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The NIMA protein kinase is hyperphosphorylated and activated downstream of p34^{cdc2}/cyclin B: coordination of two mitosis promoting kinases

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Initiation of mitosis in *Aspergillus nidulans* requires activation of two protein kinases, p34^{cdc2}/cyclin B and NIMA. Forced expression of NIMA, even when p34^{cdc2} was inactivated, promoted chromatin condensation. NIMA may therefore directly cause mitotic chromosome condensation. However, the mitosis-promoting function of NIMA is normally under control of p34^{cdc2}/cyclin B as the active G_2 form of NIMA is hyperphosphorylated and further activated by p34^{cdc2}/cyclin B when cells initiate mitosis. To see the p34^{cdc2}/cyclin B dependent activation of NIMA, okadaic acid had to be added to isolation buffers to prevent dephosphorylation of NIMA during isolation. Hyperphosphorylated NIMA contained the MPM-2 epitope and, *in vitro*, phosphorylation of NIMA by p34^{cdc2}/cyclin B generated the MPM-2 epitope, suggesting that NIMA is phosphorylated directly by p34^{cdc2}/cyclin B during mitotic initiation. These two kinases, which are both essential for mitotic initiation, are therefore independently activated as protein kinases during G_2. Then, to initiate mitosis, we suggest that each activates the other's mitosis-promoting functions. This ensures that cells coordinately activate p34^{cdc2}/cyclin B and NIMA to initiate mitosis only upon completion of all interphase events. Finally, we show that NIMA is regulated through the cell cycle like cyclin B, as it accumulates during G_2 and is degraded only when cells traverse mitosis.

Key words: *Aspergillus nidulans*/mitosis/NIMA/p34^{cdc2}/cyclin B/protein kinase

Introduction

Analysis of cell cycle mutants in *Aspergillus nidulans* has indicated that independent activation of two protein kinases is required for cells to initiate mitosis (Osmani *et al.*, 1991a). One, the ubiquitous p34^{cdc2}/cyclin B histone H1 kinase, is well established as the key determinant of mitotic initiation in many systems (Draetta, 1990; Nurse, 1990; Norbury and Nurse, 1992). The other, the NIMA protein kinase, has at present only been established as a key determinant of mitotic initiation in *A.nidulans* (Oakley and Morris, 1983; Osmani *et al.*, 1987, 1988, 1991a,b) although related kinases have been reported in organisms ranging from yeast (Jones and Rosamond, 1990) to mouse (Letwin *et al.*, 1992) and humans (Schultz and Nigg, 1993). The mammalian NIMA related kinases have been termed Nek1–3. Human Nek2 not only resembles NIMA in the catalytic domain but, like NIMA in *A.nidulans*, it is also expressed in a cell cycle dependent manner, being maximal during mitosis (Schultz *et al.*, 1994), suggesting a role in mitotic regulation. Several other genes have been identified that are also required for mitotic initiation but virtually all of these are thought to function by either directly or indirectly affecting the H1 kinase activity of p34^{cdc2}/cyclin B (Nurse, 1990). Conditional inactivation of NIMA does not prevent activation of p34^{cdc2}/cyclin B as an H1 kinase, suggesting that NIMA plays a role in mitotic initiation that does not involve activation of p34^{cdc2}/cyclin B H1 kinase activity (Osmani *et al.*, 1991a). This makes the NIMA kinase particularly important and a full molecular description of mitotic regulation requires an understanding of the relationship between the NIMA and p34^{cdc2}/cyclin B protein kinases.

Both NIMA and p34^{cdc2}/cyclin B exhibit cell cycle regulated protein kinase activity that peaks during mitosis. Both require mitotic progression in order to down-regulate their kinase activity for the next cell cycle. The regulation of p34^{cdc2}/cyclin B H1 kinase activity is well understood. During interphase, p34^{cdc2} associates with cyclin B (Evans *et al.*, 1983; Draetta *et al.*, 1988; Booher *et al.*, 1989) and is phosphorylated at Thr161 by CAK kinase and at Tyr15 by WEE1-like protein kinases. The Thr161 phosphorylation is required for activity (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Lorca *et al.*, 1992; Poon *et al.*, 1993; Solomon *et al.*, 1993) and the Tyr15 phosphorylation keeps the complex inactive (Gould and Nurse, 1989; Lundgren *et al.*, 1991). At the transition from G_2 into mitosis Tyr15 is dephosphorylated by the cdc25 tyrosine phosphatase and the p34^{cdc2}/cyclin B complex is activated as an H1 kinase (Gould and Nurse, 1989). As cells progress through mitosis cyclin B is proteolytically destroyed and the H1 kinase activity of p34^{cdc2}/cyclin B is inactivated (Glotzer *et al.*, 1991). If cells are arrested in mitosis then cyclin B is not destroyed and p34^{cdc2}/cyclin B maintains its H1 kinase activity (Murray and Kirschner, 1989).

The level of NIMA kinase activity varies through the cell cycle in a manner similar to that of the H1 kinase activity of p34^{cdc2}/cyclin B, especially with regard to the necessity to progress through mitosis to inactivate the elevated mitotic kinase levels (Osmani *et al.*, 1991b). Little is currently known about how the kinase activity of NIMA is regulated through the cell cycle but the level of *nimA* mRNA does vary through the cell cycle, peaking at mitosis (Osmani *et al.*, 1987) and *in vitro* dephosphoryla-

tion of NIMA inactivates its kinase activity (Lu *et al.*, 1993).

To address directly the relationship between p34^{cdc2}/cyclin B and NIMA during mitotic initiation we recently cloned the p34^{cdc2} gene of *A.nidulans* (designated *nimX*^{cdc2}) which is a functional homolog of fission yeast *cdc2*. Temperature sensitive alleles of *nimX* were generated by reverse genetics based on mutations defined in fission yeast *cdc2* (Osmani *et al.*, 1994). Utilizing mutations in *nimX*^{cdc2} and *nimT*^{cdc25} (O'Connell *et al.*, 1992) we have investigated how NIMA activity is regulated through the cell cycle and to define further the relationship between the two mitosis specific kinases. The results demonstrate that NIMA protein levels are regulated like a mitotic cyclin, peaking at G_2 and being destroyed upon mitotic exit. NIMA is also shown to be phosphorylated in a p34^{cdc2}/cyclin B dependent fashion during mitotic initiation. The mitotic phosphorylation of NIMA boosts its G_2 activity. Evidence is presented indicating that NIMA may be phosphorylated directly by p34^{cdc2}/cyclin B during mitotic initiation.

Results

Generation of NIMA specific antiserum and stabilization of NIMA kinase activity by okadaic acid during isolation

We have generated an antiserum (E-14) against *Escherichia coli* expressed NIMA. The antiserum specifically immunoprecipitated NIMA in an active form and allowed us to determine the level of NIMA by Western blot and to assay its kinase activity after immunoprecipitation. Preimmune serum did not precipitate significant β-casein kinase activity but immune serum precipitated β-casein kinase activity equal to that isolated using the previously characterized (Osmani *et al.*, 1991b) anti-peptide antiserum generated against the ANYRED region of NIMA (Figure 1a). The β-casein kinase activity precipitated by E-14 antiserum from extracts prepared from a strain containing the temperature sensitive *nimA5* mutation was also temperature sensitive compared with wild type extracts (Figure 1b). If extracts were precipitated with ANYRED antiserum and then probed with either ANYRED antiserum or E-14 antiserum by Western blot, we detected exactly the same bands from cell cycle staged extracts (Figure 1c) showing that the accumulated protein at G_2 and the upshifted bands seen during mitosis (see below) are also specific to NIMA (Figure 1c). Finally, overexpression of NIMA from an inducible promoter increased the abundance of a protein that migrates slightly above the 84 kDa molecular weight marker (Figure 1e) which is the same size band detected by ANYRED antiserum. These data demonstrate that the E-14 antiserum specifically detects NIMA on Western blots and can immunoprecipitate NIMA in an active form. However, to detect NIMA by Western blot from cells that do not overexpress NIMA we found it necessary first to immunoprecipitate NIMA from at least 2 mg of total protein prior to Western blot analysis.

