

The G₂/M DNA damage checkpoint inhibits mitosis through Tyr15 phosphorylation of p34^{cdc2} in *Aspergillus nidulans*

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It is possible to cause G₂ arrest in *Aspergillus nidulans* by inactivating either p34^{cdc2} or NIMA. We therefore investigated the negative control of these two mitosis-promoting kinases after DNA damage. DNA damage caused rapid Tyr15 phosphorylation of p34^{cdc2} and transient cell cycle arrest but had little effect on the activity of NIMA. Dividing cells deficient in Tyr15 phosphorylation of p34^{cdc2} were sensitive to both MMS and UV irradiation and entered lethal premature mitosis with damaged DNA. However, non-dividing quiescent conidiospores of the Tyr15 mutant strain were not sensitive to DNA damage. The UV and MMS sensitivity of cells unable to tyrosine phosphorylate p34^{cdc2} is therefore caused by defects in DNA damage checkpoint regulation over mitosis. Both the *nimA5* and *nimT23* temperature-sensitive mutations cause an arrest in G₂ at 42°C. Addition of MMS to *nimT23* G₂-arrested cells caused a marked delay in their entry into mitosis upon downshift to 32°C and this delay was correlated with a long delay in the dephosphorylation and activation of p34^{cdc2}. Addition of MMS to *nimA5* G₂-arrested cells caused inactivation of the H1 kinase activity of p34^{cdc2} due to an increase in its Tyr15 phosphorylation level and delayed entry into mitosis upon return to 32°C. However, if Tyr15 phosphorylation of p34^{cdc2} was prevented then its H1 kinase activity was not inactivated upon MMS addition to *nimA5* G₂-arrested cells and they rapidly progressed into a lethal mitosis upon release to 32°C. Thus, Tyr15 phosphorylation of p34^{cdc2} in G₂ arrests initiation of mitosis after DNA damage in *A.nidulans*.

Keywords: *Aspergillus*/DNA damage checkpoint/
nimX^{cdc2}/Tyr15 phosphorylation of p34^{cdc2}

Introduction

DNA damage causes a G₂ delay of the cell cycle in eukaryotic cells (Hartwell and Weinert, 1989; Carr, 1995; Kaufmann, 1995; Murray, 1995). This G₂ delay presumably allows cells enough time to repair damaged DNA before initiation of mitosis. The dependency of initiation of mitosis on completion of DNA damage repair is established by G₂/M DNA damage checkpoints. The first direct genetic link between the G₂ checkpoint regulation and DNA damage was demonstrated with *rad9* mutations in budding yeast. Strains carrying *rad9* mutations are

deficient in G₂ delay after DNA damage, and thus enter lethal premature mitosis in the presence of damaged DNA (Weinert and Hartwell, 1988). Many genes involved in the G₂/M DNA damage checkpoint regulation were subsequently identified in both budding and fission yeasts by screening for mutations which uncouple initiation of mitosis from G₂ delay after DNA damage (Al-Khodairy and Carr, 1992; Allen *et al.*, 1994; Weinert *et al.*, 1994). Many of these checkpoint genes have now been cloned. However, the cell cycle targets for the checkpoint regulation to bring about G₂ delay in response to DNA damage remain to be established (Carr, 1995; Murray, 1995; Lydall and Weinert, 1996).

The activation of the universally conserved p34^{cdc2} H1 kinase, the mitosis-promoting factor (MPF), is central to the timing and initiation of mitosis in all eukaryotic cells (Draetta, 1990; Nurse, 1990; Murray, 1992; Dunphy, 1994; Osmani *et al.*, 1994). The activation of p34^{cdc2} requires association with the regulatory subunit cyclinB which accumulates during late S-phase and G₂ (Evans *et al.*, 1983; Booher *et al.*, 1989), and the phosphorylation of Thr161 by CDC2-activating kinase (CAK) (Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993). During S and G₂ the p34^{cdc2}-cyclinB complex is kept in the inactive pre-MPF state through Tyr15 phosphorylation of p34^{cdc2} by Wee1/Mik1/Myt1 tyrosine kinases (Russell and Nurse, 1987; Gould and Nurse, 1989; Lungren *et al.*, 1991; Parker and Piwnicka-Worms, 1992; Mueller *et al.*, 1995). During the G₂/M transition the pre-MPF form of p34^{cdc2} is rapidly converted into the active MPF form by Tyr15 dephosphorylation catalyzed by the Cdc25 tyrosine phosphatase (Russell and Nurse, 1986; Kumagai and Dunphy, 1991). The major regulatory event for the final activation of p34^{cdc2} during mitotic initiation thus appears to be the rapid dephosphorylation at Tyr15. Therefore, Tyr15 phosphorylation of p34^{cdc2} could be a target for negative regulation by the G₂/M DNA damage checkpoint control systems.

Elevated levels of Tyr15-phosphorylated p34^{cdc2} are found to be associated with G₂-arrested cells after DNA damage in several systems (Kharbanda *et al.*, 1994; O'Connor *et al.*, 1994; Herzinger *et al.*, 1995; Barth *et al.*, 1996). However, the significance of Tyr15 phosphorylation of p34^{cdc2} in the G₂/M DNA damage checkpoint regulation remains to be established in these systems. The regulatory pathway for p34^{cdc2} tyrosine phosphorylation/dephosphorylation and its role in cell cycle regulation are best characterized in fission yeast (Russell and Nurse, 1986, 1987; Gould and Nurse, 1989; Lungren *et al.*, 1991). Fission yeast cells unable to tyrosine phosphorylate p34^{cdc2} advance mitosis and produce small 'wee' cells (Gould and Nurse, 1989; Lungren *et al.*, 1991). These mutant cells enter mitosis even when DNA replication is inhibited (Enoch and Nurse, 1990; Lungren *et al.*, 1991). Thus,

Tyr15 phosphorylation of p34^{cdc2} links initiation of mitosis to completion of DNA replication in fission yeast. However, the role of such phosphorylation in the G₂/M DNA damage checkpoint regulation is not well understood. Fission yeast cells deficient in Wee1 function are sensitive to DNA damage by UV and gamma irradiation (Al-Khodairy and Carr, 1992; Rowley *et al.*, 1992). In addition, Rowley *et al.* (1992) reported that the DNA damage checkpoint in fission yeast is mediated through the Wee1 protein kinase as fission yeast cells deficient in Wee1 function lack a significant mitotic delay after gamma irradiation. However, Barbet and Carr (1993) subsequently showed that fission yeast cells defective or lacking in Wee1 function have a normal mitotic arrest after DNA damage. The Tyr15 phosphorylation state and H1 kinase activity of p34^{cdc2} in response to DNA damage, however, were not analyzed biochemically in these studies. Thus, the role of Tyr15 phosphorylation of p34^{cdc2} in the G₂/M DNA damage checkpoint regulation remains to be firmly established in fission yeast through a combination of both biochemical and genetic studies. However, such a role in response to DNA damage has recently been established in human cells (Jin *et al.*, 1996). On the other hand, both DNA replication and DNA damage checkpoints are fully operative in budding yeast cells unable to tyrosine-phosphorylate p34^{cdc28} (Amon *et al.*, 1992; Sorger and Murray, 1992; Stueland *et al.*, 1993), conclusively demonstrating that in this system S-phase and G₂/M checkpoint regulations inhibit mitosis by some other mechanism.

