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A single p34^{cdc2} protein kinase (encoded by *nimX*^{cdc2}) is required at G_1 and G_2 in *Aspergillus nidulans*

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SUMMARY

We have cloned and sequenced a homolog of *cdc2* from *Aspergillus nidulans* that can complement the *Schizosaccharomyces pombe cdc2*-33 mutation. The gene was deleted and is required for continued nuclear DNA replication but not for mitochondrial DNA replication. Three different temperature-sensitive alleles were generated by reverse genetics. All of the mutations generate the nim phenotype of *A. nidulans*. The new gene was designated *nimX*^{cdc2} as it is not allelic to any of the other *nim* genes (*nimA* to *nimW*) of *A. nidulans*. Reciprocal shift experiments place an essential function for *nimX*^{cdc2} in G_1 and G_2. Antipeptide antibodies were generated that detect NIMX^{cdc2}, and antisera were also generated to detect NIME^{cyclinB}. The two p34^{cdc2} protein species previously detected in *A. nidulans*, p34 and p37, both precipitate using NIMX^{cdc2} C-terminus-specific antibodies but only p34 co-precipitates with NIME^{cyclinB}. Dephosphorylation of denatured p34 converts it to the p37 form, showing p37 to be the non-phosphorylated form of NIMX^{cdc2}. The phosphorylation of p34 is therefore associated with its interaction with NIME^{cyclinB}.

Key words: *Aspergillus nidulans*, p34^{cdc2}, *nimX*^{cdc2}, cyclin B, *nimE*^{cyclinB}, cell cycle regulation

INTRODUCTION

The p34^{cdc2} class of protein kinases was first identified in yeast and subsequently shown to be a highly conserved component of cell cycle regulation in all eukaryotes (for review see Draetta, 1990; Nurse, 1990; Norbury and Nurse, 1992, and for further references not included below). The level of this protein kinase catalytic unit remains constant through the cell cycle but its histone H1 kinase activity is increased at mitosis. This is achieved by inhibitory (Gould and Nurse, 1989; Lungren et al., 1991) and activating phosphorylation (Gould et al., 1991; Ducommun et al., 1991; Solomon et al., 1993; Fesquet et al., 1993; Poon et al., 1993) and binding to a class of cell cycle-regulated proteins called cyclins (Evans et al., 1983; Draetta and Beach, 1988; Booher et al., 1989). In fission and budding yeast the Cdc2/28 protein kinase is required in both G_1 and G_2 (Nurse and Bisset, 1981; Piggott et al., 1982; Reed and Wittenberg, 1990; Surana et al., 1991). In higher eukaryotes many members of this protein kinase family have been cloned (Meyerson et al., 1992) and have been termed cyclin-dependent kinases (CDKs), and evidence exists indicating that Cdk2 and Cdk3 may play a role in G_1 (Fang and Newport, 1991; Pagano, 1993; van den Heuvel and Harlow, 1993) and Cdc2 may play a role in G_2 (Th'ng et al., 1990 and van den Heuvel and Harlow, 1993) of higher eukaryotes.

Analysis of cell cycle-specific mutants of *Aspergillus nidulans* has indicated that mitosis is regulated not only by the p34^{cdc2} class of protein kinases but also by the NIMA protein kinase. The protein kinase activities of these two proteins are activated at mitosis independently of each other (Osmani et al., 1987, 1991a,b). The gene encoding p34^{cdc2} has not previously been genetically defined or cloned from *A. nidulans*, but because of its high degree of conservation its kinase activity can be followed using the *Schizosaccharomyces pombe suc1* gene product bound to Sepharose beads (p13 beads), which precipitates this class of protein from many species (Brizuela et al., 1987; Arion et al., 1988; Dunphy and Newport, 1989). In addition, p34^{cdc2} can be monitored on western blots using antisera generated against the highly conserved PSTAIR region of p34^{cdc2} (D'Urso et al., 1990). So it has been possible to study p34^{cdc2} biochemically in *A. nidulans* but not to genetically manipulate this universal component of eukaryotic cell cycle regulation.

There are potential problems associated with using p13 beads and PSTAIR antisera to study p34^{cdc2} in *A. nidulans* as there could be more than one p34^{cdc2}-related protein kinase. If p34^{cdc2}-related proteins are precipitated using p13 beads and

then probed with PSTAIR-specific antisera two bands can be resolved, a band of 37 kDa and another of 34 kDa (O'Connell et al., 1992; Lu et al., 1993). Using anti-phosphotyrosine antibodies it has been shown that at least some of the p34 species contains phosphotyrosine, and that if this phosphate is removed enzymatically, the mobility of p34 is not retarded to the p37 position (Lu et al., 1993). This observation leads to the possibility that, as in higher eukaryotes (Meyerson et al., 1992), *A. nidulans* may contain more than one member of the $p34^{cdc2}$ family of protein kinases that bind to p13 beads and are detected by PSTAIR-specific antibodies. If the two $p34^{cdc2}$-related protein kinases were functionally redundant it may also explain why a mutation in this class of gene has not been previously identified in *A. nidulans*, although other conserved components of G_2/M regulation have been.

