in G₂ by mutation in *cdc25* (data not shown).

As would be predicted, the modification to p107^{wee1} was shown to be phosphorylation. We isolated the tagged

Fig. 3. *chk1*-mediated cell cycle arrest requires *wee1*. *chk1* was expressed in *wee1Δ* cells (Russell and Nurse, 1987b) under the same conditions used for wild-type cells in Figure 2. (A) *wee1Δ* cells show similar induction of p56^{chk1} as assessed by anti-p56^{chk1} Western blotting, but are unaffected for cell cycle progression. (B) Strains with *wee* alleles of *cdc2* were analysed for sensitivity to *chk1*, showing *cdc2-1w* to be less sensitive to *chk1* than *cdc2-3w*. Cell number measurements were taken at the indicated times for both vector only and *nmt1::chk1* transformants. Cell number measurements (y-axis) are expressed as a percentage of vector-only control for each strain at the corresponding timepoint to control for different growth rates of these strains. (C) FACS analysis and DAPI staining of ethanol-fixed cells from the 16 h timepoints in (A) show no alteration in cell size or cell cycle distribution for *wee1Δ* cells. Bar, 10 μm. The generation time of *wee1Δ* grown under the conditions of media lacking thiamine and strains carrying multiple copies of the *nmt1* promoter (4 h, 20 min) accounts for the greater proportion of G₁ cells as compared with a normal FACS profile of *wee1Δ*.

p107^{wee1} on Ni-agarose from control cells and cells arrested by *chk1* overexpression. The mobility shift on p107^{wee1} prepared from cells overexpressing *chk1* was

Fig. 4. p107^{wee1} is hyperphosphorylated in *chk1*- and UV-mediated cell cycle arrests. (A) To allow detection of p107^{wee1}, the *wee1* gene was tagged by insertion of a fragment encoding six consecutive histidines, followed by three copies of the HA epitope into the *Bgl*III site of pWee1-10 (Russell and Nurse, 1987b; see Materials and methods). This construct was carried extrachromosomally under *ura4* selection. (B) Extracts were prepared from cells grown in the presence or absence of thiamine for 16 h which contained vector only (lanes 1 and 2), a single integrated copy of *nmt1::chk1* (lanes 3 and 4) or multi-copy (extrachromosomal) *nmt1::chk1* (lanes 5 and 6) as described in Figure 1. Proteins were resolved on a 6% SDS-PAGE gel, transferred to nitrocellulose and probed with the monoclonal antibody 12CA5. The Western blot shows a *chk1* dose-dependent mobility shift of p107^{wee1}. (C) Western analysis of extracts from wild-type cells, either unirradiated (lane 1), or irradiated with 200 J/m² UV-C and prepared immediately (lane 2) or after a 60 min incubation at 30°C (lane 3), shows that irradiated cells also accumulate the slower migrating form of p107^{wee1}. (D) p107^{wee1} was precipitated on Ni-NTA-agarose from vector-only control and *nmt1::chk1* cells, which had been grown in the absence of thiamine for 16 h. The precipitates were treated with the indicated units of calf intestinal phosphatase, and processed for Western blotting as in (B). The modification on p107^{wee1} was removed by this treatment, showing that it was due to phosphorylation.

reversed *in vitro* by treatment with phosphatase (Figure 4). The higher molecular weight species in the control extracts were also shown by phosphatase treatment to represent phosphorylated forms of p107^{wee1}. Thus, *chk1*-mediated cell cycle arrest is both *wee1* dependent and associated with hyperphosphorylation of p107^{wee1}.

Chk1 is a *wee1* kinase

We next asked whether p56^{chk1} could directly phosphorylate p107^{wee1}. For this purpose, we constructed a recombinant baculovirus expressing a (HIS)₆-tagged p56^{chk1}, which allowed purification from insect cell lysates

Fig. 5. p56^{chk1} is a p107^{wee1} kinase. (A) A recombinant baculovirus expressing a (his)₆-chk1 was constructed in pBlueBacHisB (Invitrogen). p56^{chk1} was detected by Western blotting of total extracts (lane 2) or cobalt-selected extracts (lane 4) of *chk1*-infected Sf21 cells, but not from extracts (lane 1) or cobalt precipitates (lane 3) of cells infected with wild-type virus. (B) Baculovirus-expressed GST-wee1K596L was prepared from Sf21 on glutathione-Sepharose (Pharmacia), and was phosphorylated by cobalt-Sepharose-precipitated p56^{chk1}, but not by cobalt-Sepharose-selected precipitates of cells infected with wild-type virus. In this experiment, p56^{chk1} was eluted from cobalt-Sepharose, but was equally active when both proteins remained bound to beads. (C) Phosphoamino acid analysis of labelled GST-wee1K596L in (B) shows that phosphorylation by p56^{chk1} occurs exclusively on serine residues. The positions of standards run with the assay are shown.