In *Aspergillus nidulans*, coordinate activation of two mitosis-promoting kinases is required for the initiation of mitosis (Osmani *et al.*, 1991a; Ye *et al.*, 1995). One is the universally conserved p34^{cdc2} H1 kinase encoded by the *nimX* gene (Osmani *et al.*, 1994). The regulatory pathway for p34^{cdc2} activation is also conserved in *A. nidulans* (Osmani *et al.*, 1991a, 1994; O'Connell *et al.*, 1992; P.Ramos, X.Ye, R.Fincher, S.Osmani and L.Ellis, in preparation). The other essential mitosis-promoting kinase is the NIMA kinase which is currently well characterized only in *A. nidulans* (for review, see Osmani and Ye, 1996). However, indirect evidence indicates that a NIMA-like mitotic pathway may also exist in fission yeast, *Xenopus* and human cells (O'Connell *et al.*, 1994; Lu and Hunter, 1995). Unlike most mitotic regulatory functions which regulate p34^{cdc2} H1 kinase activity, NIMA kinase is required for initiation of mitosis independently of the regulation of p34^{cdc2} H1 kinase activity (Osmani *et al.*, 1991a).

We recently uncovered two overlapping S-phase checkpoint mechanisms over the initiation of mitosis in *A. nidulans* (Ye *et al.*, 1996). The first, the slowing of S-phase checkpoint, monitors the rate of DNA replication and, when DNA replication is slowed, prevents mitosis temporarily through Tyr15 phosphorylation of p34^{cdc2}. Thus, when DNA replication is slowed, cells deficient in tyrosine phosphorylation of p34^{cdc2} enter mitosis prematurely. The second, the S-phase arrest checkpoint, requires both BIME function and Tyr15 phosphorylation of p34^{cdc2} which in combination inactivate NIMA kinase and cause inhibition of mitosis when DNA replication is actually halted. Thus, cells lacking either Tyr15 phosphorylation of p34^{cdc2} (Ye *et al.*, 1996) or the function of BIME (Osmani *et al.*, 1988, 1991b; James *et al.*, 1995) have a limited capacity to initiate mitosis when DNA replication

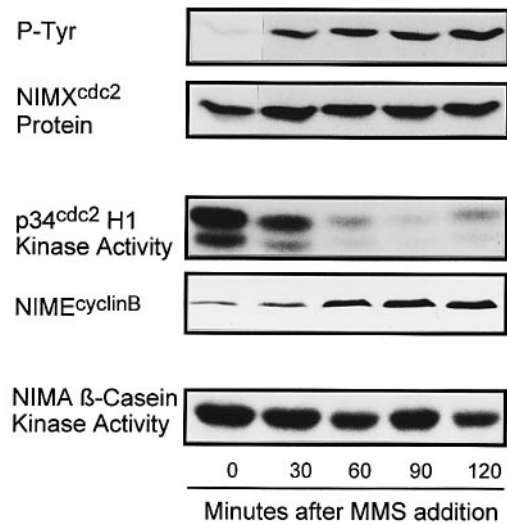


Fig. 1. Effects of DNA damage by MMS on p34^{cdc2} and NIMA kinases. MMS (0.04%) was added to an exponentially growing wild-type (strain R153) culture. Samples were taken at 30 min intervals after MMS addition, and NIMA and p34^{cdc2} kinase activities were analyzed. The level of Tyr15-phosphorylated p34^{cdc2} was determined by Western blotting using a monoclonal anti-phosphotyrosine antibody after immunoprecipitation of p34^{cdc2}. After P-Tyr detection the blot was stripped and the protein level of p34^{cdc2} in the immunoprecipitates was detected using E-77 anti-NIMX^{cdc2} antisera.

is stopped. Only cells lacking both Tyr15 phosphorylation of p34^{cdc2} and the function of BIME are able to activate NIMA precociously and initiate mitosis effectively from S-phase in the absence of DNA replication (Ye *et al.*, 1996).

In this study we examined the relationship between Tyr15 phosphorylation of p34^{cdc2} and G₂ delay caused by DNA damage in *A. nidulans*. We found that *A. nidulans* cells unable to Tyr15-phosphorylate p34^{cdc2} were deficient in the DNA damage-mediated G₂ delay and entered mitosis prematurely after DNA damage. Consequently, only dividing cells deficient in Tyr15 phosphorylation of p34^{cdc2} were sensitive to DNA damage. Thus, Tyr15 phosphorylation of p34^{cdc2} in G₂ alone links initiation of mitosis to completion of DNA damage repair in *A. nidulans*.

Results

MMS increases the level of Tyr15-phosphorylated p34^{cdc2}

Methyl methanesulfonate (MMS) causes effective DNA damage in *A. nidulans* (Kafer and Mayor, 1986), the addition of 0.01% MMS to germinating wild-type conidia delaying the first mitosis for more than 4 h. The first mitosis of germinating conidiospores normally occurs between 5.5 and 6.5 h after germination at 32°C, whereas the first mitosis of MMS-treated germinating conidiospores did not occur until 9–10 h (data not shown, see also Figure 3). This suggests that a DNA damage checkpoint mechanism operates in *A. nidulans*, the activation of which causes a delay of entry into mitosis.

To investigate if either (or both) of the mitosis-producing kinases, p34^{cdc2} or NIMA, are targets of the DNA damage checkpoint mechanism, we first determined the biochemical effects of addition of MMS to an exponentially growing wild-type culture. Addition of MMS only slightly inhibited NIMA kinase activity (Figure 1). We previously

demonstrated that NIMA is a target for the negative regulation by S-phase checkpoints via both Tyr15 phosphorylation of p34^{cdc2} and the function of BIME (Ye *et al.*, 1996). Activation of S-phase checkpoints by the DNA synthesis inhibitor hydroxyurea (HU) rapidly inactivates the NIMA kinase (Ye *et al.*, 1996). Here, lack of strong inhibition of NIMA by DNA damage suggests that NIMA is not a primary target for the DNA damage checkpoint system.

In contrast, addition of MMS caused rapid Tyr15 phosphorylation of p34^{cdc2} (Figure 1), the level of phosphorylation increasing markedly 30 min after MMS addition. Even though the level of NIME^{cyclinB} increased, p34^{cdc2} H1 kinase activity was reduced after MMS addition (Figure 1). Thus, the DNA damage checkpoint could delay mitosis by inhibition of p34^{cdc2} through Tyr15 phosphorylation.

Dividing cells deficient in Tyr15 phosphorylation of p34^{cdc2} are sensitive to UV irradiation and MMS because they enter mitosis prematurely with damaged DNA

If the DNA damage checkpoint is indeed mediated through Tyr15 phosphorylation of p34^{cdc2}, dividing cells unable to undergo such phosphorylation should be sensitive to DNA-damaging agents, as cells deficient in DNA damage checkpoint regulation would be unable to restrain cell cycle progression and would enter lethal premature mitosis in the presence of damaged DNA. Non-dividing cells deficient in p34^{cdc2} Tyr15 phosphorylation, on the other hand, should not be sensitive to DNA-damaging agents as they would not be able to undergo a lethal premature mitosis but would be able to repair damaged DNA. We recently generated a *nimX^{cdc2AF}* mutant strain, in which p34^{cdc2} cannot be tyrosine-phosphorylated, using a two-step gene replacement technique after *in vitro* mutagenesis of *nimX^{cdc2}* (Ye *et al.*, 1996). We therefore checked the sensitivity of dividing and non-dividing *nimX^{cdc2AF}* cells to UV irradiation. Wild-type and DNA damage repair-deficient *uvsH4^{rad18}* (Kafer and Mayor, 1986; Yoon *et al.*, 1995) strains were used as controls.

When UV irradiation was applied to quiescent conidiospores, the *nimX^{cdc2AF}* mutant strain was no more sensitive to UV irradiation than the wild-type (Figure 2A). The *nimX^{cdc2AF}* conidiospores must therefore be able to repair DNA damage during the process of germination before they initiate mitosis after entering the cell cycle from G₁ arrest. On the other hand, the *uvsH4^{rad18}* strain, as previously reported (Kafer and Mayor, 1986), was extremely sensitive to UV irradiation, and was completely killed by a 100 J/m² dose which only slightly affected the wild-type and *nimX^{cdc2AF}* strains (Figure 2A). The data suggest that, unlike the DNA repair-deficient *uvsH4^{rad18}* strain, the *nimX^{cdc2AF}* mutant has no deficiency in DNA damage repair mechanisms.