Surprisingly, we have found that addition of the phosphatase inhibitor okadaic acid (OA) to HK extraction buffer (which already contains the following phosphatase inhibitors: 5 mM EDTA, 15 mM EGTA, 1 mM sodium vanadate, 10 mM sodium fluoride, 15 mM PNPP, 60 mM β-glycerophosphate) can markedly increase the level of NIMA kinase activity recovered after isolation. Addition of OA to HK extraction buffer modified the pattern of NIMA activation observed during *nimT23*^{cdc25} block/release experiments. Without addition of OA, low NIMA kinase activity was isolated from exponentially growing cells (Figure 1f, −OA). This level markedly increased at the G_2 arrest point, did not increase consistently at mitosis after release of the G_2 arrest, and decreased as cells progressed into the next cell cycle. This is the pattern of activation we have previously reported (Osmani *et al.*, 1991a). The addition of OA to HK extraction buffer caused stabilization of NIMA kinase activity in the exponential sample and in the mitotic sample (Figure 1f, Ex and 30′+). Thus, when OA is used during isolation, significant activation of NIMA is seen to be dependent upon activation of p34^{cdc2}/cyclin B by NIMT^{cdc25} (Figure 1f, +OA; see also Figures 3 and 6), an activation step we had previously not observed (Osmani *et al.*, 1991a). Addition of OA had no effect on the level of NIMA protein recovered (data not shown), further indicating that OA prevented dephosphorylation and inactivation of NIMA during isolation.

Active NIMA protein accumulates at the G_2 arrest point of nimT23^{cdc25} *and is hyperphosphorylated and further activated as cells enter mitosis*

As addition of OA to HK extraction buffer was able to significantly stabilize the kinase activity of NIMA during isolation, we re-addressed the issue of whether NIMA is phosphorylated or activated after activation of p34^{cdc2}/cyclin B. For the rest of the studies described proteins were isolated in the presence of OA to prevent inactivation of NIMA during isolation. Temperature shift experiments were repeated numerous times (Figures 2 and 3) using a temperature sensitive allele of *nimT*^{cdc25} (*nimT23*^{cdc25}). Cells were grown to early log phase at 30°C before being arrested in G_2 at the restrictive temperature (42°C). They were then returned to 30°C to allow cells synchronously to enter and complete mitosis. As previously reported, p34^{cdc2}/cyclin B H1 kinase activity increased upon release of *nimT23*^{cdc25} to permissive temperature as cells entered mitosis. This elevated level of H1 kinase activity was maintained if mitotic progression was halted by adding nocodazole before downshift (Figure 2). The stabilization of H1 kinase activity upon arrest in mitosis correlated with the stabilization of NIME$^{\text{cyclin B}}$ protein which, as expected, was degraded upon mitotic progression in the absence of nocodazole (Figure 2).

Upon shift to 42°C we observed an initial rapid inactivation and degradation of NIMA in both wild type cells (Ye *et al.*, unpublished) and the *nimT23*^{cdc25} strain (Figure 3). At the G_2 arrest point of *nimT23*^{cdc25} a significant accumulation of NIMA protein and a 4-fold increase in the level of NIMA protein kinase activity were observed (Figure 3). NIMA protein was phosphorylated at this arrest point as its mobility during SDS−PAGE was increased upon enzymatic dephosphorylation (Figure 1d, G_2 NIMA). Upon release into mitosis NIMA kinase activity was further increased 2-fold. In addition, the already phosphorylated NIMA was hyperphosphorylated as cells entered mitosis causing a mobility decrease for mitotic NIMA during

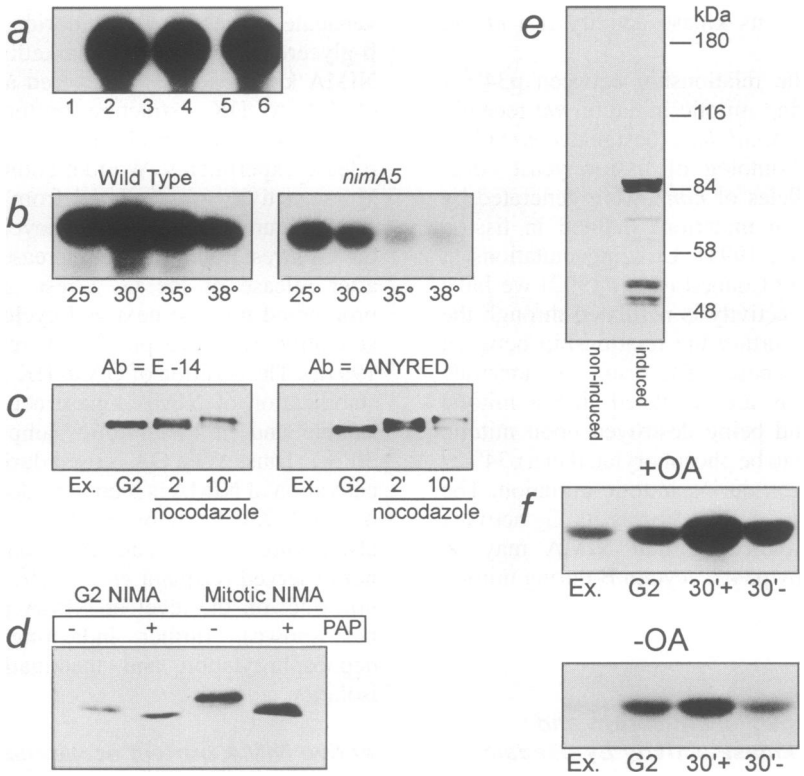

Fig. 1. Characterization of NIMA antisera and modification of NIMA. (**a**) Preimmune (lanes 1 and 3) and immune sera E-14 (lanes 2 and 4) raised against the bacterially expressed NIMA in two rabbits were compared with the previously characterized (Osmani *et al.*, 1991b) anti-NIMA ANYRED peptide preimmune (lane 5) and immune serum (lane 6) for their ability to immunoprecipitate NIMA in an active form. NIMA kinase activity was assayed using β-casein and [γ-^{32}P]ATP as substrate. Phosphorylated β-casein was resolved by SDS−PAGE and detected by autoradiography. (**b**) Wild type NIMA and temperature sensitive mutant NIMA5 were immunoprecipitated with E-14 antiserum and assayed for NIMA kinase activity at the temperatures indicated. (**c**) Western blot detection of NIMA protein immunoprecipitated with NIMA ANYRED anti-peptide serum and detected with E-14 (left) and ANYRED antisera (right). Protein extracts were made from exponentially growing cells (Ex.), cells arrested in G_2 by the *nimA5* mutation (G_2) and cells released into mitosis in the presence of nocodazole as indicated. (**d**) NIMA immunoprecipitated from *nimT23* G_2 arrested cells (G_2 NIMA) and cells released into mitosis in the presence of nocodazole (Mitotic NIMA) were treated with potato acid phosphatase (PAP) as indicated before separation by SDS−PAGE. NIMA was visualized by Western blotting using E-14 antiserum. (**e**) Western blot analysis of NIMA overexpressed in a strain of *A.nidulans* containing multiple copies of *nimA* under the control of the ethanol-inducible *alcA* promoter (Osmani *et al.*, 1988). Total protein extracts (200 μg) of pre-induced cells and cells induced in ethanol medium for 2.5 h were separated in 7.5% SDS−PAGE and detected by Western blotting with E-14 antiserum. Molecular weight standards (kDa) are indicated on the right. (**f**) Cell cycle staged cells were obtained by temperature shift using *nimT23*. Cells were ground in HK extraction buffer and NIMA was isolated by immunoprecipitation in the presence or absence of 1 μM OA and assayed for NIMA β-casein kinase activity. Ex., exponentially growing cells; G_2, cells arrested for 3 h at 42°C. Cells were released from the G_2 arrest point for 30 min in the presence (30′+) and absence (30′−) of nocodazole.

