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

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Chk1 is a wee1 kinase in the G₂ DNA damage checkpoint ensuring 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 p34cdc2. Studies in mammalian cells have correlated induction of inhibitory tyrosine 15 (Y15) phosphorylation on p34cdc2 with the response to DNA damage. However, genetic studies in fission yeast have suggested that the major Y15 kinase, p107wee1, 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 p107wee1 is hyperphosphorylated in cells delayed by chkl overexpression or UV irradiation, and that p56mik1 can phosphorylate p107wee1 directly in vitro. These observations suggest that in response to DNA damage p107wee1 is phosphorylated by p56mik1 in vivo, and this results in maintenance of Y15 phosphorylation and hence G₂ delay. In the absence of wee1, other Y15 kinases, such as p66mik1, may partially substitute for p107wee1 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/ p34cdc2/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, p34cdc2 (Nurse, 1990). In the fission yeast Schizosaccharomyces pombe, as in all eukaryotes, this requires association of p34cdc2 with its regulatory B-type cyclin subunit and phosphorylation on threonine 167 of p34cdc2 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 p34cdc2 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 p34cdc2 Y15 phosphorylation is less clear. Cells lacking the major Y15 kinase encoded by wee1, p107wee1, delay cell cycle progression following DNA damage and yet are still
In the G1 DNA damage checkpoint, it has been shown in the yeast S. cerevisiae (Nurse, 1990). Many proteins involved in this checkpoint have been identified, and these data show that in fission yeast, as 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 delay is unknown, and it is possible that this delay is mediated by a conserved kinase p53/p56^lyn (Kharbanda et al., 1994b). Furthermore, in the G1 DNA damage checkpoint, it has been shown that overexpression of a mutant cdk4 allele (Cdk4^P17) causes cell cycle progression to 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 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 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/m^2^ 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 reappeared 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

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**Fig. 1.** DNA damage-induced cell cycle delay is associated with maintenance of p34^cdc2 Y15 phosphorylation. Early G2 cells (972K) were collected by centrifugal elution. Half were irradiated with 100 J/m^2^ UV-C, the other half were left as unirradiated controls. (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 antibody directed against the PSTAIRE region of p34^cdc2 (a kind gift from Dr. H.Nishitani) as a loading control.
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\(^{cdcl}\) 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\(^{cdcl}\) by Y15 phosphorylation.

\textbf{Chk1 promotes cell cycle arrest associated with inhibition of p34\(^{cdcl}\) 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 \textit{chk1}, which encodes a protein kinase, p56\(^{chkl}\), that is essential for the G2 DNA damage checkpoint. There is a gain-of-function caused by overexpression of \textit{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 \textit{chk1} can rescue the radiation sensitivity caused by lack of some checkpoint functions. Furthermore, p56\(^{chkl}\) 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\(^{chkl}\) acts near the end of the G2 DNA damage checkpoint signal transduction pathway, downstream of the other identified components, and possibly close to p34\(^{cdcl}\).

We wished to determine whether the cell cycle arrest caused by \textit{chk1} overexpression was equivalent to that involving activation of p56\(^{chkl}\) 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 \textit{chk1} from the \textit{mmn1} promoter in wild-type cells. Between 10 and 12 h growth in the absence of thiamine, which is required for derepression of the promoter (Maudrell, 1993), we observed a cessation of cell division and loss of p34\(^{cdcl}\) kinase activity, which was confirmed to be concomitant with induction of p56\(^{chkl}\) by Western blotting (Figure 2).

Unlike the more transient effects of evoking the G2 DNA damage checkpoint by UV irradiation, continued overexpression of \textit{chk1} led to cells becoming highly elongated, as they were maintained in a G2 arrest with inactive p34\(^{cdcl}\). Consistent with this, strains arrested by \textit{chk1} overexpression accumulated significantly higher levels of Y15-phosphorylated p34\(^{cdcl}\) than did unarrested controls (Figure 2). Thus \textit{chk1} overexpression has a similar effect to evoking the G2 DNA damage checkpoint: cells are arrested in G2 because p34\(^{cdcl}\) is maintained inactive by Y15 phosphorylation, and continuation of \textit{chk1} overexpression is, therefore, analogous to a constitutively acting checkpoint arrest.