using cobalt-Sepharose affinity chromatography (Figure 5). A previously characterized baculovirus expressing catalytically inactive p107^{wee1} as a GST fusion protein (GST-wee1K596L) (Parker *et al.*, 1993) was used to prepare a substrate for *in vitro* kinase assays on glutathione-Sepharose. These assays showed that p56^{chk1} could directly phosphorylate p107^{wee1}, and phosphoamino acid analysis showed that this occurred exclusively on serine residues (Figure 5). Control experiments confirmed that p56^{chk1} was unable to phosphorylate GST. We consistently observed low level labelling of GST-wee1K596L incubated either alone or with cobalt-Sepharose preparations of extracts infected with wild-type baculovirus. This may be due either to residual activity of the mutant kinase or to a low level of contamination.

The *in vitro* phosphorylated p107^{wee1} did not undergo the mobility shift which was seen in the fission yeast extracts, which may be because *in vitro* we are observing phosphorylation by a single kinase (p56^{chk1}). In the fission yeast extracts, we were observing phosphorylation of p107^{wee1} in the background of other potential phosphorylation events such as those described for phosphorylation by p67^{nim1/cdr1}, a mitosis-specific kinase, and auto-phosphorylation of p107^{wee1} itself (Featherstone and Russell, 1991; Coleman *et al.*, 1993; Parker *et al.*, 1993; Tang *et al.*, 1993). The lack of the mobility shift caused

Fig. 6. Phosphorylation of p107^{wee1} by p56^{chk1} does not alter its Y15 kinase activity. (A) Baculovirus-expressed GST-wee1 and GST-wee1K596L were incubated with p56^{chk1} bound to cobalt-Sepharose beads or incubated with control beads as described in Figure 5. In both cases, wee1 proteins were phosphorylated by p56^{chk1}. (B) These preparations of GST-wee1 and GST-wee1K596L were then assayed for Y15 kinase activity using baculovirus-expressed p34^{cdc2}(K33R) isolated in complexes with GST-cyclin B on glutathione-Sepharose as described (Coleman *et al.*, 1993; Parker *et al.*, 1993). Reactions were electrophoresed on a 10% SDS-PAGE gel, blotted to nitrocellulose, probed with antibodies to phosphotyrosine and subsequently reprobed with anti-p34^{cdc2} (PSTAIR) to control for loading. These assays showed that only wild-type GST-wee1 was active as a Y15 kinase, and this activity was not altered after phosphorylation by p56^{chk1}.

by p56^{chk1} phosphorylation of p107^{wee1} may also be due to the effects of the GST portion of the fusion protein used in these experiments.

The observations from these *in vitro* kinase assays are fully consistent with the genetic observations showing p56^{chk1} functions through p107^{wee1}, and that up-regulation of p56^{chk1} by either overexpression or DNA damage results in hyperphosphorylation of p107^{wee1} *in vivo*. They confirm that p107^{wee1} and p56^{chk1} can interact directly, and thus establish a link between a member of the signal transduction pathway controlling the DNA damage checkpoint and the core cell cycle machinery.

Phosphorylation of p107^{wee1} by p56^{chk1} does not alter its Y15 kinase activity

Evoking a checkpoint which functions through p107^{wee1}-dependent Y15 phosphorylation of p34^{cdc2} could have either of two effects on p107^{wee1} activity: either it could maintain G₂ levels of this kinase in cells that, in the absence of DNA damage, would have entered mitosis, or it may stimulate its activity to enhance cell cycle arrest further. Our observation of maintenance of Y15 phosphorylation during a G₂ DNA damage checkpoint arrest (Figure 1) suggests the former may result. In order to assess whether *in vitro* phosphorylation of p107^{wee1} by p56^{chk1} altered its activity as a Y15 kinase, we performed similar assays to those described above, but used baculovirus-expressed active GST-wee1 as the substrate (Parker *et al.*, 1992). We observed that p56^{chk1} also phosphorylated active GST-wee1, resulting in [³²P]ATP labelling above that due to autophosphorylation (Figure 6). When we assayed the Y15 kinase activity of these two forms of active GST-wee1, we found no change in p107^{wee1} activity caused by p56^{chk1} phosphorylation when assayed by Western blotting for the presence of phosphotyrosine on p34^{cdc2} (Figure 6). As expected, the mutant p107^{wee1}