In contrast, germinating *nimX^{cdc2AF}* conidiospores were sensitive to UV irradiation (Figure 2B). In this experiment conidiospores were first allowed to germinate for 4.5 h before UV irradiation was applied, and by which time they had entered the first cell cycle. As the *nimX^{cdc2AF}* mutant cells attempt the first mitosis earlier than the wild-type (Ye *et al.*, 1996; see also Figure 3), we checked whether the difference in UV sensitivity between the wild-

type and the *nimX^{cdc2AF}* mutant strains was caused by the different cell cycle stages at which the UV irradiation was applied. However, when germinating conidiospores of the wild-type strain were irradiated by UV at various times after germination, their survival rate was very similar to that shown in Figure 2B. Thus, UV sensitivity of dividing *nimX^{cdc2AF}* mutant cells is likely to be caused specifically by deficient DNA damage checkpoint regulation.

As MMS addition to a growing culture caused rapid Tyr15 phosphorylation and reduced the H1 kinase activity of p34^{cdc2} (Figure 1), we expected that the *nimX^{cdc2AF}* mutant cells would also be sensitive to MMS. As shown in Figure 2C and D, the *nimX^{cdc2AF}* strain was indeed much more sensitive to MMS than the wild-type, measured either as reduction in survival rate or in colony size in the presence of various concentrations of MMS incorporated into the medium.

To confirm directly that the *nimX^{cdc2AF}* mutant was deficient in the DNA damage checkpoint control, we determined the kinetics of entry into mitosis of germinating conidiospores in the presence of a low concentration of MMS. In the absence of MMS the chromosome mitotic index (CMI%) of the wild-type strain began to rise at 5 h and peaked at 6.5 h after germination (Figure 3). Addition of MMS to the germinating medium of the wild-type strain completely inhibited mitosis for up to 7 h after germination (Figure 3).

As previously demonstrated (Ye *et al.*, 1996), *nimX^{cdc2AF}* mutant cells entered mitosis earlier than the wild-type cells (Figure 3). Addition of MMS delayed but did not stop the *nimX^{cdc2AF}* mutant cells progressing into mitosis (Figure 3). By 7 h after germination, >50% of the *nimX^{cdc2AF}* mutant cells entered mitosis, whereas none of the wild-type cells attempted mitosis in the presence of MMS (Figure 3). These data show that the *nimX^{cdc2AF}* mutant strain is deficient in the DNA damage checkpoint regulation, as it entered mitosis prematurely in the presence of damaged DNA, thus exhibiting increased sensitivity to DNA-damaging agents.

Addition of MMS, however, did cause a delay of progression into mitosis in the *nimX^{cdc2AF}* germinating conidiospores (Figure 3). Such delay may be caused by non-specific cytotoxic effects of MMS, although addition of MMS may have activated additional checkpoint mechanisms. As germinating conidiospores enter the cell cycle from G₁, candidates for such additional checkpoint mechanisms are the G₁/S checkpoints which transiently delay entry into S-phase after DNA damage, as demonstrated in the budding yeast (Siede *et al.*, 1993, 1994; Lydall and Weinert, 1996).

We demonstrated previously that Thr14 phosphorylation cooperates with Tyr15 phosphorylation in the negative regulation of p34^{cdc2} in response to the S-phase checkpoint control (Ye *et al.*, 1996). Thus, the strain bearing both T14A and Y15F mutations of *nimX^{cdc2}* demonstrates greater sensitivity to HU than does the strain with only the Y15F mutation (Ye *et al.*, 1996). To investigate whether Thr14 phosphorylation of p34^{cdc2} also had a role in the DNA damage checkpoint regulation, we compared the UV sensitivity of germinating conidiospores derived from various mutant strains (Ye *et al.*, 1996). The strain bearing the T14A mutation alone showed a wild-type level of sensitivity to UV (Figure 2E). Unlike the sensitivity to

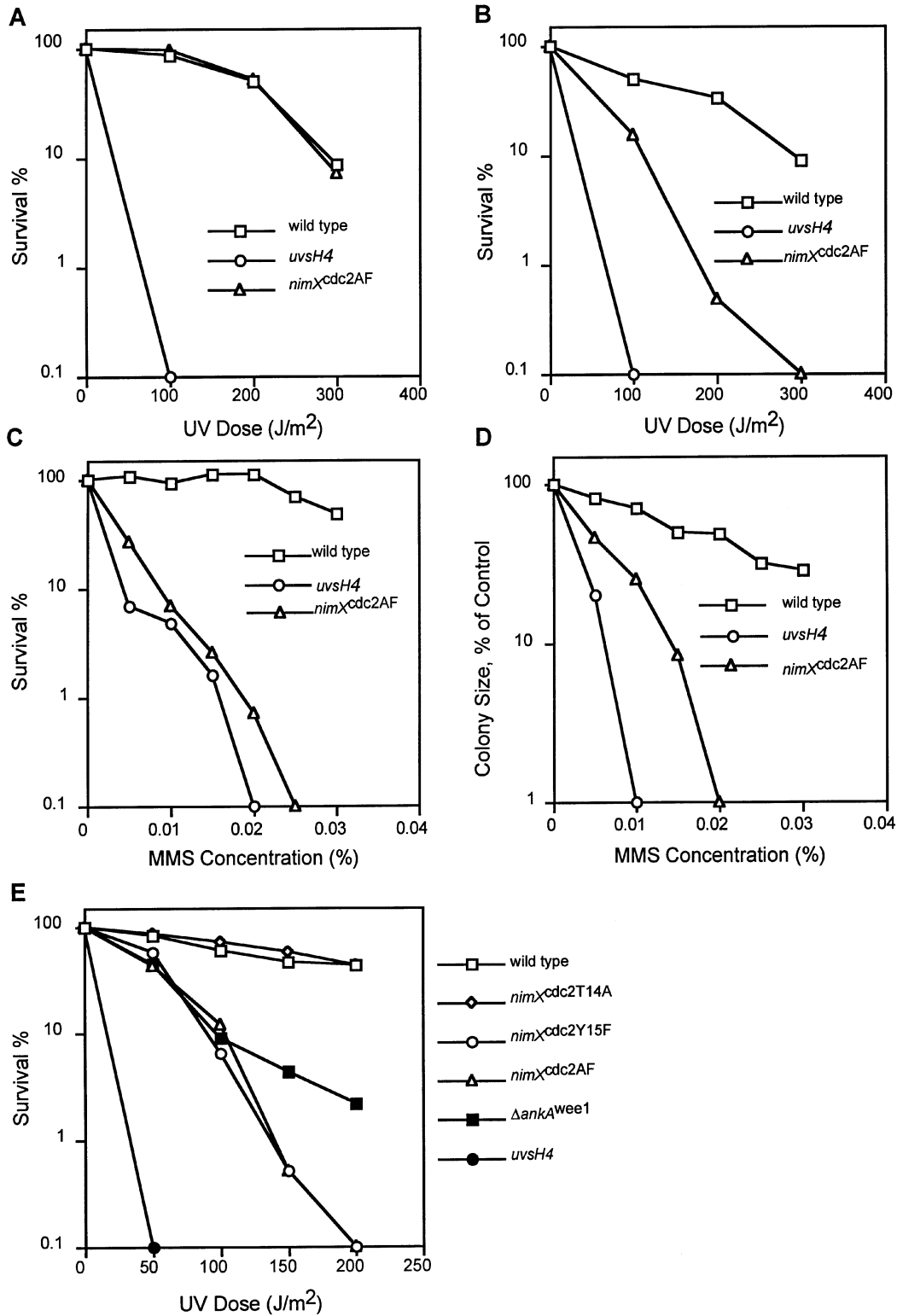


Fig. 2. UV and MMS sensitivity of strains deficient in Tyr15 phosphorylation of p34^{cdc2}. Conidiospores (250 spores/plate) of a wild-type and *nimXcdc2AF* strain were spread out onto YAG plates. The plates were either immediately UV irradiated (A) or incubated at 32°C for 4.5 h to allow spore germination and were then UV irradiated (B). After UV irradiation the plates were incubated at 32°C for 2 days for colony formation. To test sensitivity of the strains to MMS, conidiospores (250 spores/plate) were spread out on YAG plates containing various concentrations of MMS (C). The survival rate of the strains after DNA damage either by UV irradiation or by MMS incorporated into the medium was expressed as percentage of colonies produced by control conidiospores in the absence of treatment. In addition the strains were spot-inoculated onto MMS-containing YAG plates to assess colony growth (D). The colony size was measured 3 days after inoculation and effect of MMS on colony growth was expressed as a percentage of colony size of their respective controls without MMS. The survival rate of various mutant strains affecting the inhibitory phosphorylation status of p34^{cdc2} after UV irradiation (E). UV irradiation was applied 4.5 h after germination as for (B).