SDS−PAGE. This is most clearly seen for cells arrested in mitosis by nocodazole (Figure 3) and in the samples shown in Figure 6. The decreased mobility of NIMA seen upon the transition from G_2 into mitosis was caused by phosphorylation as enzymatic dephosphorylation of mitotic NIMA increased its mobility (Figure 1d, mitotic NIMA). These data indicate that G_2 arrest allowed accumulation of partially phosphorylated and partially activated NIMA protein. Full activation and hyperphosphorylation of NIMA then occurred as cells entered mitosis after activation of p34^{cdc2}/cyclin B by *nimT23*^{cdc25} mediated tyrosine dephosphorylation.

Upon mitotic progression the activity of NIMA was decreased (Figures 3 and 6). If cells were arrested in a pseudometaphase state, by addition of nocodazole before release of the G_2 arrest, then NIMA kinase activity remained at an elevated level. Upon mitotic progression NIMA was degraded but if cells were arrested in mitosis then NIMA protein remained stable in its hyperphosphorylated form (Figure 3). Thus changes in NIMA kinase activity upon mitotic progression were, at least in part, caused by variation in the level of NIMA protein. The data indicate that both NIME$^{\text{cyclin B}}$ and NIMA are degraded upon mitotic progression and that both are stabilized if cells are arrested in a mitotic state by nocodazole.

Hyperphosphorylation and final activation of NIMA at mitosis occurs after activation of p34^{cdc2} H1 kinase

A partially synchronous mitosis was generated using a temperature sensitive allele of *nimX*^{cdc2} in block/release experiments (Figure 4). At the arrest point of *nimX3*^{cdc2} the H1 kinase activity of p34^{cdc2} was low, and NIMA kinase activity was absent due to the inability of NIMA activity to recover from heat shock in this strain. This is because expression of *nimA* requires a non-mitotic form of p34^{cdc2} after NIMA is degraded due to heat shock (Ye *et al.*, unpublished). Release to permissive temperature led to a synchronous nuclear division starting at 50 min

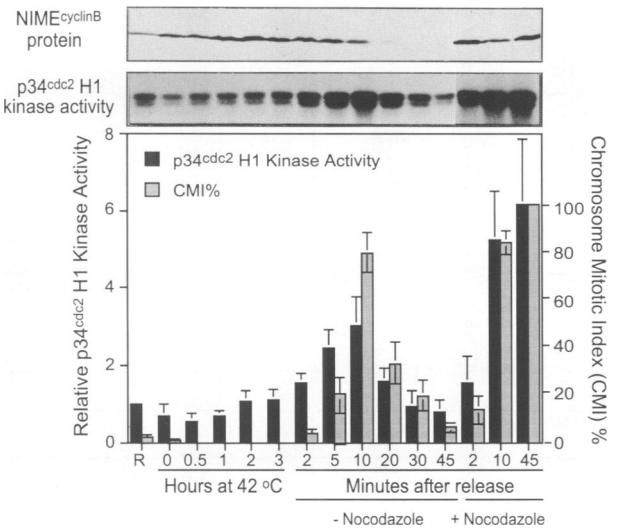

Fig. 2. *nimT23*^{cdc25} G_2 block/release: effects on p34^{cdc2}/cyclin B. SO53 *nimT23* strain grown to early log phase at permissive temperature of 30°C was blocked at the restrictive temperature of 42°C for 3 h and then released into mitosis by downshift to 30°C in the presence or absence of nocodazole. Samples were taken before temperature shift (R, exponentially growing random sample) and at various time points as indicated after temperature upshift and temperature downshift to monitor chromosome mitotic index, the level of NIME$^{cyclin\ B}$, and p34^{cdc2}/cyclin B H1 kinase activity. The graph represents means of data obtained from six such block/release experiments and representative Western blot and kinase activity autoradiographic data are shown. The standard error of means is indicated by vertical bars.

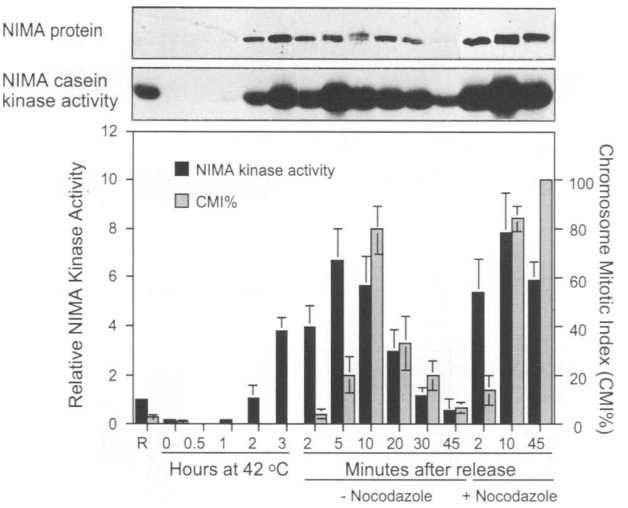

Fig. 3. *nimT23*^{cdc25} G_2 block/release: effects in NIMA. Samples were the same as in Figure 2 but NIMA β-casein kinase activity and NIMA protein levels were determined.

and peaking at 60 min. This partially synchronous mitosis occurred after activation of H1 kinase activity. NIMA protein and NIMA kinase activity began to accumulate as p34^{cdc2} H1 kinase activity increased at 30 min and a mobility shift of NIMA was apparent as p34^{cdc2} H1 kinase activity peaked at the 50 min time point. NIMA kinase activation occurred at 40 min, before it was hyperphosphorylated, and it was further activated at the 60 min time point. The kinetics of activation of p34^{cdc2} H1 kinase and the subsequent effects on NIMA indicate that activation of p34^{cdc2} H1 kinase occurs before final activation and hyperphosphorylation of NIMA.

p34^{cdc2}/cyclin B phosphorylates NIMA in vitro *and generates the MPM-2 antigen on NIMA*