(GST-*wee1*K596L) had no detectable Y15 kinase activity. These data were confirmed in other experiments by ^{32}P labelling of p34^{cdc2}, and also by assays of p34^{cdc2} histone H1 kinase subsequent to phosphorylation by p107^{wee1} (data not shown). Therefore, although p107^{wee1} is phosphorylated by p56^{chk1}, this does not affect p107^{wee1} kinase activity *in vitro*.

Discussion

Although many proteins involved in eliciting a G₂ cell cycle arrest in response to DNA damage have been identified, little is known about how they interact to send signals to the core cell cycle regulators. Further, we have not known what the ultimate effect of evoking this checkpoint is on the p34^{cdc2}-cyclin B complexes. In this study we have described experiments which address both of these issues.

By Western blot analysis of extracts prepared from irradiated G₂ cells, we showed that p34^{cdc2} is maintained in its inactive, Y15-phosphorylated form. This was maintained in cells despite the fact that they had fulfilled the cell size and completion of S-phase requirements for entry into mitosis. These experiments were performed at a radiation dose which causes little effect in terms of viability on wild-type cells, which recover due to the transient cell cycle delay induced by the checkpoint. This delay is, however, not long enough to see a significant accumulation of high levels of the Y15-phosphorylated form of p34^{cdc2}. These data are consistent with those described for mammalian cells which have correlated p34^{cdc2} Y15 phosphorylation with DNA damage-induced G₂ delay (Kharbanda *et al.*, 1994a). Furthermore, other groups have demonstrated recently that Y15 phosphorylation is crucial in the G₂ DNA checkpoints of *Aspergillus nidulans* (Ye *et al.*, 1997) and fission yeast (N.Rhind and P.Russell, personal communication), and in a G₂ checkpoint resulting from irradiation of human cells in S-phase (Jin *et al.*, 1996).

In our experiments, the cells are already in G₂ when they suffer DNA damage, and so our data would indicate that, under these conditions, the checkpoint functions to hold the cells in G₂ and prevent the onset of mitosis whilst the DNA is repaired. This maintenance of p34^{cdc2} inhibition by Y15 phosphorylation rather than induction of an additional arrest mechanism is in contrast to that described for vertebrate cells in G₁: here, the potentially active cdk2-cyclin E complexes are inhibited by induction of the p21 cdk inhibitor (Harper *et al.*, 1993; Macleod *et al.*, 1995). A role for inhibition of G₁ cdks by tyrosine phosphorylation in G₁ checkpoints has also been shown by mutation in cdk4 (Tereda *et al.*, 1995). Furthermore, cdc25 phosphatases acting in G₁ have the ability, when overproduced, to override G₁ checkpoints in cellular transformation (Galaktionov *et al.*, 1995). Thus, inhibition of cdks by tyrosine phosphorylation appears to be generally important in checkpoints responding to DNA damage.

Although we had shown that p34^{cdc2} Y15 phosphorylation is associated with G₂ checkpoint arrest, we asked whether this was a result of activation of the checkpoint. To answer this question, we chose to investigate the effects of up-regulation of the p56^{chk1} kinase by overexpression. This line of investigation was particularly useful as it

allowed investigation of the cell cycle arrest phenotype as a constitutive event in the absence of DNA damage. From these experiments, we established that overexpression of *chk1* also resulted in G₂ cell cycle arrest with p34^{cdc2} inactivated by Y15 phosphorylation. Importantly, we established that this arrest mechanism functioned directly through *wee1* rather than through another p34^{cdc2} inhibitory mechanism: in the absence of *wee1*, cells were completely non-responsive to the effects of *chk1* overexpression. The fact that these cells retained their *wee* phenotype rules out the possibility that p56^{chk1} could be acting alternatively through inhibition of p80^{cdc25}, as this would suppress the effects of lacking p107^{wee1}. Furthermore, interactions with *wee* alleles of *cdc2* confirmed the requirement for *wee1* in this arrest, and that this was at the level of p107^{wee1}-mediated p34^{cdc2} Y15 phosphorylation.