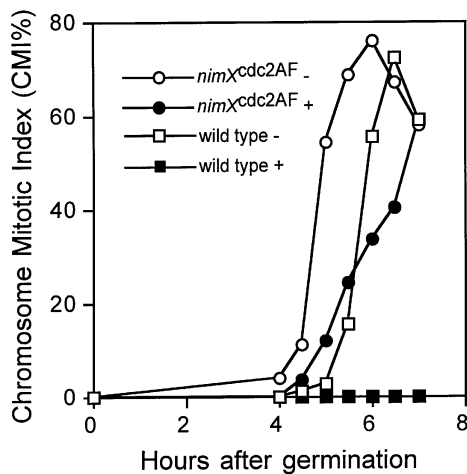


Fig. 3. *nimXcdc2AF* mutations promote mitosis prematurely in the presence of DNA damage. Conidiospores of the wild-type and *nimXcdc2AF* strains were germinated in YG medium containing 5 μ g/ml nocodazole and 0.01% MMS. Nocodazole was added to trap cells in mitosis in order to facilitate the determination of the rate at which cells entered mitosis. Samples were taken at the time intervals indicated, fixed and stained with DAPI. CMI% is shown as percentage of germinated spores with condensed chromatin. -, absence of MMS; +, presence of MMS.

HU, the strain bearing both T14A and Y15F mutations did not have more UV sensitivity than did the strain with the Y15F mutation alone, and similar results were obtained using MMS as the DNA-damaging agent (data not shown). Thus, Thr14 phosphorylation of p34^{cdc2} appears to have no role in the DNA damage checkpoint regulation, at least in response to UV and MMS. As previously demonstrated for HU sensitivity of the strains (Ye *et al.*, 1996), MMS sensitivity caused by the Tyr15 mutation of p34^{cdc2} is also dominant over the endogenous wild-type *nimXcdc2*. The strain with Δ *ankA*^{wee1} was also found to be UV (Figure 2E) and MMS sensitive (data not shown). Thus the DNA damage checkpoint in *A.nidulans* in response to UV and MMS is likely to be mediated through Tyr15 phosphorylation of p34^{cdc2}.

DNA damage by MMS delays entry into mitosis during *nimT23cdc25* block-release by delaying Tyr15 dephosphorylation of p34^{cdc2}

The major DNA damage checkpoint in both budding and fission yeasts operates during the G₂/M transition preventing initiation of mitosis in the presence of damaged DNA (Hartwell and Weinert, 1989; Carr, 1995; Lydall and Weinert, 1996). To address the role of Tyr15 phosphorylation of p34^{cdc2} in the G₂/M DNA damage checkpoint control specifically, we utilized G₂-specific temperature-sensitive mutations to synchronize cells at G₂ by temperature upshift before causing DNA damage with MMS, and then observed the relationship between Tyr15 phosphorylation of p34^{cdc2} and the initiation of mitosis upon return of the cells to permissive temperature.

Inactivation of NIMT^{cdc25}, a homolog of the fission yeast p34^{cdc2}-specific tyrosine phosphatase Cdc25, blocks cells at G₂ (Figure 4B, -MMS). At the arrest point, p34^{cdc2}-cyclinB accumulates as inactive pre-MPF in which p34^{cdc2} is phosphorylated at both Tyr15 and Thr161 (Figure 4A and C, G₂ sample). In exponentially-growing cells,

p34^{cdc2} is present in two states, seen as two bands on Western blots (Figure 4A and C, EX. cells). The faster-migrating band of p34^{cdc2} is associated with NIME^{cyclinB} and is phosphorylated at Thr161 or at both Thr161 and Tyr15 (Osmani *et al.*, 1994; Ye *et al.*, 1996). Upon release to permissive temperature, cells entered a rapid and synchronous mitosis with peak CMI at 10–15 min (Figure 4B, -MMS; Ye *et al.*, 1995) as p34^{cdc2} became rapidly tyrosine-dephosphorylated and activated. As previously reported (Ye *et al.*, 1995), NIMA and NIME^{cyclinB} accumulated at the *nimT23cdc25* G₂ arrest point, and the NIMA kinase became fully activated and hyperphosphorylated after activation of p34^{cdc2} by tyrosine dephosphorylation (Figure 4A). As cells progressed through mitosis, both NIMA and NIME^{cyclinB} were degraded, leading to the down-regulation of the two mitosis-promoting kinases. When NIME^{cyclinB} was degraded, some p34^{cdc2} became dephosphorylated at Thr161 and was thus converted to the non-phosphorylated slower-migrating band on Western blot (Figure 4A and C).

MMS was added to the *nimT23cdc25* G₂-arrested cells for 75 min to cause transient DNA damage and then removed from the culture by fresh medium exchange as cells were down-shifted to release the G₂ block. Addition of MMS to *nimT23cdc25* G₂-arrested cells markedly delayed entry into mitosis after return to 32°C (Figure 4B, + MMS). This mitotic delay was correlated with delayed activation of p34^{cdc2} by tyrosine dephosphorylation (Figure 4C) and p34^{cdc2} remained Tyr15-phosphorylated for 1 h compared with only 5 min without MMS addition (Figure 4A). NIME^{cyclinB} accumulated at the *nimT23cdc25* G₂ arrest point and remained stable during the MMS-induced mitotic delay (Figure 4C). As p34^{cdc2} became transiently activated by Tyr15 dephosphorylation between 60 and 70 min after release (Figure 4C) cells concurrently underwent a largely synchronous mitosis with peak CMI at 70 min (Figure 4B). Then, the level of NIME^{cyclinB} was reduced, and p34^{cdc2} kinase activity was down-regulated, as cells progressed through mitosis (Figure 4B and C). These data clearly demonstrate a strong correlation between G₂ delay and Tyr15 phosphorylation of p34^{cdc2} after DNA damage caused by addition of MMS.