The data presented above indicate that NIMA is phosphorylated in a p34^{cdc2}/cyclin B dependent manner during initiation of mitosis. We investigated the possibility that NIMA is in fact phosphorylated by p34^{cdc2}/cyclin B directly. A kinase negative version of NIMA (NIMA^{K-}) was synthesized in *E.coli* and soluble protein extracts were prepared from induced or non-induced bacteria harboring the NIMA^{K-} expression plasmid. The extracts were used as the substrate in kinase reactions in the presence and absence of *A.nidulans* p34^{cdc2}/cyclin B immunopurified using affinity purified C-terminal specific antibodies (Figure 5). The induced NIMA^{K-} protein was phosphorylated by p34^{cdc2}/cyclin B (Figure 5a). A similar result was obtained using p13 purified p34^{cdc2} or p34^{cdc2}/cyclin B immunoprecipitated with antiserum specific for NIME$^{cyclin\ B}$ (data not shown). If we precipitated from cells containing inactive p34^{cdc2} (cells first arrested in S-phase with HU then shifted to restrictive temperature for the *nimX3*^{cdc2} mutation) no NIMA phosphorylating activity was observed (data not shown), indicating that the phosphorylation of NIMA was specific to p34^{cdc2}. The level of soluble NIMA^{K-} expression in *E.coli* was very low, and could not be distinguished after Coomassie Blue staining (data not shown), but this low level of NIMA was readily and specifically phosphorylated by p34^{cdc2}/cyclin B, indicating that it was a good substrate for p34^{cdc2}/cyclin B. Bacterially produced kinase positive NIMA or NIMA immunoprecipitated from *Aspergillus* failed to phosphorylate NIMA^{K-} (data not shown).

The *in vivo* phosphorylation of NIMA that occurred upon activation of p34^{cdc2}/cyclin B in *A.nidulans* caused a mobility shift of NIMA to a higher molecular weight form (Figures 3 and 6). The *in vitro* phosphorylation of NIMA^{K-} by p34^{cdc2}/cyclin B also caused NIMA^{K-} protein to change mobility to a higher molecular weight form (Figure 5b, right panel).

It has been reported that phosphorylation by mitotic p34^{cdc2}/cyclin B may generate the MPM-2 antigen (Westendorf *et al.*, 1994), an epitope containing a phosphorylated residue recognized by the MPM-2 monoclonal antibody first generated against mitotic HeLa cells (Davis *et al.*, 1983). We therefore tested the ability of p34^{cdc2}/cyclin B to generate the MPM-2 antigen on bacterially produced NIMA^{K-}. NIMA^{K-} was phosphorylated by *A.nidulans* p34^{cdc2}/cyclin B and then probed by Western blotting using either NIMA specific antiserum or MPM-2 monoclonal antibody. All of the full length NIMA^{K-} protein was phosphorylated by p34^{cdc2}/cyclin B on at least one residue as all of it moved to a higher molecular weight form after phosphorylation (Figure 5b, right panel). Only the NIMA^{K-} protein with upshifted mobility caused by p34^{cdc2}/cyclin B phosphorylation was detected by the MPM-2 monoclonal antibody (Figure 5b, left panel). This demonstrates that p34^{cdc2}/cyclin B phosphorylates NIMA^{K-} *in vitro* and generates the MPM-2 antigen.

The MPM-2 antigen is generated on NIMA after activation of p34^{cdc2}/cyclin B in vivo

We have shown that *in vitro* phosphorylation of NIMA^{K-} by p34^{cdc2}/cyclin B generates the MPM-2 antigen

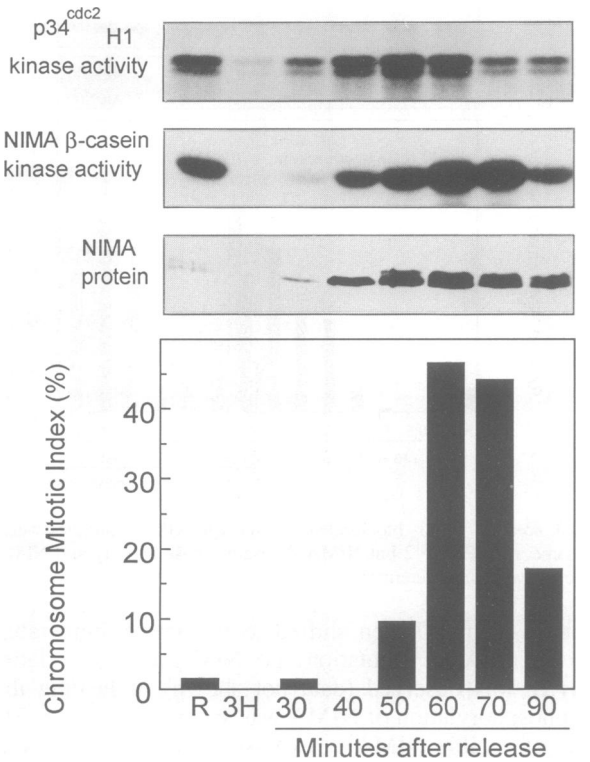

Fig. 4. Block/release of ts$^-$ p34^{cdc2} and effects on NIMA. Exponentially growing cells (R) containing the *nimX3*^{cdc2} mutation were shifted to the restrictive temperature of 42°C for 3 h (3H) and then downshifted to permissive temperature (30°C) for an additional 90 min. Cells were harvested at the time points indicated to determine p34^{cdc2} H1 kinase activity, NIMA β-casein kinase activity, NIMA protein levels and the chromosome mitotic index of the culture.

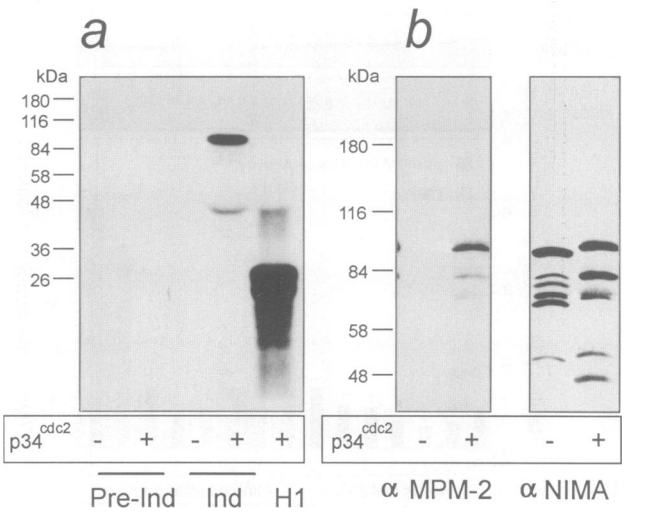

Fig. 5. *Aspergillus* p34^{cdc2}/cyclin B phosphorylates NIMA *in vitro* and generates the MPM-2 antigen on NIMA. (**a**) Total soluble protein extracts made from *E.coli* harboring NIMA^{k-} expression plasmid before (Pre-Ind) and after induction (Ind) were incubated with [γ-^{32}P]ATP in KAB buffer in the presence (+) or absence (−) of the immunoprecipitated p34^{cdc2}/cyclin B H1 kinase. Histone H1 was used as a control substrate as indicated. Phosphorylation of NIMA by p34^{cdc2}/cyclin B was detected by autoradiography. (**b**) Phosphorylation of NIMA by p34^{cdc2}/cyclin B was also determined by Western blot analysis first with the MPM-2 monoclonal antibody (α MPM-2, left panel) and then with NIMA antiserum (α NIMA, right panel) .