\textbf{Chk1 overexpression elicits a cell cycle arrest through wee1}

\textit{wee1} encodes the major p34\(^{cdcl}\) Y15 kinase in fission yeast, p107\(^{weel}\) (Russell and Nurse 1987b; Lundgren et al., 1991). We therefore asked whether \textit{chk1} mediates its arrest on cell cycle progression via p107\(^{weel}\). If this were the case, then a \textit{wee1}\(\Delta\) mutant should be defective in its response to \textit{chk1} overexpression. We tested this hypothesis by overexpressing \textit{chk1} in a strain (\textit{wee1}\(\Delta\)) lacking the p107\(^{weel}\) kinase. Under the same conditions as the wild-type controls, \textit{wee1}\(\Delta\) strains overexpressing \textit{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 \textit{wee1}, cells are completely non-responsive to overexpression of \textit{chk1}. These data show that the \textit{chk1}-mediated cell cycle arrest acts through \textit{wee1}. If p56\(^{chkl}\) were alternatively acting by inhibiting the p80\(^{cdcl}\) phosphatase, then we would have observed a suppression of the ‘\textit{wee}’ phenotype in these cells, which was clearly not the case. This is in keeping with the biochemical studies showing p80\(^{cdcl}\) levels to be low in G2 and activated on entry into mitosis (Moreno et al., 1990; Dunphy, 1994).

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

\textbf{p107\(^{weel}\) is hyperphosphorylated in cells arrested by DNA damage or \textit{chk1} overexpression}

Given the requirement for \textit{p107\(^{weel}\)} for \textit{chk1}-mediated cell cycle arrest, we decided to assay p107\(^{weel}\) for modifications consistent with a direct interaction between p107\(^{weel}\) and p56\(^{chkl}\). Previous studies have shown that p107\(^{weel}\) is present at concentrations too low to be detected by conventional antibodies (Alique et al., 1994). Detection of p107\(^{weel}\) 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 \textit{wee1} from its endogenous promoter, which was engineered to allow detection and isolation of p107\(^{weel}\): 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 added conditions which would preserve any post-translational modifications (Figure 4). That the tagged form of \textit{wee1} was functional was confirmed by complementation of
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\textsubscript{wee1} migrated predominantly as a single band with a smearing of higher molecular weight species. However, when chk1 was overexpressed, p107\textsubscript{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\textsubscript{wee1} was dependent on the derepression of the promoter. A similar distribution of p107\textsubscript{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\textsubscript{chk1} (data not shown). In these strains, a substantial proportion of p107\textsubscript{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\textsubscript{wee1} which is associated with cell cycle arrest and maintenance of p34\textsubscript{cdc2} Y15 phosphorylation. In no case did we observe a mobility shift for all the p107\textsubscript{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\textsubscript{chk1} in vivo, and may reside in a separate cellular compartment.

Overexpression of chk1 has a similar effect on p34\textsubscript{cdc2} Y15 phosphorylation as did DNA damage. We therefore assayed the effect of DNA damage on p107\textsubscript{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 by chk1 overexpression from a multi-copy plasmid. Even under repressing conditions, the nmt1 promoter directs 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\textsubscript{wee1} and Y15-phosphorylated p34\textsubscript{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\textsubscript{wee1} was shown to be phosphorylation. We isolated the tagged
Chk1 is a wee1 kinase

We next asked whether p56chk1 could directly phosphorylate p107wee1. For this purpose, we constructed a recombinant baculovirus expressing a (HIS)6-tagged p56chk1, which allowed purification from insect cell lysates 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 p107wee1. Thus, chk1-mediated cell cycle arrest is both wee1 dependent and associated with hyperphosphorylation of p107wee1.

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Phosphorylation of p107 wee1 itself (Featherstone and caused by p56 chk1 phosphorylation when assayed by extracts, which may be because yeast extracts, we were observing phosphorylation of active GST–wee1, resulting in [32P]ATP labelling above catalytically inactive p107wee1 as a GST fusion protein. A previously characterized baculovirus expressing the core cell cycle machinery. incubated either alone or with cobalt–Sepharose prepara-

Our observation of maintenance of Y15 phospho-

The in vitro phosphorylated p107weel 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 (p56chk1). In the fission yeast extracts, we were observing phosphorylation of p107weel in the background of other potential phosphorylation events such as those described for phosphorylation by p67nim1/cdr1, a mitosis-specific kinase, and auto-

by p56chk1 phosphorylation of p107weel 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 p56chk1 functions through p107weel, and that up-regulation of p56chk1 by either overexpression or DNA damage results in hyperphosphorylation of p107weel in vivo. They confirm that p107weel and p56chk1 can interact directly, and thus establish a link between a member of the signal transduc-

Phosphorylation of p107weel by p56chk1 does not alter its Y15 kinase activity

Evoking a checkpoint which functions through p107weel-dependent Y15 phosphorylation of p34cdc2 could have either of two effects on p107weel 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 phosphory-