Inactivation of p34^{cdc2} by Tyr15 phosphorylation after DNA damage by MMS prevents rapid entry into mitosis upon release of the *nimA5* mutation

Previous studies have demonstrated that NIMA is required for mitotic initiation by a mechanism that does not involve activation of p34^{cdc2} H1 kinase activity (Osmani *et al.*, 1991a; Ye *et al.*, 1996). For example, inactivation of NIMA does not prevent full activation of p34^{cdc2} H1 kinase activity and inactivation of NIMA prevents mitotic initiation even when p34^{cdc2} is fully activated and cannot be Tyr15 phosphorylated (Ye *et al.*, 1996; Figures 5A and 6). In fact, upon release of cells from the *nimA5* G₂ arrest point, the levels of p34^{cdc2} H1 kinase activity are seen to decrease as cells are entering mitosis (Figure 5A). We therefore asked whether the activated p34^{cdc2} present at the *nimA5* G₂ arrest point is inactivated upon DNA damage by MMS. As can be seen in Figure 5B, and as expected, p34^{cdc2} activity increased when cells were arrested in G₂ due to inactivation of NIMA. However, upon addition of MMS, p34^{cdc2} activity was severely inhibited and it was

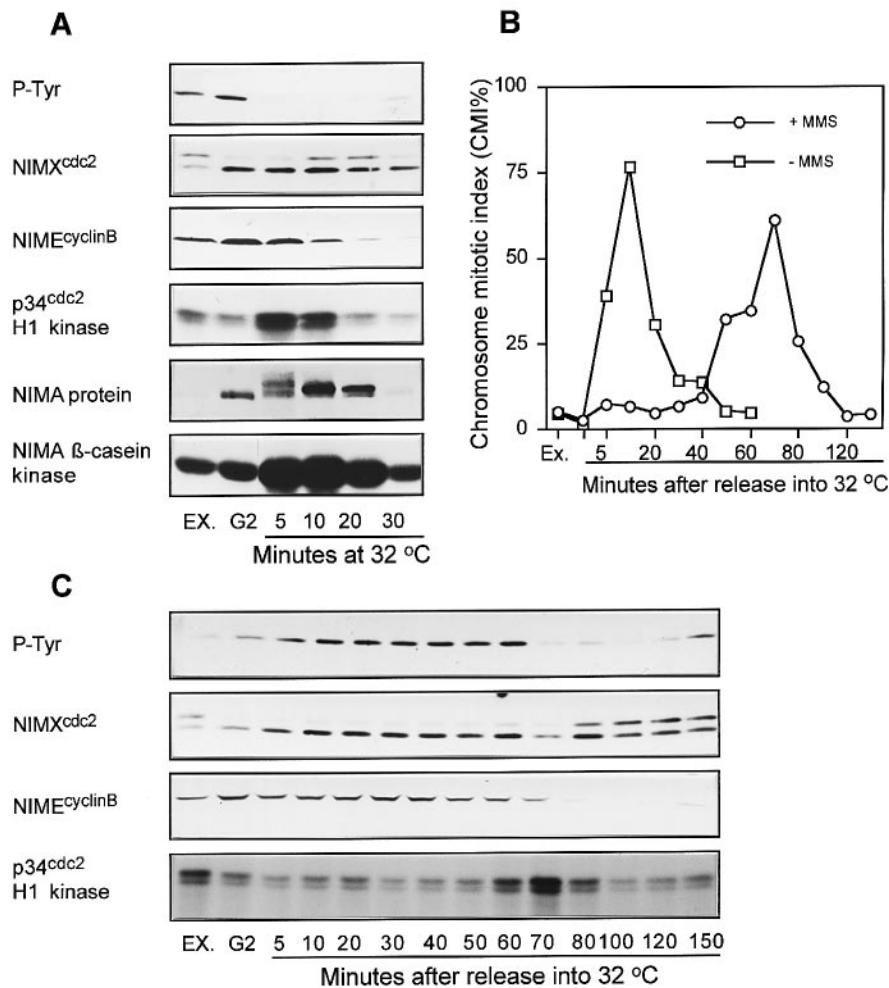


Fig. 4. *nimT23cdc25* block–release experiment in the presence and absence of MMS. (A) *nimT23cdc25* block–release in the absence of MMS addition. (B) Chromosome mitotic index (CMI%) during *nimT23cdc25* block–release with or without MMS addition. (C) *nimT23cdc25* block–release in the presence of MMS addition. An early log phase culture of *nimT23cdc25* was upshifted to the restrictive temperature of 42°C to inactivate NIMT^{cdc25} and cause cell cycle arrest at the *nimT23cdc25* G₂ arrest point. MMS was added to the G₂-arrested culture to a final concentration of 0.04% at 2.5 h after temperature upshift and was removed from the culture 75 min after addition by exchange with fresh medium prewarmed to 42°C. The culture was then released from the G₂ arrest into permissive temperature of 32°C. For the control culture in the absence of MMS, cells were blocked for the same period of time (3.25 h) at 42°C as for the MMS-treated culture before release into permissive temperature to allow the initiation of synchronous mitosis. Samples were taken at the time points indicated for CMI%, and for biochemical analysis.

Tyr15-phosphorylated (Figure 5B). Normally, release of cells from the *nimA5* G₂ arrest point allows cells rapidly to enter mitosis (Figure 5A). However, after MMS addition there was a long G₂ delay as p34^{cdc2} remained Tyr15-phosphorylated and largely inactivated. These cells eventually entered a partially synchronous mitosis as the level of Tyr15 phosphorylation decreased (Figure 5B). During the G₂ delay caused by MMS addition, the level of NIME^{cyclinB} increased and virtually all p34^{cdc2} was phosphorylated on the activating Thr161 site (faster-migrating band on NIMX^{cdc2} Western blot, Figure 5B). Thus, the G₂ delay after DNA damage is not mediated through regulation of either NIME^{cyclinB} levels or CAK activity but instead correlates well with Tyr15 phosphorylation of p34^{cdc2}.

In summary, *nimT23cdc25* and *nimA5* mutations arrest cells in G₂ at restrictive temperature either before (*nimT23cdc25*) or after (*nimA5*) activation of p34^{cdc2} and generate rapid synchronous mitosis upon release into permissive temperature. Addition of MMS to *nimT23cdc25* and *nimA5* G₂-arrested cells caused a marked delay of

entry into mitosis after release of the G₂ arrests. This mitotic delay caused by MMS was correlated with Tyr15 phosphorylation of p34^{cdc2}.

Cells unable to tyrosine-phosphorylate p34^{cdc2} lack the G₂ delay after DNA damage and enter mitosis prematurely

To demonstrate directly whether Tyr15 phosphorylation of p34^{cdc2} was responsible for the DNA damage-induced G₂ delay, we repeated the *nimA5* block–release experiments with or without MMS addition using *nimA5* + Δ *ankA*^{wee1} and *nimA5* + *nimXcdc2AF* double mutants. As expected, the double mutants were still arrested in G₂ at the restrictive temperature for the *nimA5* mutation, although p34^{cdc2} cannot be Tyr15-phosphorylated (Ye *et al.*, 1996; Figure 6). Upon release into permissive temperature, the double-mutant strains both rapidly underwent a similar synchronous mitosis (Figure 6, –MMS). This G₂ arrest using the *nimA5* mutation therefore enabled us to analyze directly the consequence of lack of p34^{cdc2} tyrosine