(Figure 5b) and that *in vivo* NIMA is hyperphosphorylated upon activation of p34^{cdc2}/cyclin B during mitotic initiation (Figures 3 and 4). If the *in vitro* phosphorylation of NIMA^{K-} by p34^{cdc2}/cyclin B to generate the MPM-2 antigen reflects the *in vivo* situation then NIMA should be phosphorylated and become MPM-2-reactive after p34^{cdc2}/cyclin B is activated during mitotic initiation. We therefore determined the MPM-2 reactivity of NIMA during a *nimT23*^{cdc25} block/release induced synchronous mitosis (Figure 6). Arrest in G_2 caused accumulation of NIMA in an active but not hyperphosphorylated state. After 5 min at the permissive temperature the accumulated NIMA had undergone a mobility upshift as revealed by SDS−PAGE. Concomitantly the NIMA protein became reactive to the MPM-2 monoclonal antibody. NIMA remained MPM-2-reactive at the 5, 7 and 10 min time points. At the 15 min time point, although NIMA protein was still present in the upshifted form and the peak mitotic index occurred at this time point, NIMA was not MPM-2-reactive. This suggests that NIMA is transiently phosphorylated on a site(s) that generates an epitope detected by MPM-2 after NIMT^{cdc25} activation of p34^{cdc2}/cyclin B. Comparing the kinetics of NIMA phosphorylation at the MPM-2 site with the chromosome mitotic index indicates that the site is phosphorylated early during initiation of mitosis. NIMA protein was markedly reduced by 20 min as the mitotic index began to fall.

The level of p34^{cdc2}/cyclin B H1 kinase activity increased at 2 min upon release of the *nimT23*^{cdc25} arrest. At this time point very little NIMA was upshifted or reactive to MPM-2. However, at 5 min virtually all NIMA was upshifted and was MPM-2 positive. This suggests that if p34^{cdc2}/cyclin B is the kinase that phosphorylates NIMA to generate the MPM-2 epitope, which seems likely, then it cannot phosphorylate NIMA immediately upon its activation as an H1 kinase. The level of NIME$^{cyclin\ B}$ also decreased as cells initiated mitosis but its degradation preceded the degradation of NIMA (Figure 6), suggesting that these two mitotically degraded proteins may not be degraded by exactly the same mechanism.

Basal NIMA kinase activity is not dependent on p34^{cdc2}/cyclin B

Since expression of *nimA* normally requires p34^{cdc2} function (Ye *et al.*, unpublished) we constructed a strain (RP2) containing several copies of inducible *nimA* driven by the *alcA* promoter and a ts$^-$ allele of *nimX*^{cdc2}. This strain causes cell cycle arrest at restrictive temperature due to mutant p34^{cdc2} and cannot grow on *alcA*-inducing carbon sources, such as ethanol, as the induction of high levels of NIMA causes lethal premature mitotic events (Osmani *et al.*, 1988). To determine if phosphorylation of NIMA by p34^{cdc2} in *A.nidulans* is essential for NIMA kinase activity we first arrested RP2 at the restrictive temperature to impair p34^{cdc2} function and cause cell cycle arrest and also left some cells at the permissive temperature. The cultures were then transferred to *alcA* inducing medium to allow expression of *nimA* from the *alcA* promoter. NIMA was then isolated and assayed (Figure 7a). Upon shift to inducing medium, NIMA protein was induced and high levels of NIMA protein kinase activity were detected in both control cells (Figure 7a, 32°C+ETOH) and those arrested in the cell cycle by inactivation of *nimX3*^{cdc2} (Figure 7a, 42°C+ETOH). As the level of NIMA kinase

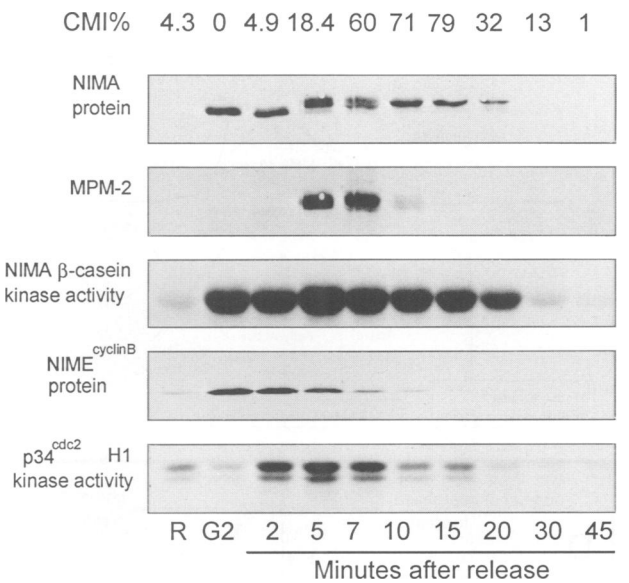

Fig. 6. NIMA becomes transiently MPM-2-reactive at mitosis upon activation of p34^{cdc2}/cyclin B after release from the *nimT23*^{cdc25} G_2 arrest point. Exponentially growing SO53 *nimT23*^{cdc25} cells (R) were blocked in late G_2 at the restrictive temperature of 42°C for 3 h (G_2) and released synchronously into mitosis at the permissive temperature of 30°C. Cells were harvested at the time points indicated. The percentage of nuclei in mitosis (chromosome mitotic index, CMI%) was calculated by counting mitotic figures after staining cells with DAPI and NIMA protein, NIME$^{cyclin\ B}$ protein and NIMA MPM-2 reactivity were analyzed by immunoblotting. NIMA β-casein kinase activity and p34^{cdc2} H1 kinase activity are shown after autoradiography of ^{32}P incorporation into β-casein and histone H1.

activity induced in cells containing a functional p34^{cdc2} kinase was the same as that induced in cells in which p34^{cdc2} was functionally inactivated, and had low H1 kinase activity (data not shown), it strongly indicates that phosphorylation of NIMA by p34^{cdc2} is not essential for NIMA kinase activity in *A.nidulans*. Cells were also pre-synchronized in S-phase with hydroxyurea (HU) before imposing the restrictive temperature to arrest cells specifically in G_2 with inactive p34^{cdc2} and NIMA was still induced in an active form (data not shown). It is also known that NIMA induced in *E.coli* is active and phosphorylated (Lu *et al.*, 1993), further supporting the notion that NIMA does not need to be phosphorylated by p34^{cdc2} in order to have a basal level of kinase activity.

NIMA can promote chromatin condensation when p34^{cdc2} is inactivated

High levels of NIMA induction are able to promote chromatin condensation and spindle formation even if cells are arrested in S-phase (Osmani *et al.*, 1988). To determine if high levels of NIMA kinase activity could promote chromatin condensation in the absence of p34^{cdc2} function we fixed cells and then stained them with DAPI (Figure 7b and c). By 90 min of induction, >90% of the cells had condensed chromatin (Figure 7c), one of the hallmarks of mitosis. Thus, although lack of p34^{cdc2} function caused a slight delay in the increase in chromosome mitotic index caused by induction of NIMA (data not shown), increased levels of NIMA were able to promote mitotic events independent of p34^{cdc2}.