Through Western blot analysis, we showed that p107^{wee1} became hyperphosphorylated when cells were arrested by *chk1* overexpression or UV-induced DNA damage. Together with the genetic observations, these data strongly implicated p107^{wee1} as a direct target of p56^{chk1}. Evidence for this model was gained by *in vitro* kinase assays using baculovirus-expressed proteins, which showed that p56^{chk1} can phosphorylate p107^{wee1} directly on serine residues. Although these data strongly suggest that p56^{chk1} is phosphorylating p107^{wee1} directly *in vivo*, we do not have direct evidence for this. Due to the complexity of phosphorylation events on p107^{wee1}, this issue can only be resolved by mapping the *in vitro* phosphorylation sites, and assessing the function of these by mutation studies *in vivo*. Such experiments are underway.

The phosphorylation of p107^{wee1} by p56^{chk1} did not affect its *in vitro* Y15 kinase activity, despite the fact that p56^{chk1} clearly affects both p107^{wee1} and Y15 phosphorylation. These data should be compared with published work concerning the checkpoint function associated with fission yeast *rad24*, which encodes a 14-3-3 protein (Ford *et al.*, 1994). Analysis of *chk1* overexpression has shown it to act downstream of all checkpoint mutants with the exception of *rad24*. Strains deleted for *rad24* are only partially responsive to *chk1*, becoming delayed but not arrested in cell cycle progression. Importantly, however, *rad24*Δ cells exhibit a *wee* phenotype, and thus also have a defect in cell cycle progression accelerating them through G₂. These phenotypes could be related if *rad24*Δ strains are defective in some aspect of p107^{wee1} control, which in turn makes them less responsive to p56^{chk1}-mediated signals. In view of these data, it is noteworthy that a physical interaction between murine homologues of p107^{wee1} and 14-3-3 proteins have been observed *in vitro* and *in vivo*, without an effect on p107^{wee1} activity (R.Honda and H.Yasuda, personal communication). Further, 14-3-3 proteins have been implicated in subcellular localization and protein-protein interactions (Burbelo and Hall, 1995) through association with phosphoserine residues, which were the sites of phosphorylation of p107^{wee1} by p56^{chk1}. Both the *wee* phenotype and insensitivity to *chk1* of *rad24*Δ could, therefore, be a result of inappropriate localization or protein-protein interactions of p107^{wee1} when the *rad24*-encoded 14-3-3 protein is not present, and not a result of alteration of its intrinsic kinase activity.

Based on the genetic and biochemical experiments

Fig. 7. Model for $p56^{chk1}$ function in the G_2 DNA damage checkpoint. Irradiation of a cell in G_2 results in DNA damage. This damage must be detected, but the molecular nature of the detection mechanism is unknown. Subsequent to detection, the checkpoint is activated through a signal transduction cascade leading to activation of $p56^{chk1}$. $p56^{chk1}$, perhaps through a mechanism requiring the *rad24*-encoded 14-3-3 protein, then phosphorylates $p107^{wee1}$. The hyperphosphorylated form of $p107^{wee1}$ can then maintain phosphorylation of $p34^{cdc2}$ on Y15 for the duration of the checkpoint delay. Maintenance of Y15 phosphorylation results in maintenance of G_2 , even though the normal cell size and completion of S-phase requirements for mitosis have been fulfilled.

performed in fission yeast, and the *in vitro* phosphorylation experiments with recombinant protein, we propose a model for cell cycle arrest following DNA damage in G_2 : as a consequence of DNA damage, $p56^{chk1}$ is activated and maintains $p34^{cdc2}$ Y15 phosphorylation by phosphorylating $p107^{wee1}$ (Figure 7). This phosphorylation does not affect the *in vitro* $p107^{wee1}$ kinase activity, but does affect the *in vivo* function of $p107^{wee1}$, perhaps by affecting its intracellular localization, interaction with $p34^{cdc2}$ or interaction with negative regulatory proteins functioning at the G_2 -M transition. This leads to the activity of $p107^{wee1}$ being maintained after genome damage beyond the point when an appropriate cell size is achieved and cells would normally enter mitosis. In vertebrates, a similar mechanism has been proposed for the DNA replication checkpoint (McGowan and Russell, 1995). This checkpoint clearly functions through Y15 phosphorylation (Enoch *et al.*, 1992), but is not associated with an increase in $p107^{wee1}$ activity above that observed in normal G_2 cells (McGowan and Russell, 1995; Mueller *et al.*, 1995). Although the DNA replication and DNA damage checkpoints have overlapping components, $p56^{chk1}$ is not involved in the replication checkpoint, suggesting that separable but overlapping pathways are involved in G_2 checkpoint controls. A good candidate for a protein carrying out an analogous function to $p56^{chk1}$ in the fission yeast DNA replication checkpoint is $p52^{cds1}$ (Murakami and Okayama, 1995). This protein kinase is required for cell cycle delay following a block to DNA replication, but not following DNA damage. At this stage, we do not know the mechanism by which DNA damage is detected, or how this results in activation of $p56^{chk1}$. However, it has been shown that $p56^{chk1}$ becomes phosphorylated after DNA damage (Walworth and Bernards, 1996), and this may play some role in its activation.