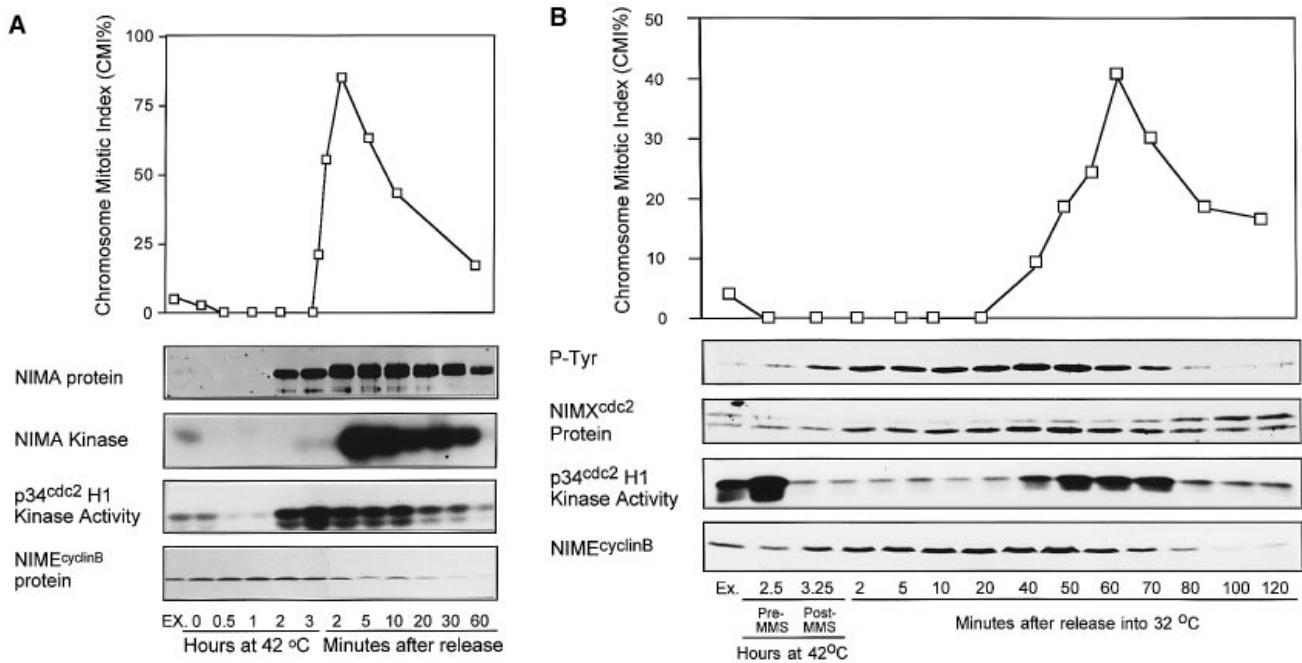


Fig. 5. *nimA5* block-release experiment in the presence and absence of MMS. *nimA5* block-release with or without MMS was carried out as for *nimT23^{cdc25}* block-release described in Figure 4. (A) *nimA5* block-release in the absence of MMS addition. (B) *nimA5* block-release in the presence of MMS addition.

phosphorylation during a synchronized mitosis in the presence of damaged DNA.

Addition of MMS to *nimA5* G₂-arrested cells markedly delayed entry into mitosis (Figure 6). If the *anka^{wee1}* gene was deleted, this G₂ delay was partially overcome (Figure 6) and a partially synchronous mitosis of the *nimA5* + Δ *anka^{wee1}* strain occurred ~30 min earlier than the *nimA5* single mutant strain. However, if the putative inhibitory phosphorylation sites, T14 and Y15, of p34^{cdc2} (*nimX^{cdc2}*) were mutated to non-phosphorylatable A and F residues respectively (*nimX^{cdc2AF}*), this G₂ delay was then completely overcome (Figure 6). Furthermore, p34^{cdc2} H1 kinase activity remained high after MMS addition in the *nimX^{cdc2AF}* mutant cells (data not shown). Thus, the G₂ delay after DNA damage was mediated through Tyr15 phosphorylation of p34^{cdc2}.

The partial override of the MMS-induced G₂ delay by Δ *anka^{wee1}* suggests the existence of a *mik1* homolog in *A.nidulans*. This likelihood is further indicated as Δ *anka^{wee1}* only partially complements the *nimT23^{cdc25}* mutation, whereas non-Tyr15-phosphorylated p34^{cdc2} (*nimX^{cdc2Y15F}* or *nimX^{cdc2AF}*) completely complements *nimT23^{cdc25}* (P.Ramos, X.Ye, R.Fincher, S.Osmani and L.Ellis, in preparation).

To determine the consequence of the premature mitosis caused by *nimX^{cdc2AF}* after DNA damage in the above block-release experiments, we compared the viability of the single *nimA5* and the *nimA5* + *nimX^{cdc2AF}* double-mutant cells. As shown in Figure 7, cells of either the *nimA5* or *nimA5* + *nimX^{cdc2AF}* double-mutant strains remained viable after *nimA5* block-release in the absence of MMS treatment. With the addition of MMS, the viability of the single *nimA5* mutant cells was slightly reduced. In contrast, a major reduction in viability of the double-mutant cells was observed. Thus, *A.nidulans* cells unable to tyrosine-phosphorylate p34^{cdc2} are deficient in the

G₂/M DNA damage checkpoint control. The mutant cells undergo premature lethal mitosis in the presence of damaged DNA and thus exhibit increased sensitivity to DNA-damaging agents.

Discussion

In this study we have demonstrated that *A.nidulans* cells have a DNA damage checkpoint mechanism operating during the G₂/M transition to restrain progression into mitosis if cellular DNA is damaged. Furthermore, we have established that this G₂/M DNA damage checkpoint is mediated through Tyr15 phosphorylation of p34^{cdc2} to bring about G₂ delay of entry into mitosis. A role for the inhibitory phosphorylation of p34^{cdc2} in radiation-induced G₂ arrest has also recently been demonstrated in human cells (Jin *et al.*, 1996). In *A.nidulans* the NIMA kinase is also required for the initiation of mitosis and has a role in the S/M checkpoint regulation (Ye *et al.*, 1996). However, we found that NIMA does not have a role in the G₂/M DNA damage checkpoint regulation. The relationship between the G₂/M DNA damage checkpoint regulation and Tyr15 phosphorylation of p34^{cdc2} is shown in Figure 8.

The cellular level of Tyr15-phosphorylated p34^{cdc2} is regulated by two opposing enzymatic activities. The Wee1 tyrosine kinase specifically phosphorylates Tyr15, and Cdc25 tyrosine phosphatase specifically dephosphorylates Tyr15 of p34^{cdc2}. In *A.nidulans* the Tyr15 phosphorylation/dephosphorylation of p34^{cdc2} (NIMX^{cdc2}) is carried out by ANKA^{wee1} and NIMT^{cdc25}, homologs of fission yeast Wee1 and Cdc25 respectively (O'Connell *et al.*, 1992; P.Ramos, X.Ye, R.Fincher, S.Osmani and L.Ellis, in preparation). The activities of Wee1 and Cdc25 are cell cycle-regulated (Izumi *et al.*, 1992; Kumagata and Dunphy, 1992; Tang *et al.*, 1993; for a review, see Maller, 1994). During

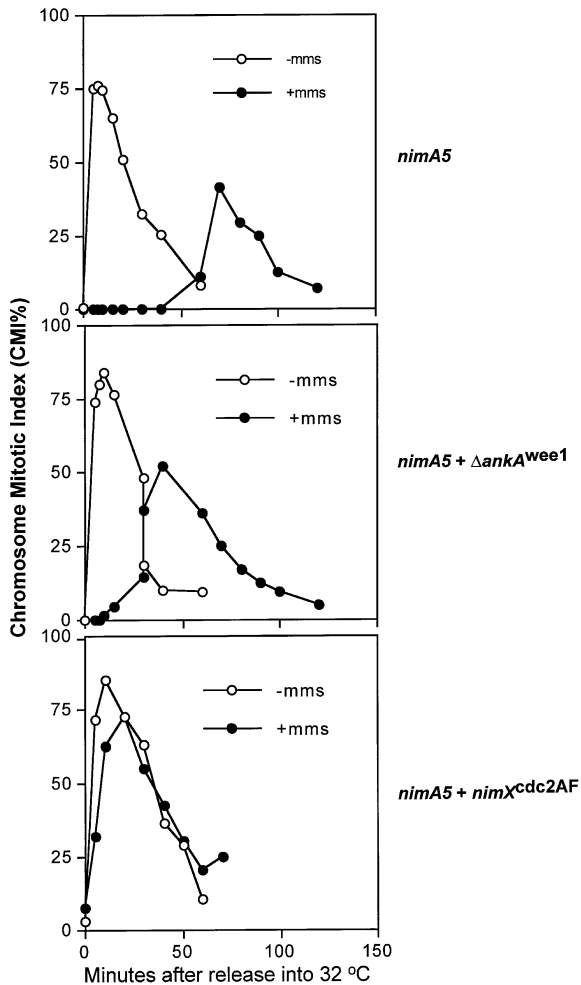


Fig. 6. Chromosome mitotic index (CMI%) of the *nimA5*, and *nimA5 + ΔankA^{wee1}* or *nimA5 + nimX^{cdc2AF}* double-mutant strains during *nimA5* block–release in the presence or absence of MMS addition. Conidiospores of the *nimA5*, and *nimA5 + ΔankA^{wee1}* or *nimA5 + nimX^{cdc2AF}* double-mutant strains were germinated for 8 h at the restrictive temperature of 42°C for the *nimA5* mutation. MMS was then added to the culture and then removed by medium exchange 75 min after addition; cells were then released immediately into permissive temperature. Cell samples were taken at the indicated time points after release, fixed and stained with DAPI. Chromosome mitotic index was determined as percentage of cells with condensed chromatin.