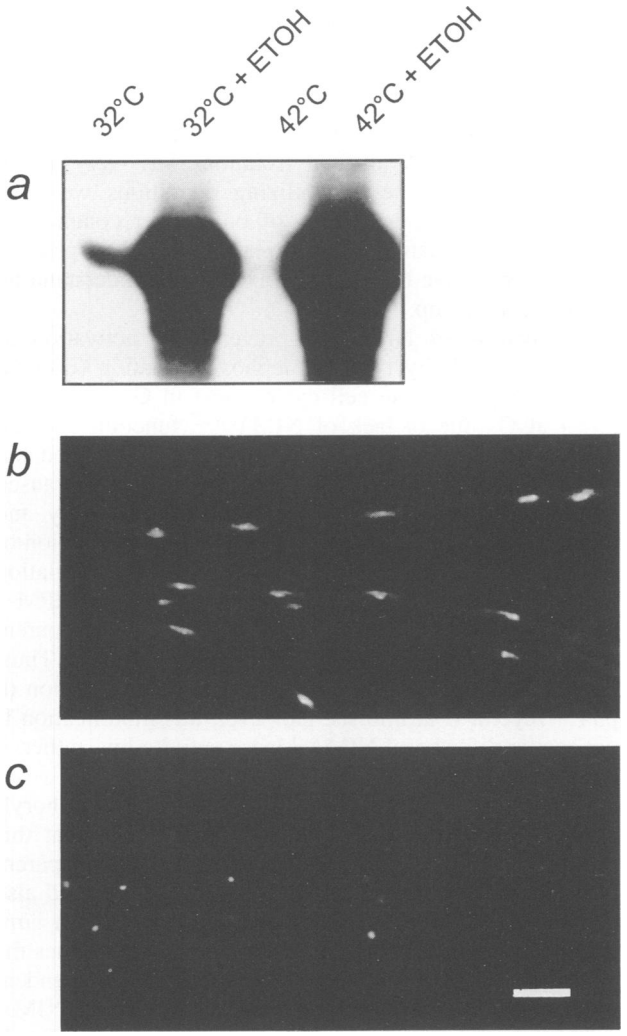

Fig. 7. Induced expression of NIMA causes chromatin condensation in the absence of p34^{cdc2} function. (**a**) A strain (RP2) containing *alcA::nimA* and ts$^-$ p34^{cdc2} (*nimX3*^{cdc2}) was grown to early log phase in liquid minimum medium with acetate as carbon source (*alcA*-repressing) at 32°C. Half of the culture was blocked for 3 h at 42°C to inactivate p34^{cdc2} and samples were taken (a, 32 and 42°C). The culture was then washed with prewarmed (32 and 42°C, respectively) fresh medium without carbon source by filtration through Miracloth, transferred to ethanol (*alcA*-inducing) medium at 32°C (a, 32°C+ETOH) or 42°C (a, 42°C+ETOH) and incubated for an additional 2.5 h. NIMA β-casein kinase activity was assayed after immunoprecipitation of NIMA and ^{32}P-labelled β-casein is shown after autoradiography. (**b** and **c**) Cells treated as described above at the restrictive temperature (b) or after induction of NIMA at the restrictive temperature (c) are shown after fixing and staining with DAPI to show nuclear morphology of DNA. Bar represents ~5 μm.

Discussion

The major finding of this work concerns the role of p34^{cdc2}/cyclin B in the activation of NIMA during initiation of mitosis. We had previously found no role for p34^{cdc2}/cyclin B in the activation of NIMA (Osmani *et al.*, 1991a). However, we have since found that the kinase activity of NIMA is exceptionally sensitive to inactivation by dephosphorylation during isolation procedures used to assay this kinase. In past work we utilized buffer conditions that should prevent dephosphorylation during isolation. Many phosphatase inhibitors were incorporated into isola-

tion buffers and the temperature of samples was kept below 4°C. In spite of these precautions, NIMA was still susceptible to inactivation by dephosphorylation during isolation (Figure 1f, and X.S.Ye and S.A.Osmani, unpublished). Upon addition of OA to isolation buffers, the stability of NIMA during isolation was very much improved. Under these stabilizing conditions we have been able to reconfirm some of our earlier conclusions regarding the relationship between NIMA and $p34^{cdc2}$/cyclin B and have been able to extend our understanding of this relationship.

Inactivation of $NIMT^{cdc25}$ prevents the activation of $p34^{cdc2}$/cyclin B by tyrosine dephosphorylation (Osmani *et al.*, 1991a) causing cell cycle arrest in G_2. Cell cycle arrest at G_2 due to lack of $NIMT^{cdc25}$ function allowed accumulation of NIMA protein in a phosphorylated but partially activated form. Release of this G_2 arrest caused a further 2-fold increase in NIMA kinase activity and hyperphosphorylation of NIMA protein after activation of $p34^{cdc2}$/cyclin B by $NIMT^{cdc25}$. The hyperphosphorylation of NIMA correlated with the generation of the MPM-2 antigen on NIMA and caused an increase in the apparent molecular weight of NIMA during SDS−PAGE. Thus, NIMA is modified by phosphorylation after activation of $p34^{cdc2}$/cyclin B at mitosis. However, this modification is not required for basal NIMA kinase activity but, rather, it boosts its G_2 level of kinase activity.

In vitro, we found that NIMA was readily phosphorylated by $p34^{cdc2}$/cyclin B and, as seen *in vivo*, that this phosphorylation caused an increase in the apparent molecular weight of NIMA during SDS−PAGE and also generated the MPM-2 antigen on NIMA. As the *in vitro* phosphorylation of NIMA by $p34^{cdc2}$/cyclin B mimics the characteristic of the *in vivo*, $p34^{cdc2}$/cyclin B dependent phosphorylation of NIMA, it strongly suggests that NIMA could be phosphorylated directly by $p34^{cdc2}$/cyclin B during the initiation of mitosis. Alternatively, NIMA could be phosphorylated by a kinase that is activated by $p34^{cdc2}$/cyclin B during mitotic initiation. Either way, our data demonstrate that NIMA is phosphorylated after activation of $p34^{cdc2}$/cyclin B during initiation of mitosis. Our current understanding of the regulation of NIMA, and the role of $p34^{cdc2}$/cyclin B, is shown in Figure 8a.

As NIMA is phosphorylated and activated downstream from $p34^{cdc2}$/cyclin B, it could be argued that the role of the $p34^{cdc2}$/cyclin B pathway of mitotic regulation is to activate NIMA. In this model, mitotic initiation would be regulated by a phosphorylation cascade with NIMA being the final activated kinase downstream from $p34^{cdc2}$/cyclin B. NIMA would then phosphorylate all substrates required for mitotic initiation. However, given the ability of $p34^{cdc2}$/cyclin B to phosphorylate many mitotically relevant substrates (Moreno and Nurse, 1990; Nigg, 1991) this scenario seems unlikely. A more likely model involves NIMA kinase activity being boosted by $p34^{cdc2}$/cyclin B-mediated phosphorylation during initiation of mitosis and then NIMA feeding back to $p34^{cdc2}$/cyclin B to allow final functional activation of $p34^{cdc2}$/cyclin B. For example, this may involve allowing $p34^{cdc2}$/cyclin B access to its substrates in the nucleus or other specific subcellular localization.