In this study we describe the molecular cloning and characterization of a functional homolog of *S. pombe* $p34^{cdc2}$ and examine its role in the cell cycle of *A. nidulans*.

MATERIALS AND METHODS

Isolation and characterization of *A. nidulans cdc2/28*-like gene

Two degenerate primers, GGAATTCCGARGGNACNTAYGGNGTNGTNTAYAA and CGGGATCCCGARNARRTTYTGNGGYTTNARRTC, were used at 250 µM under standard PCR conditions for *Taq* polymerase (Perkin Elmer) using *A. nidulans* and *S. pombe* DNA as templates. PCR was carried out as follows, 5 minutes at 94°C then 35 cycles of 1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C with a final extension of 10 minutes at 72°C. For identification of *Escherichia coli* cells harboring cosmids of interest a small portion of frozen bacterial cells was first boiled in PCR buffer lacking *Taq* polymerase for 10 minutes prior to addition of enzyme and PCR performed as described above. The PCR product was cloned as an *Eco*RI and an *Eco*RI-*Bam*HI fragment into M13 and sequenced. The PCR fragment was used as a probe to isolate cDNA and the cDNA was cloned and sequenced as described previously (Osmani et al., 1988; O'Connell et al., 1992).

Complementation of *S. pombe cdc2-33*

Media and molecular methods for *S. pombe* were as described by Moreno et al. (1991). Strain ED628 (*cdc2-33; leu1-32; ura4-294*) was a kind gift from Dr Peter Fantes and the thiamine-repressible expression vector pREP1 a kind gift from Dr Kinsey Maundrell (Maundrell, 1993). $pREP1cdc2^{asp}$ was constructed by cloning a 1.2 kb *Xma*I cDNA fragment of $nimX^{cdc2}$ into the *Xma*I site of pREP1Δ ATG, which had been constructed by digesting pREP1 with *Nde*I and *Bam*HI, and religating after blunting with Klenow. $pREP1cdc2^{asp}$ was introduced into *cdc2-33* cells and leu^+ transformants were selected at 25°C on minimal medium containing 2 µM thiamine. Transformants were then streaked in the presence and absence of thiamine and grown at 25°C and 37°C.

Generation of mutations in $nimX^{cdc2}$

A genomic 2.5 kb *Pst*I-*Xba*I fragment from cosmid W15D10 was cloned into the vector pRG3 (Waring et al., 1989) and mutated using the Transformer site-directed mutagenesis kit using conditions specified by the manufacturer (Clontech, Palo Alto, CA). Oligonucleotides used for the mutagenesis were as follows, GCCTCTATTTCCT**A**GTGACTCGGA for the G225S mutation, CAAGCCTCTA**C**TTCCTGGTGA for the F223L mutation and CATGCATCCA**C**ACTTCCAGCA for the Y306H mutation. In each case the selection primer was GGGATCCTCTTGACGCGATGG, which destroys the *Xba*I site of the plasmid. All mutations were confirmed by sequence analysis. The mutated plasmid DNA was used to transform strain GR5 (*wA3; pyroA4; pyrG89*) to uracil/uridine prototrophy. After streaking to single colony three times, transformants were spot inoculated onto plates containing 1 mg/ml 5-fluoroorotic acid (5-FOA) plus 10 mM uridine and uracil. Transformants that readily produced fans of growth able to grow in the presence of 5-FOA were tested for temperature sensitivity by testing spores from the 5-FOA-resistant fans for growth at 32°C and 42°C. In this way temperature-sensitive strains were identified. These were again streaked to single colony three times before crossing to strain A122 (*riboA1; yA2; nicB8*). Progeny from this cross were isolated that were temperature-sensitive and carried the following markers: SO58 = $nimX^{cdc2}$G225S; *wA3; nicB8; pyroA4.* SO64 = $nimX^{cdc2}$F223L; *wA3;nicB8; riboA1.* SO65 = $nimX^{cdc2}$Y306H; *wA3; pyroA4; riboA1.*