If $p107^{wee1}$ is a target of the G_2 DNA damage checkpoint, how can strains completely lacking *wee1* arrest in response to irradiation (Barbet and Carr, 1993)? The answer to this is likely to lie in the redundancy of Y15 kinases in fission yeast (Lundgren *et al.*, 1991). We suggest that $p107^{wee1}$ is a target of the checkpoint, but not the only target. At least one other Y15 kinase, $p66^{mik1}$, is likely to play some role. The radiation sensitivity of *wee1* Δ cells indicates that a *wee1*-independent arrest involving *mik1* is not sufficient to maintain wild-type levels of viability after DNA damage. When both kinases are absent, all G_2 controls are lost and cells enter mitosis constitutively, leading to a lethal mitotic catastrophe phenotype. Although

wee1 and *mik1* have overlapping functions, *mik1* Δ mutants do not exhibit a *wee* phenotype, suggesting that $p66^{mik1}$ plays a minor role in G_2 control in the presence of $p107^{wee1}$. It is also important to note that the viability of *wee1* Δ cells, even when unirradiated, requires all known G_2 checkpoint functions, including *chk1* (Al-Khodairy *et al.*, 1994), which may also act through *mik1* under these conditions. This indicates that checkpoint functions are constitutively active in these cells, and may act to up-regulate $p66^{mik1}$ so as to prevent premature entry into mitosis. Our data show that Y15 phosphorylation plays a role in the G_2 DNA damage checkpoint functioning through $p56^{chk1}$, but we cannot rule out additional regulatory mechanisms.

The data presented here provide a direct link between a G_2 checkpoint function and a core cell cycle regulator. This link establishes a firm basis on which to build this pathway, which although it has many identified members, still lacks the biochemical links required to understand this important biological phenomenon.

Materials and methods

Fission yeast methods

All strains are derivatives of wild-type *S.pombe* 972h⁻. Standard procedures for propagation, transformation and extract preparation were used. Small G_2 cells prepared from synchronous cultures were derived by centrifugal elutriation. For UV irradiation, cells were re-inoculated into pre-warmed (30°C) medium for a recovery period of 20 min, and then filtered in aliquots onto PVDF membranes. Half the membranes were then irradiated with 100 J/m² UV-C (254 nm), following which both populations of cells were washed into pre-warmed medium. Cell cycle progression was followed by septation indices, which were estimated by phase contrast microscopy every 10 min, and increases in cell number in samples fixed in 3.7% formaldehyde for cell counting in a Sysmex K-1000 cell counter.

Detection and dephosphorylation of $p107^{wee1}$ from fission yeast

In order to detect $p107^{wee1}$ by Western blotting, a tagging fragment corresponding to six consecutive histidines and three copies of the influenza virus HA epitope (RSHHHHHHGGRIFFYPYDPYAGY-PYDVPDYAGSYDPYDPAQAQCGRS) was inserted into the *Bgl*II site of *pWee1*-10 (Russell and Nurse, 1987b). This construct rescues the temperature-sensitive allele *wee1*-50, and was carried extrachromosomally under *ura4* selection. For detection of $p107^{wee1}$, proteins were resolved on a 6% SDS-PAGE gel, transferred to nitrocellulose and probed with the monoclonal antibody 12CA5. For dephosphorylation of $p107^{wee1}$, protein extracts were prepared from boiled cells in 8 M urea, 100 mM NaH₂PO₄, 10 mM Tris, 60 mM β -glycerophosphate, 100 μ M Na orthovanadate, 15 mM p-Nitrophenyl Phosphate (PNPP), 1% Triton X-100, pH 8, and $p107^{wee1}$ was affinity isolated on Ni-NTA-agarose (Qiagen). Beads were washed extensively in extraction buffer, and urea

removed by dilution batch washing. Precipitates were washed three times in CIP buffer (50 mM Tris, 5 mM MgCl₂, pH 8), treated with the indicated units of calf intestinal phosphatase (Gibco-BRL) for 30 min at 37°C and processed for Western blotting.