interphase, the Wee1 kinase activity is high, whereas Cdc25 phosphatase activity is low. Thus, p34^{cdc2} H1 kinase accumulates in the Tyr15-phosphorylated, inactive pre-MPF form during interphase. During the G₂/M transition Cdc25 phosphatase activity is abruptly activated and Wee1 kinase inactivated, thus leading to rapid Tyr15 dephosphorylation and activation of p34^{cdc2} H1 kinase. It is believed that Wee1, Cdc25 and p34^{cdc2} H1 kinase are all in a feedback loop in which activation of p34^{cdc2} further activates itself through feedback activation of Cdc25 and inhibition of Wee1 (King *et al.*, 1994; Maller, 1994). However, this regulatory circuit of p34^{cdc2} activation is apparently not essential during a normal cell cycle progression in *A.nidulans* as cells deficient in Tyr15 phosphorylation of p34^{cdc2} are viable (Ye *et al.*, 1996 and the present study).

At present, it is not understood how the DNA damage

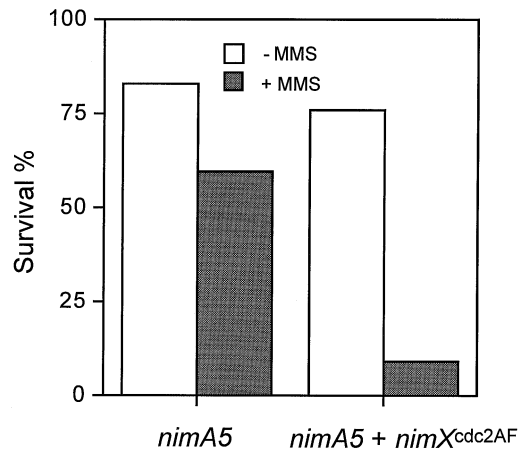


Fig. 7. Cell viability of the *nimA5* single and the *nimA5 + nimX^{cdc2AF}* double-mutant strains during a *nimA5* block–release in the presence or absence of MMS addition. The *nimA5* temperature block–release and MMS addition were carried out exactly as described for Figure 6. After release from the *nimA5* G₂ arrest MMS was removed and germlings were plated out on YAG plates for colony formation. Cell viability was then assessed as percentage of colonies formed by the same number of conidiospores without temperature block–release and MMS addition.

DNA damage checkpoint

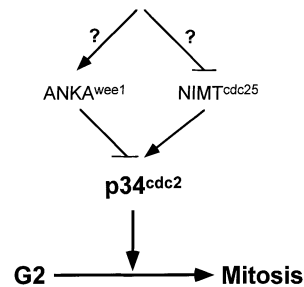


Fig. 8. Model showing the relationship between Tyr15 phosphorylation of p34^{cdc2} and the initiation of mitosis mediated by the G₂/M DNA damage checkpoint.

checkpoint in *A.nidulans* regulates p34^{cdc2} activation to keep p34^{cdc2} Tyr15-phosphorylated during response to DNA damage. This could be achieved by either activating the ANKA^{wee1} kinase or inactivating the NIMT^{cdc25} phosphatase, or both. Certainly ANKA^{wee1} kinase is required for the G₂/M DNA damage checkpoint regulation because ANKA^{wee1} is the major tyrosine kinase that phosphorylates p34^{cdc2} at Tyr15 (P.Ramos, X.Ye, R.Fincher, S.Osmani and L.Ellis, in preparation). Moreover, ΔANKA^{wee1} cells are DNA damage checkpoint-deficient and are sensitive to DNA-damaging agents. However, whether the DNA damage checkpoint regulates ANKA^{wee1} kinase activity directly is currently not known.

The results of *nimT23^{cdc25}* block–release with MMS addition suggest that NIMT^{cdc25} may be inactivated by the DNA damage checkpoint. Normally NIMT^{cdc25} is very rapidly activated upon release from *nimT23^{cdc25}* G₂ arrest as p34^{cdc2} becomes Tyr15-dephosphorylated and activated within 5 min after release (Figure 4A). However, p34^{cdc2} remains highly Tyr15-phosphorylated for 1 h after release if MMS is added to cause transient DNA damage to the *nimT23^{cdc25}* G₂-arrested cells (Figure 4C). If activation of NIMT^{cdc25} is indeed prevented during this temperature

block–release in response to DNA damage, then p34^{cdc2} would remain Tyr15-phosphorylated. The inactivation of Cdc25C phosphatase activity has been implicated in G₂ arrest in response to DNA damage in human cells (O’Conor *et al.*, 1994; Barth *et al.*, 1996). However, we cannot exclude the possibility that high activation of ANKA^{wee1} after DNA damage could act to counteract the normal activation of NIMT^{cdc25}.

At the *nimA5* G₂ arrest point, p34^{cdc2} is already activated by Tyr15 dephosphorylation (Figure 5A; Osmani *et al.*, 1991a). According to the positive feedback loop scheme of p34^{cdc2} activation (King *et al.*, 1994; Maller, 1994), activated p34^{cdc2}, as seen at the *nimA5* G₂ arrest point, would generate fully activated NIMT^{cdc25} but inactive ANKA^{wee1}. If this regulatory scheme is active in *A.nidulans* then the ANKA^{wee1} kinase would be inactive at the *nimA5* G₂ arrest point as p34^{cdc2} is activated. DNA damage would then have to lead to some activation of ANKA^{wee1}, or an equivalent kinase, in order to cause Tyr15 phosphorylation of p34^{cdc2}. In addition to the studies presented here, DNA damage by MMS can also effectively cause Tyr15 phosphorylation and inactivation of p34^{cdc2} in cells blocked in mitosis (X.Ye, R.Fincher, A.Tang and S.Osmani, unpublished data). This suggests that in *A.nidulans* checkpoint systems that monitor successful progression through mitosis could also be mediated through inactivation of p34^{cdc2} by Tyr15 phosphorylation. It will be of interest to see how ANKA^{wee1} and NIMT^{cdc25} are regulated in response to DNA damage at different periods of the cell cycle to ascertain their respective roles in the inhibition of p34^{cdc2} at different cell cycle stages.

We recently demonstrated that Tyr15 phosphorylation of p34^{cdc2} has a role in two overlapping S-phase checkpoint mechanisms in *A.nidulans*, one involving both Tyr15 phosphorylation of p34^{cdc2} and the function of BIME (Ye *et al.*, 1996). How is Tyr15 phosphorylation of p34^{cdc2} involved in both the S-phase checkpoint and the G₂/M DNA damage checkpoint regulation? The simplest explanation is that although cells may use different mechanisms to detect unreplicated DNA and DNA damage, the signals generated in response to such detections may converge upon the same cell cycle target, that is, inhibition of p34^{cdc2} by Tyr15 phosphorylation. In addition, these two checkpoint mechanisms may actually overlap as they both monitor, and respond to, the state of DNA, and therefore may share common components of a checkpoint signal transduction pathway leading to Tyr15 phosphorylation of p34^{cdc2}. This hypothesis is supported by the observations that *A.nidulans* cells bearing mutations in *uvsB* or *uvsD*, which were originally isolated as UV irradiation-sensitive mutants (Jansen, 1970; Fortuin, 1971), are deficient not only in S-phase checkpoint control but also in the G₂/M DNA damage checkpoint control (X.Ye, A.Tang, R.Fincher and S.Osmani, unpublished results). Similarly, several genes have been identified in fission yeasts that are required for both S-phase and G₂/M checkpoint controls (Carr, 1995) and these functions may also influence the Tyr15 phosphorylation state of p34^{cdc2}, although this has not as yet been tested. Although tyrosine phosphorylation of p34^{cdc28} has no role in S-phase or DNA damage checkpoint controls in budding yeast, several genes which have overlapping functions in both S-phase and G₂/M DNA damage checkpoint controls have also been identified in

this system (Murray, 1995; Lydall and Weinert, 1996). The target of these genes to cause cell cycle arrest after DNA damage or inhibition of DNA replication remains to be identified.