In this type of model (Figure 8b), regulatory signals generated by checkpoints (Hartwell and Weinert, 1989)

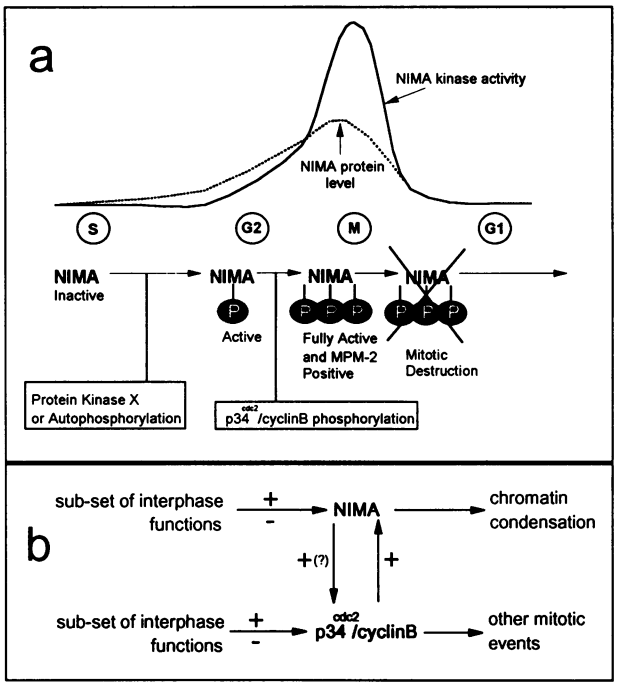

Fig. 8. (**a**) The role of $p34^{cdc2}$/cyclin B in the phosphorylation and activation of NIMA. The level of NIMA protein (dotted line) is shown compared with the level of NIMA kinase activity through the cell cycle. The cell cycle times are not to scale as mitosis normally takes just 5 min (5% of the cell cycle time) during the cell cycle. The phosphorylation state of NIMA and the role of $p34^{cdc2}$/cyclin B is shown at different stages of the cell cycle. (**b**) Coordination between NIMA and $p34^{cdc2}$/cyclin B. NIMA, although active in G_2, is normally phosphorylated and further activated by $p34^{cdc2}$/cyclin B during mitotic initiation. However, if NIMA is overexpressed it can cause chromatin condensation without activation of $p34^{cdc2}$/cyclin B suggesting that NIMA plays a direct role in chromosome condensation that is normally under control of $p34^{cdc2}$. We speculate that NIMA in turn plays a role in the function of $p34^{cdc2}$ to ensure that the two kinases are functionally coordinated upon completion of all interphase events. However, in the absence of NIMA function $p34^{cdc2}$/cyclin B is fully activated as an H1 kinase (Osmani *et al.*, 1991a). How (or if) NIMA positively affects $p34^{cdc2}$/cyclin B mitotic promoting activity is currently unknown.

that need to prevent the initiation of mitosis, such as those generated by damaged DNA or unreplicated DNA, or heat shock, can prevent initiation of mitosis by stopping activation of either the $p34^{cdc2}$/cyclin B or NIMA pathways of mitotic regulation. For instance, under conditions when DNA is damaged, initiation of mitosis may be held in check due to inactivation of $p34^{cdc2}$/cyclin B with little effect on NIMA. Under other conditions, such as heat shock, NIMA is inactivated and, although $p34^{cdc2}$/cyclin B is still active, mitosis is not initiated, suggesting that mitosis is held in check under these conditions due to lack of NIMA (Ye *et al.*, unpublished). However, to ensure that both kinases are then activated coordinately, one component in the activation of each kinase would have to depend on the activation of the other kinase (Figure 8b). We have shown here that this is true for final activation of NIMA by $p34^{cdc2}$/cyclin B. We are currently trying to find experimental evidence for the positive effect of NIMA on $p34^{cdc2}$/cyclin B function to complete this regulatory circuit.

To confirm that phosphorylation of NIMA by $p34^{cdc2}$/cyclin B is not necessary for NIMA kinase activity in

A.nidulans we induced production of NIMA from an inducible promoter after cells were arrested in the cell cycle by inactivation p34^{cdc2} using the temperature sensitive *nimX3*^{cdc2} mutation. In the absence of p34^{cdc2} function induced NIMA had kinase activity. This clearly demonstrates that basal NIMA kinase activity is independent of p34^{cdc2} although it is known that NIMA needs to be phosphorylated in order to have kinase activity (Lu *et al.*, 1993). The induction of high levels of NIMA in the absence of p34^{cdc2} function still caused chromatin condensation even though cells were arrested at interphase of the cell cycle. This indicates that the mitotic promoting activity of NIMA, although normally dependent on p34^{cdc2}/cyclin B, can be made independent of p34^{cdc2} by overexpression. This result further suggests that one role of p34^{cdc2}/cyclin B is to boost NIMA kinase activity during G_2/M. It also suggests that NIMA plays a direct role in the condensation of chromosomes during mitosis and that this function of NIMA is normally activated by p34^{cdc2}/cyclin B. Similar effects of NIMA overexpression have been observed in both fission yeast and human cells (O'Connell *et al.*, 1994) as NIMA induction causes chromatin condensation in both these systems.

One obvious experiment that should enable us to determine if NIMA can promote all of mitosis in the absence of p34^{cdc2} is to induce NIMA when p34^{cdc2} function is impaired. We have shown that overexpressed NIMA can cause chromatin condensation in the absence of p34^{cdc2} function; what about spindle formation? Unfortunately, the process of temperature shifts and carbon source changes required to do this type of experiment has very detrimental effects on current protocols for immunofluorescence in *A.nidulans*. A negative finding is therefore of limited value. Further studies will be required to establish if NIMA can promote spindle formation in the absence of p34^{cdc2}/cyclin B function. However, we stress that NIMA normally needs to be activated by p34^{cdc2}/cyclin B before it can promote mitotic events. This is most clearly demonstrated by the lack of any mitotic events seen in cells arrested in G_2 by the *nimT23*^{cdc25} temperature sensitive mutation. These cells cannot condense chromatin or form a mitotic spindle, even though NIMA has accumulated in an active form. Only when the *nimT23*^{cdc25} mutation is released are cells able to phosphorylate and further activate NIMA and then enter mitosis.

We have also shown that NIMA behaves like a mitotic cyclin. It accumulates during G_2 and is degraded upon mitotic progression but is stabilized if cells are arrested in mitosis. The mitotic destruction of NIMA causes inactivation of its kinase activity, just as the kinase activity of the p34^{cdc2}/cyclin B is inactivated during mitosis by degradation of cyclin B. Thus the periodicity of NIMA kinase activity during the cell cycle is regulated not only by phosphorylation but also at the level of protein accumulation and destruction. The functional significance of the mitotic destruction of NIMA is addressed in the accompanying paper (Pu and Osmani, 1995).

MPF activity has been found to reside not only in the p34^{cdc2}/cyclin B complex but also in other, as yet undefined, proteins in *Xenopus* eggs (Kuang *et al.*, 1991). These non-p34^{cdc2}/cyclin B forms of MPF can be immunodepleted using the MPM-2 monoclonal antibody (Kuang *et al.*, 1989), showing that some proteins containing the MPM-2 epitope have MPF activity. One form of non-p34^{cdc2}/cyclin B MPF may be the Cdc25 tyrosine phosphatase as this protein, like NIMA, is phosphorylated, becomes upshifted in SDS−PAGE and becomes MPM-2 positive during mitosis (Kuang *et al.*, 1994). As NIMA contains the MPM-2 epitope during mitosis its homologs may correspond to another non-p34^{cdc2}/cyclin B form of MPF that reacts to the MPM-2 antibody.

In summary, our data begin to define a regulatory circuit in which initiation of mitosis is first regulated by independent activation of the kinase activity of two protein kinases, p34^{cdc2}/cyclin B and NIMA. Then, the mitosis promoting function of each kinase is coordinated because the final function of each kinase is dependent on the other. This may explain why either kinase activity alone can be significantly activated without promoting a complete mitosis.