Baculovirus expression

The *chk1* open reading frame was amplified by PCR to include *Bgl*III sites at codon 2 and directly after the termination codon, and cloned into pBlueBacHisB (Invitrogen). DNA sequencing was performed (ABI) to confirm the integrity of the amplified sequence. A recombinant virus was then constructed using the linear transfection module (Invitrogen) as recommended, and was propagated at an m.o.i. of 5, with extracts prepared 2–3 days post-infection. Recombinant p56^{chk1} was isolated from Sf21 lysates prepared in 250 mM NaCl, 50 mM Tris pH 8, 10% glycerol, 0.1% NP-40, 50 mM NaF, 10 mM Na pyrophosphate, 100 μM Na orthovanadate, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, leupeptin and pepstatin, on cobalt–Sephacrose (Talon, Clontech) as recommended. The other recombinant viruses used here for expression of wee1, cyclin B and cdc2 have been described (Parker *et al.*, 1993).

Protein kinase assays

Fission yeast p34^{cdc2} kinase assays using histone H1 as a substrate were performed on immunoprecipitates using a monoclonal antibody directed against the *cdc13*-encoded B-type cyclin. Assays were quantified by PhosphorImager analysis (Molecular Dynamics). For phosphorylation of p107^{wee1} by p56^{chk1}, baculovirus-expressed proteins were selected on affinity reagents (cobalt–Sephacrose or glutathione–Sephacrose), washed extensively in extraction buffer, and finally in kinase assay buffers. Assays were performed in 50 mM Tris, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 10 μM ATP, with 10 μCi of 3000 Ci/mmol [³²P]ATP. Reactions were incubated for 30 min at 30°C, and terminated by boiling in SDS–PAGE buffer. Samples were run on an 8% SDS–PAGE gel, stained, dried and exposed to film. Assays were quantified by PhosphorImager analysis (Molecular Dynamics). Control experiments confirmed that p56^{chk1} was unable to phosphorylate GST. For phospho-amino acid analysis, ³²P-labelled GST–wee1K596L was transferred to PVDF and incubated in 5.7 M HCl at 100°C for 2 h. Reactions were lyophilized, resuspended with phospho-amino acid standards and electrophoresed at pH 3.5 on a thin-layer cellulose plate. For assays of p107^{wee1} activity, p34^{cdc2}(K33R) was isolated in complexes with GST–cyclin B on glutathione–Sephacrose, and GST–wee1 kinase assays were performed as described (Coleman *et al.*, 1993; Parker *et al.*, 1993). Reactions were electrophoresed on a 10% SDS–PAGE gel, blotted to nitrocellulose, probed with antibodies to phosphotyrosine and subsequently reprobed with anti-p34^{cdc2} (PSTAIR) to control for loading. Western blots were quantified by densitometry (Molecular Dynamics).

Antibodies

Polyclonal antisera against p56^{chk1} were raised in rabbits immunized with full-length bacterially expressed, histidine-tagged p56^{chk1}. Procedures for protein expression, isolation, immunization and affinity purification were as described (O'Connell *et al.*, 1994). The affinity-purified sera were then used at a concentration of 1:100 on crude extracts. That this specifically detected p56^{chk1} was confirmed by comparing extracts of wild-type-, *chk1Δ*- and *chk1*-overexpressing cells. For detection of Y15-phosphorylated p34^{cdc2}, a phospho-specific rabbit polyclonal antibody (New England Biolabs) was used at a concentration of 1:250. For detection of epitope-tagged p107^{wee1}, the 12CA5 monoclonal antibody was used at a concentration of 40 μg/ml. For detection of total p34^{cdc2}, a rabbit polyclonal antiserum directed against the PSTAIR region (a kind gift of Dr Hideo Nishitani) was used at a concentration of 1:10 000.