In addition to identifying the G₂/M DNA damage checkpoint system mediated through Tyr15 phosphorylation of p34^{cdc2}, the present results also suggest that *A.nidulans* cells may have a p34^{cdc2} Tyr15 phosphorylation-independent DNA damage checkpoint system operating in earlier stages of the cell cycle. This hypothesis is based on the observation that although non-Tyr15-phosphorylated p34^{cdc2} mutant cells completely overcome the MMS-induced G₂ delay (Figure 6), the same mutant cells show a delay of progression into mitosis when germinated from G₁ in the presence of MMS (Figure 3). There are at least two possible levels of control that may mediate this delay after DNA damage that do not rely on Tyr15 phosphorylation of p34^{cdc2}. In mammalian cells, p53 is known to be required for the DNA damage-induced G₁ arrest, partly through a mechanism involving p53-dependent induction of the CDK inhibitor p21 (Kaufmann, 1995; Lydall and Weinert, 1996). In addition, DNA damage may cause a slowing or arrest of DNA replication which could then impose S-phase checkpoint regulation over mitosis (Lamb *et al.*, 1989; Paulovich and Hartwell, 1995). If DNA damage does lead to impaired DNA replication in *A.nidulans* it is unlikely that this would lead to our observed delay in mitosis in the *nimX^{cdc2AF}* mutant strain germinated in the presence of MMS, as the slowing of S-phase checkpoint over mitosis is defective in this strain (Ye *et al.*, 1996). If germination in MMS leads to an arrest of the initiation of S-phase, or causes its delay, then the arrest of S-phase checkpoint could be operative as this level of control involves not only Tyr15 phosphorylation of p34^{cdc2} but also the function of *bimE* (Ye *et al.*, 1996). Future studies will determine if *bimE* plays a role in DNA damage checkpoint control in G₁ and S-phase.

In conclusion, *A.nidulans* cells have a G₂/M DNA damage checkpoint mechanism and the major cell cycle target for this checkpoint regulation is Tyr15 phosphorylation of p34^{cdc2}. In response to DNA damage the checkpoint control rapidly inhibits cell cycle progression into mitosis by inactivating p34^{cdc2} H1 kinase through Tyr15 phosphorylation, presumably allowing time for DNA damage repair in G₂ before initiation of mitosis. Having established the cell cycle targets for the S-phase checkpoints (Ye *et al.*, 1996) and for the DNA damage checkpoint we can now begin to delineate the signal transduction pathways leading to Tyr15 phosphorylation of p34^{cdc2} in response to inhibition of DNA replication and to DNA damage in *A.nidulans*.

Materials and methods

Aspergillus nidulans strains and general techniques

A.nidulans strains used in this study were R153 (*pyroA4*; *wA3*); SO53 (*nimT23^{cdc25}*; *wA2*); SO54 (*nimA5*; *wA2*); Δ AnkA^{wee1}(Δ ankA^{wee1}; *pyrG89*; *pyr4+*; *pyroA4*; *wA3*); FRY2 (*nimA5*; Δ ankA^{wee1}; *pyrG89*; *pyr4+*; *yA2*); FRY20 (*pNIG6-nimX^{cdc2AF}*; *pyr4+* *pyroA4*; *pyrG89*; *wA3*); FRY20-1 (*nimX^{cdc2AF}*; *pyroA4*; *pyrG89*; *wA3*); FRY24 (*pNIG6-nimX^{cdc2Y15F}*; *pyr4+*; *pyroA4*; *pyrG89*; *wA3*); FRY25 (*pNIG6-nimX^{cdc2T14A}*; *pyr4+*; *pyroA4*; *pyrG89*; *wA3*); AT27 (*nimX^{cdc2AF}*; *nimA5*; *pyroA4*; *riboA2*; *wA3*); A329 (*adE20*; *biA1*; *wA3*; *uvsH4*; *methG1*; *pyroA4*). Media and general techniques for culture, protein extraction,

protein immunoprecipitation, NIMA and p34^{cdc2} kinase assays, Western blotting, and DAPI staining for chromosome mitotic index determination were as previously described (Osmani *et al.*, 1987, 1991a, 1994; Oakley and Osmani, 1993; Ye *et al.*, 1995).

Sensitivity test to UV irradiation and MMS

Non-dividing and dividing cells deficient in Tyr15 phosphorylation of p34^{cdc2} were tested for sensitivity to UV irradiation. Conidiospores (dormant in a quiescent G₀ state) were suspended in 0.2% Tween-20 and plated out on YAG plates (250 spores/plate). The plates were then irradiated immediately with UV using a microprocessor-controlled UV crosslinker (FB-UVXL-1000; Fisher Biotech, Pittsburgh, PA, USA) to determine UV sensitivity of non-dividing cells. To determine UV sensitivity of dividing cells, conidiospores on YAG plates were first allowed to germinate for 4.5 h at 32°C before UV irradiation. By this time the germinated spores had entered the cell cycle and were about to undergo the first mitosis. UV-irradiated germlings were then incubated in a 32°C incubator for 2 days for colony formation. The survival rate after UV irradiation was determined as a percentage of colonies produced by control conidiospores without UV irradiation.

In MMS (Aldrich, St Louis, MO, USA) sensitivity tests, various concentrations of MMS were incorporated into YAG medium. To determine the survival rate in the presence of MMS, conidiospores (250 spores/plate) were plated out on YAG plates containing MMS. After a 2 day incubation at 32°C, the survival rate was determined as the percentage of colonies produced by control conidiospores on YAG plates containing no MMS. MMS sensitivity was also measured by reduction in colony growth. In this case conidiospores were spot-inoculated with tooth-picks onto YAG plates containing MMS.

nimT23^{cdc25} and *nimA5* temperature block–release

nimT23^{cdc25} and *nimA5* mutant cells were first grown to early log phase at 32°C and were then rapidly upshifted to the restrictive temperature of 42°C. After G₂ arrest at 42°C, cells were released into synchronous mitosis by temperature downshift to 32°C. To determine the effect of MMS on the rapid synchronous mitosis generated by *nimT23^{cdc25}* and *nimA5* block–release, MMS (0.04%) was added to the G₂-arrested cells 2.5 h after temperature upshift to 42°C. After incubation at 42°C for a further 75 min, MMS was removed from the culture by medium exchange and cells were then downshifted to permissive temperature of 32°C.

Cell viability assay after *nimA5* block–release

nimA5 mutant conidiospores were germinated at 42°C for 8 h in YG medium containing 0.1% agar to prevent clumping. The germinating spores were arrested in the first cell cycle at the *nimA5* G₂ arrest point. MMS (0.04%) was added to the *nimA5* G₂-arrested germlings for 75 min to cause DNA damage and was then removed from the culture by medium exchange with fresh YG containing no agar. The germlings were then released from *nimA5* G₂ arrest into the permissive temperature of 32°C. The germlings were resuspended in 0.2% Tween-20, plated out (250 germlings/plate), and incubated at 32°C for colony formation. Cell viability was assessed as the percentage of colonies produced by 250 conidiospores without *nimA5* temperature block–release and MMS addition.

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