Fig. 5. p56chk1 is a p107weel kinase. (A) A recombinant baculovirus expressing a (his)6-chk1 was constructed in pBlueBacHisB (Invitrogen). p56chk1 was detected by Western blotting of total extracts (lane 2) or cobalt-selected extracts (lanes 3 and 4) of cobalt-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 p56chk1, but not by cobalt–Sepharose-selected precipitates of cells infected with wild-type virus. In this experiment, p56chk1 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 p56chk1 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 p107weel 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 p56chk1 could directly phosphorylate p107weel, and phosphoamino acid analysis showed that this occurred exclusively on serine residues (Figure 5). Control experiments confirmed that p56chk1 was unable to phosphorylate GST. We consistently observed low level labelling of GST–wee1K596L incubated either alone or with cobalt–Sepharose prepa-

They confirm that p107weel and p56chk1 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 p107weel by p56chk1 does not alter its Y15 kinase activity

Fig. 6. Phosphorylation of p107weel by p56chk1 does not alter its Y15 kinase activity. (A) Baculovirus-expressed GST–wee1 and GST– wee1K596L were incubated with p56chk1 bound to cobalt–Sepharose beads or incubated with control beads as described in Figure 5. In both cases, wee1 proteins were phosphorylated by p56chk1. (B) These preparations of GST–wee1 and GST–wee1K596L were then assayed for Y15 kinase activity using baculovirus-expressed p34cdc2(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 probed with anti-p34cdc2 (PSTAIRE) 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 p56chk1.
(GST–wee1K596L) had no detectable Y15 kinase activity. These data were confirmed in other experiments by 32P-labelling of p34\(^{cd2}\), and also by assays of p34\(^{cd2}\) 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 G\(_2\) 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\(^{cd2}\)-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 G\(_2\) cells, we showed that p34\(^{cd2}\) 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\(^{cd2}\). These data are consistent with those described for mammalian cells which have correlated p34\(^{cd2}\) Y15 phosphorylation with DNA damage-induced G\(_2\) delay (Kharbanda et al., 1994a). Furthermore, other groups have demonstrated recently that Y15 phosphorylation is crucial in the G\(_1\) DNA checkpoints of Aspergillus nidulans (Ye et al., 1997) and fission yeast (N.Rhind and P.Russell, personal communication), and in a G\(_2\) checkpoint resulting from irradiation of human cells in S-phase (Jin et al., 1996).

In our experiments, the cells are already in G\(_2\) when they suffer DNA damage, and so our data would indicate that, under these conditions, the checkpoint functions to hold the cells in G\(_2\) and prevent the onset of mitosis whilst the DNA is repaired. This maintenance of p34\(^{cd2}\) inhibition by Y15 phosphorylation rather than induction of an additional arrest mechanism is in contrast to that described for vertebrate cells in G\(_1\): 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 G\(_1\) cdks by tyrosine phosphorylation in G\(_1\) checkpoints has also been shown by mutation in cdk4 (Tereda et al., 1995). Furthermore, cdc25 phosphatases acting in G\(_1\) have the ability, when overproduced, to override G\(_1\) 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\(^{cd2}\) Y15 phosphorylation is associated with G\(_2\) 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 G\(_2\) cell cycle arrest with p34\(^{cd2}\) inactivated by Y15 phosphorylation. Importantly, we established that this arrest mechanism functioned directly through wee1 rather than through another p34\(^{cd2}\) 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\(^{cd2}\), 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\(^{cd2}\) 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, rad24Δ cells exhibit a wee phenotype, and thus also have a defect in cell cycle progression accelerating them through G\(_2\). These phenotypes could be related if rad24Δ 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, with 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 rad24Δ 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
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 G2: as a consequence of DNA damage, p56\text{chk1} is activated and maintains p34\text{cdcl2} Y15 phosphorylation by phosphorylating p107\text{wee1} (Figure 7). This phosphorylation does not affect the in vivo p107\text{wee1} kinase activity, but does affect the in vitro function of p107\text{wee1}, perhaps by affecting its intracellular localization, interaction with p34\text{cdcl2} or interaction with negative regulatory proteins functioning at the G2-M transition. This leads to the activity of p107\text{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\text{wee1} activity above that observed in normal G2 cells (McGowan and Russell, 1995; Mueller et al., 1995). Although the DNA replication and DNA damage checkpoints have overlapping components, p56\text{chk1} is not involved in the replication checkpoint, suggesting that separable but overlapping pathways are involved in G2 checkpoint controls. A good candidate for a protein carrying out an analogous function to p56\text{chk1} in the fission yeast DNA replication checkpoint is p52\text{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\text{chk1}. However, it has been shown that p56\text{chk1} becomes phosphorylated after DNA damage (Walworth and Bernards, 1996), and this may play some role in its activation.