Acknowledgements

The authors wish to thank Dr H.Piwica-Worms for providing baculoviruses, and Drs R.Honda and H.Yasuda; and N.Rhind and P.Russell for communication of results prior to publication. We are also grateful to Dr D.Germain (PMCI) for critical reading of the manuscript and many useful discussions, and to Drs J.Hayles (ICRF), J.Corrêa-Bordes (ICRF), R.Pearson (PMCI) and R.Ramsey (PMCI) for much helpful advice. We are also most grateful to Mrs Liz Eaton for much assistance in trans-global communications. H.V. is a recipient of an Australian Postgraduate Award, and M.O. is a Special Fellow of the Leukemia Society of America.

References

- Aligue,R., Akhavan-Niak,H. and Russell,P. (1994) A role for hsp90 in cell cycle control: wee1 tyrosine kinase activity requires interaction with hsp90. *EMBO J.*, **13**, 6099–6106.
- Al-Khodairy,F., Fotou,E., Sheldrick,K.S., Griffiths,D.J.F., Lehman,A.R. and Carr,A.M. (1994) Identification and characterisation of new elements involved in checkpoint and feedback controls in fission yeast. *Mol. Biol. Cell*, **5**, 147–160.
- Barbet,N.C. and Carr,A.M. (1993) Fission yeast *wee1* protein kinase is not required for DNA damage-dependent mitotic arrest. *Nature*, **364**, 824–827.
- Burbelo,P.D. and Hall,A. (1995) Hot numbers in signal transduction. *Curr. Biol.*, **5**, 95–96.
- Coleman,T.R., Tang,Z. and Dunphy,W.G. (1993) Negative regulation of the wee1 protein kinase by direct action of the nim1/cdr1 mitotic inducer. *Cell*, **72**, 919–929.
- Dulic,V., Kaufmann,W.K., Wilson,S.J., Tlsty,T.D., Lees,E., Harper,J.W., Elledge,S.J. and Reed,S.I. (1994) p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell*, **76**, 1013–1023.
- Dunphy,W.G. (1994) The decision to enter mitosis. *Trends Cell Biol.*, **4**, 202–207.
- Enoch,T. and Nurse,P. (1990) Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. *Cell*, **60**, 665–673.
- Enoch,T., Carr,A. and Nurse,P. (1992) Fission yeast genes involved in coupling mitosis to completion of DNA replication. *Genes Dev.*, **6**, 2035–2046.
- Featherstone,C. and Russell,P. (1991) Fission yeast p107^{wee1} mitotic inhibitor is a tyrosine/serine kinase. *Nature*, **349**, 808–811.
- Ford,J.C., Al-Khodairy,F., Fotou,E., Sheldrick,K.S., Griffiths,D.J.F. and Carr,A.M. (1994) 14-3-3 protein homologs required for the DNA damage checkpoint in fission yeast. *Science*, **265**, 533–535.
- Forsburg,S.L. (1993) Comparison of *Schizosaccharomyces pombe* expression systems. *Nucleic Acids Res.*, **21**, 2955–2956.
- Galaktionov,K., Lee,A.K., Eckstein,J., Draetta,G., Meckler,J.M.L. and Beach,D. (1995) CDC25 phosphatases as potential human oncogenes. *Science*, **269**, 1575–1577.
- Harper,J.W., Adami,G.R., Wei,N., Keyomarsi,K. and Elledge,S.J. (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, **75**, 805–816.
- Hartwell,L. and Weinert,T. (1989) Checkpoints: controls that ensure the order of cell cycle events. *Science*, **246**, 629–634.
- Humphrey,T. and Enoch,T. (1995) Keeping mitosis in check. *Curr. Biol.*, **5**, 376–379.
- Jin,P., Gu,Y. and Morgan,D.O. (1996) Role of inhibitory CDC2 phosphorylation in radiation-induced G2 arrest in human cells. *J. Cell Biol.*, **134**, 963–970.
- Kharbanda,S., Saleem,A., Datta,R., Yuan,Z., Weichselbaum,R. and Kufe,D. (1994a) Ionizing radiation induces rapid tyrosine phosphorylation of p34^{cdc2}. *Cancer Res.*, **54**, 1412–1414.
- Kharbanda,S., Yuan,Z., Rubin,E., Weichselbaum,R. and Kufe,D. (1994b) Activation of the src-like p56/p53^{lyn} tyrosine kinase by ionizing radiation. *J. Biol. Chem.*, **269**, 20739–20743.
- Lundgren,K., Walworth,N., Booher,R., Dembski,M., Kirschner,M. and Beach,D. (1991) mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. *Cell*, **64**, 1111–1122.
- Macleod,K.F., Sherry,N., Hannon,G., Beach,D., Tokino,T., Kinzler,K., Vogelstein,B. and Jacks,T. (1995) p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes Dev.*, **9**, 935–944.
- Maundrell,K. (1993) Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene*, **123**, 127–130.
- McGowan,C.H. and Russell,P. (1995) Cell cycle regulation of human WEE1. *EMBO J.*, **14**, 2166–2175.
- Millar,J., McGowan,C., Lenaers,G., Jones,R. and Russell,P. (1991) p80^{cdc25} mitotic inducer is the tyrosine phosphatase that activates p34^{cdc2} kinase in fission yeast. *EMBO J.*, **10**, 4301–4309.
- Moreno,S., Nurse,P. and Russell,P. (1990) Regulation of mitosis by cyclic accumulation of p80^{cdc25} mitotic inducer in fission yeast. *Nature*, **344**, 549–552.
- Moreno,S., Klar,A. and Nurse,P. (1991) Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.*, **194**, 795–823.
- Mueller,P.R., Coleman,T.R. and Dunphy,W.G. (1995) Cell cycle regulation of a *Xenopus* wee1-like kinase. *Mol. Biol. Cell*, **6**, 119–134.