Materials and methods

Aspergillus strains and general techniques

Aspergillus nidulans strains used were R153 (*wA3; pyroA4*), SO54 (*nimA5; wA2*), SO53 (*nimT23; wA2*), SO65 (*nimX3; wA3; pyroA4; riboA1*), 18D (*alcA::nimA; fwA1; benA22; pabaA1*) and RP2 (*nimX3; alcA::nimA; fwA1; riboA1; pabaA1*). Growth of strains, cell cycle blocks and DAPI staining techniques for microscopy were as described previously (Osmani *et al.*, 1987, 1994).

Preparation of cell extracts

Aspergillus mycelia grown in YG were harvested and ground in HK extraction buffer as described previously (Osmani *et al.*, 1991a), except that 1 μM OA was incorporated into the extraction buffer and the concentration of sodium vanadate was increased to 1 mM. For MPM-2 detection of NIMA, mycelia were ground in a modified EB buffer containing 10 μg/ml leupeptin, 10 μg/ml trypsin−chymotrypsin inhibitor, 10 μg/ml aprotinin, 10 μg/ml *N*-tosyl-phenylalanine chloromethylketone, 2 mM *Nα-p*-tosyl-L-arginine methyl ester, 5 mM benzamindine, 2 mM phenylmethylsulfonyl fluoride, 80 mM β-glycerophosphate, 20 mM EGTA (pH 7.5), 50 mM NaF, 1 mM ATP, 2 mM dithiothreitol, 1 μM OA and 10 nM microcystin.

Antibodies

Antibodies raised against the C-terminal peptide sequence, GSSYYSGRARRNGFHC, of p34^{cdc2} of *A.nidulans*, the bacterially expressed and purified NIME$^{cyclin\ B}$, and the ANYRED peptide, CGRANYREDASLRSSG, of NIMA, were described previously (Osmani *et al.*, 1991b, 1994). To generate antisera against the full length NIMA kinase, the coding sequence of NIMA cDNA was amplified by PCR with forward primer, 5′-CGGGGTACCACAATGGCAATCGCACTG-3′ and reverse primer, 5′-ATAATGATGCGGCCGCCCTCAAGTTGCGAATCAC-3′, and cloned into an IPTG T7 inducible expression vector, pET21a (Novagen), as a *Bgl*II−*Not*I fragment to give rise to pET21a-NIMA, which has a T7 leader sequence at its N-terminus and a His-tag at its C-terminus. BL21(DE3) pLysS cells harboring pET21a-NIMA were induced for 4 h at 20°C in 0.4 mM IPTG. The bacterially expressed NIMA accumulated mostly in an insoluble form and NIMA was His-tag purified from both the soluble and insoluble *E.coli* fractions. Polyclonal antibodies were elicited in two New Zealand white male rabbits by injecting 500 μg of the NIMA protein purified from soluble and insoluble fractions in a 1:1 ratio using the Ribi adjuvant system (Ribi Immunochem Research, Montana) according to the manufacturer's instructions. Half of the NIMA protein injected was denatured by SDS−heat treatment. The initial injection was followed by two booster injections at 13 day intervals.

Immunoprecipitations

For NIMA kinase assays and Western blot analyses, NIMA was immunoprecipitated with 1 μl NIMA antiserum/mg protein extract made as described above. The protein extracts were incubated with antibodies on ice for 2 h with periodic mixing every 30 min. Then 1 mg protein

A–Sepharose beads (Pharmacia) per μl antibody were added and incubated for 30 min at 4°C on a rocking shaker. The immuno-complex was collected by centrifugation and washed three times with HK buffer and twice with KAB buffer (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol). Immunoprecipitation of $p34^{cdc2}$/cyclin B for H1 kinase assay was done as previously described (Osmani *et al.*, 1994).

Kinase assays

$p34^{cdc2}$/cyclin B H1 kinase and NIMA kinase assays were carried out under conditions described recently (Lu *et al.*, 1993; Osmani *et al.*, 1994). The labeled substrates were resolved on 12% SDS–acrylamide gels and quantified by scanning on an AMBIS 4000 radio analytical imaging detector.

Western blot analysis

To determine the level of NIMA protein through the cell cycle, protein extracts were immunoprecipitated with NIMA antibodies. The immunoprecipitates were boiled for 5 min in Laemmli sample buffer, separated by 7.5% SDS–PAGE and electroblotted onto nitrocellulose. Blots were blocked overnight in 5% BSA in TBST (20 mM Tris pH 7.5, 500 mM NaCl, 0.05% Tween-20 and 0.02% sodium azide). NIMA antisera at a 1:200 dilution in the above 5% BSA solution were incubated for 2 h. The blots were washed four times in TBST, for 10 min each, and then were incubated for 40 min with protein A–peroxidase conjugate (1:50 000 dilution) in 3% non-fat dry milk in TBST. The blots were washed four times in TBST for 5 min each wash, and developed using an enhanced chemiluminescence detection system (ECL) from Amersham according to the manufacturer's recommendations. The level of $NIME^{cyclin\ B}$ protein in extracts was determined by direct immunoblot analysis of 100 μg total proteins separated by 10% SDS–PAGE using the ECL detection reagents as previously described (Osmani *et al.*, 1994).

Production of kinase negative NIMA in E.coli and in vitro phosphorylation by $p34^{cdc2}$/cyclin B

NIMA produced in *E.coli* has kinase activity using β-casein as an artificial substrate and is extensively autophosphorylated on multiple sites (Lu *et al.*, 1993). To determine whether NIMA is a substrate for $p34^{cdc2}$, we produced a kinase negative version of NIMA in bacteria by changing the putative ATP binding lysine 40 to methionine in pET21a-NIMA construct using the Transformer Site-directed Mutagenesis system (Clontech, CA) to give pET21a-$NIMA^{k-}$. The mutation was confirmed by DNA sequencing. The mutated NIMA had no kinase activity and was not phosphorylated in bacteria. Soluble bacterial protein was made in HK buffer after induction of bacteria harboring the expression plasmid. $p34^{cdc2}$/cyclin B was immunoprecipitated from mitotic *Aspergillus* extracts with affinity-purified $p34^{cdc2}$ C-terminal peptide antiserum, $NIME^{cyclin\ B}$ antiserum, or precipitated with p13 beads. Immunoprecipitates using preimmune sera and immunoprecipitates from extracts containing inactive $p34^{cdc2}$ were used as controls. *In vitro* phosphorylation of NIMA by $p34^{cdc2}$/cyclin B was determined in three ways. First, we tested whether NIMA could be labeled by $p34^{cdc2}$/cyclin B using [γ-^{32}P]ATP; second, we determined whether phosphorylation of NIMA by $p34^{cdc2}$/cyclin B could cause a mobility shift after SDS–PAGE by Western blot; third, we tested if phosphorylation of NIMA by $p34^{cdc2}$/cyclin B would generate an MPM-2 antigen by Western blot analysis using the MPM-2 monoclonal antibody.

MPM-2 immunoblot analysis

For MPM-2 detection, nitrocellulose blots were blocked in 20% calf serum in TBST overnight. To protect the MPM-2 antigen, 10 nM microcystin was included in the blocking solution. The blots were incubated for 1 h with MPM-2 antibody diluted 1:500 in 20% calf serum in TBST. The blots were then washed four times in 1× TBST, for 10 min each, and incubated with secondary antibody solution (rabbit antimouse horse radish peroxidase conjugate, 1:50 000 dilution in 20% calf serum TBST) for 1 h. After three 5 min washes in TBST, the blots were processed for ECL detection as described above.

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