If p107\text{wee1} is a target of the G2 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\text{wee1} is a target of the checkpoint, but not the only target. At least one other Y15 kinase, p66\text{mik1}, is likely to play some role. The radiation sensitivity of wee1\Delta 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 G2 controls are lost and cells enter mitosis constitutively, leading to a lethal mitotic catastrophe phenotype. Although wee1 and mik1 have overlapping functions, mik1\Delta mutants do not exhibit a wee phenotype, suggesting that p66\text{mik1} plays a minor role in G2 control in the presence of p107\text{wee1}. It is also important to note that the viability of wee1\Delta cells, even when unirradiated, requires all known G3 checkpoint functions, including chk1 (Al-Khodair 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\text{mik1} so as to prevent premature entry into mitosis. Our data show that Y15 phosphorylation plays a role in the G2 DNA damage checkpoint functioning through p56\text{chk1}, but we cannot rule out additional regulatory mechanisms.

The data presented here provide a direct link between a G2 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* 972r-. Standard procedures for propagation, transformation and extract preparation were used. Small G2 cells prepared from synchronous cultures were derived by centrifugal elutriation. For UV irradiation, cells were re-innucleated into pre-warmed (30°C) medium for a recovery period of 20 min, and then filtered in aliquots on 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 seption 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\text{wee1} from fission yeast**

In order to detect p107\text{wee1} by Western blotting, a tagging fragment corresponding to six consecutive histidines and three copies of the influenza virus HA epitope (RSHHIIHHHGRFFYPYDPDYAGY-PYDPDYASYYPYDPDVAQGGR) was inserted into the 4\betaHI 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\text{wee1}, proteins were resolved on a 6% SDS-PAGE gel, transferred to nitrocellulose and probed with the monoclonal antibody 12CA5. For dephosphorylation of p107\text{wee1}, protein extracts were prepared from boiled cells in 8 M urea, 100 mM NaH2PO4, 10 mM Tris, 60 mM β-glycerophosphate, 100 μM Na orthovanadate, 15 mM p-Nitrophenyl Phosphate (PNPP), 1% Triton X-100, pH 8, and p107\text{wee1} was affinity isolated on Ni-NTA-agarose (Qiagen). Beads were washed extensively in extraction buffer, and urea

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**Fig. 7.** Model for p56\text{chk1} function in the G2 DNA damage checkpoint. Irradiation of a cell in G2 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\text{chk1}. p56\text{chk1}, perhaps through a mechanism requiring the rad24-encoded 14-3-3 protein, then phosphorylates p107\text{wee1}. The hyperphosphorylated form of p107\text{wee1} can then maintain phosphorylation of p34\text{cdcl2} on Y15 for the duration of the checkpoint delay. Maintenance of Y15 phosphorylation results in maintenance of G2, even though the normal cell size and completion of S-phase requirements for mitosis have been fulfilled.
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 chkl open reading frame was amplified by PCR to include BglII 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 m.o.i. of 5, with extracts prepared 2–3 days post-infection. Recombinant p56Δchkl was isolated from S21 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-Sepharose (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 p34cdc2 kinase assays using histone H1 as a substrate were performed on immunoprecipitates using a monoclonal antibody directed against the ccld13-encoded B-type cyclin. Assays were quantified by Phospholager analysis (Molecular Dynamics). For phosphorylation of p107weel by p56Δchkl baculovirus-expressed proteins were selected on affinity reagents (cobalt–Sepharose or glutathione–Sepharose), 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 1 μCi of 3000 Ci/mmol [β³²P]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 Phospholager analysis (Molecular Dynamics). Control experiments confirmed that p56Δchkl was unable to phosphorylate GST. For phosphorylation of γ-Raf acid activation, 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 phosphoamino acid standards and electrophoresed at pH 3.5 on a thin-layer cellulose plate. For assays of p107weel activity, p34cdc2 (K33R) was isolated in complexes with GST–cyclin B on glutathione–Sepharose, and GST–weel 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-p34cdc2 (PSTAIRE) to control for loading.

Western blots were quantified by densitometry (Molecular Dynamics).

**Antibodies**

Polyclonal antisera against p56Δchkl were raised in rabbits immunized with full-length bacterially expressed, histidine-tagged p56Δchkl. 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 p56Δchkl was confirmed by comparing extracts of wild-type, chklΔΔ and chkl1-overexpressing cells. For detection of Y15-phosphorylated p34cdc2, a phospho-specific rabbit polyclonal antibody (New England Biolabs) was used at a concentration of 1:250. For detection of epitope-tagged p107weel, the 12CA5 monoclonal antibody was used at a concentration of 40 μg/ml. For detection of total p34cdc2, a rabbit polyclonal antisera directed against the PSTAIR region (a kind gift of Dr Hideo Nishitani) was used at a concentration of 1:10000.

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