- Murakami,H. and Okayama,H. (1995) A kinase from fission yeast responsible for blocking mitosis in S phase. *Nature*, **374**, 817–819.
- Nurse,P. (1975) Genetic control of cell size at cell division in yeast. *Nature*, **256**, 547–551.
- Nurse,P. (1990) Universal control mechanism regulating onset of M-phase. *Nature*, **344**, 503–508.
- Nurse,P. (1994) Ordering S phase and M phase in the cell cycle. *Cell*, **79**, 547–550.
- O'Connell,M.J., Norbury,C. and Nurse,P. (1994) Premature chromatin condensation upon accumulation of NIMA. *EMBO J.*, **13**, 4926–4937.
- Parker,L.L., Atherton-Fessler,S. and Piwnica-Worms,H. (1992) p107^{wee1} is a dual-specificity kinase that phosphorylates p34^{cdc2} on tyrosine 15. *Proc. Natl Acad. Sci. USA*, **89**, 2917–2921.
- Parker,L.L., Walter,S.A., Young,P.G. and Piwnica-Worms,H. (1993) Phosphorylation and inactivation of the mitotic inhibitor wee1 by the *nim1/cdr1* kinase. *Nature*, **363**, 736–738.
- Russell,P. and Nurse,P. (1987a) The mitotic inducer *nim1*⁺ functions in a regulatory network of protein kinase homologs controlling the initiation of mitosis. *Cell*, **49**, 569–576.
- Russell,P. and Nurse,P. (1987b) Negative regulation of mitosis by *wee1*⁺, a gene encoding a protein kinase homolog. *Cell*, **49**, 559–567.
- Tang,Z., Coleman,T.R. and Dunphy,W.G. (1993) Two distinct mechanisms for negative regulation of the Wee1 protein kinase. *EMBO J.*, **12**, 3427–3436.
- Tereda,Y., Tatsuka,M., Jinno,S. and Okayama,H. (1995) Requirement for tyrosine phosphorylation of cdk4 in G1 arrest induced by ultraviolet irradiation. *Nature*, **376**, 358–362.
- Tyers,M., Tokiwa,G. and Futcher,B. (1993) Comparison of the *Saccharomyces cerevisiae* G₁ cyclins—CLN3 may be an upstream activator of CLN1, CLN2 and other cyclins. *EMBO J.*, **12**, 1955–1968.
- Walworth,N.C. and Bernards,R. (1996) rad-dependent response of the *chk1*-encoded protein kinase at the DNA damage checkpoint. *Science*, **271**, 353–356.
- Walworth,N., Davey,S. and Beach,D. (1993) Fission yeast *chk1* protein kinase links the rad checkpoint pathway to *cdc2*. *Nature*, **363**, 368–371.
- Ye,X.S., Fincher,R.R., Tang,A. and Osmani,S.A. (1997) The G₂/M DNA damage checkpoint inhibits mitosis through Tyr15 phosphorylation of p34^{cdc2} in *Aspergillus nidulans*. *EMBO J.*, **16**, 182–192.

Received on October 21, 1996; revised on November 19, 1996