Molecular deletion of $nimX^{cdc2}$ from *A. nidulans*

A 9.0 kb genomic *Sac*II fragment of cosmid W15D10 was cloned into vector pBluescript SK^+ and this plasmid was digested with *Pst*I and religated to produce plasmid pNIG4 (insert 4.2 kb). Plasmid pNIG4 was cut with *Bam*HI and *Sma*I to remove 0.986 kb of $nimX^{cdc2}$, which was replaced with the *pyrG* gene as a 1.36 kb *Sca*I-*Bam*HI fragment to yield pNIG7. The insert of pNIG7 was isolated as a *Sac*II-*Pst*I fragment by freeze-fracture from SeaPlaque agarose and used to transform strain GR5 to uridine/uracil prototrophy. Putative heterokaryons were identified by streaking conidia from primary transformants in the presence and absence of uridine/uracil. Heterokaryons were confirmed by Southern blot analysis as described previously (Osmani et al., 1988). Confirmed heterokaryons were propagated by transferring small samples of mycelia to selective medium periodically.

Antibody production and protein analysis

Antibodies were generated against the C-terminal peptide sequence of $NIMX^{cdc2}$, amino-GSSYYSGRARRNGFHC-amide, covalently coupled to *Diphtheria* toxoid via the cysteine residue. The peptide-carrier conjugate was then injected into two sheep at 100 nmoles of peptide per injection on days 1 and 14. Both sheep were bled on day 35 to obtain hyperimmune antipeptide serum, which was pooled and affinity-purified using a column of the specific peptide coupled to Thiopropyl-Sepharose 6B. All of these procedures, from peptide synthesis to affinity purification of antibodies, were carried out by Chiron Mimotope Pty. Ltd., 11 Duerdin St, Clayton, Victoria 3168, Australia. To generate antisera against $NIME^{cyclinB}$ a cDNA fragment of *nimE* encoding the entire $NIME^{cyclinB}$ open reading frame was generated by PCR using as forward primer, GCCGGATCCATGAATGAAAACGACGAGAAT, and reverse primer, CGGAATTCGCGTTGTCTTTGATGGAGTTGTA. The fragment was cloned as a *Bam*HI-*Eco*RI fragment into expression vector pET-21a and protein induced and HIS tagged purified from *E. coli* as described by the manufacturer in the pET system manual (Novagen. Inc.).

Polyclonal antibodies were elicited in four New Zealand White male rabbits by injecting 500 µg of protein using the Ribi adjuvant system (Ribi Immunochem Research, Montana) following the manufacturer's protocols. Half of the immunogen protein was denatured by SDS-heat treatment prior to injection. For western blotting samples were separated by SDS-PAGE and electroblotted (Idea Scientific Company, Minneapolis) to nitrocellulose (Amersham Hybond-ECL or Schleicher & Schuell). Western analysis was performed according to an optimized protocol for ECL (Amersham Life Sciences). First antibody dilutions were from 1:100 to 1:1000 and blots were visualized by ECL after a second incubation in horseradish peroxidase-linked protein A at a 1:50,000 dilution.

For protein kinase assays 50 µg protein, isolated as described previously (Osmani et al., 1991b), was immunoprecipitated with 5 µl preimmune or immune serum or affinity-purified antibodies. Assays were

carried out as described previously but the ATP concentration was doubled as was the concentration of histone H1 (Osmani et al., 1991b).

For dephosphorylation, proteins were first affinity-purified using p13 beads, which were re-suspended in 2% SDS, 100 mM DTT and boiled for 2 minutes. A sample was then diluted 20-fold using PAP phosphatase buffer as described by the manufacturer (Boeringer Mannheim); 0.1 unit PAP was added to half of the sample and was incubated for 2.5 hours at 32°C prior to analysis by western blotting.

RESULTS

Molecular cloning and sequence analysis of a functional p34^{cdc2} homolog from *A. nidulans*

To clone p34^{cdc2}-related protein kinases from *A. nidulans* two degenerate primers were designed, based on a comparison of p34^{cdc2} homologs from several species, and used in PCR experiments with *A. nidulans* genomic DNA, cDNA and DNA isolated from a chromosome II-specific cosmid library (Brody et al., 1991) used as template. *S. pombe* genomic DNA was used as a positive control. From each of the *A. nidulans* DNA samples a band slightly larger than that amplified from the *S. pombe* DNA was generated (data not shown). As the chromosome II-specific DNA proved positive we repeated the PCR using 10 pooled DNA samples each derived from 40 individual cosmids. Three of these pools again generated the expected size of PCR product. In a final round of PCR, using individual cosmids in bacterial cells as a source of template, a single cosmid (W15D10) was identified that generated the positive PCR product (data not shown).

A λgt10 cDNA library (Osmani et al., 1988) was screened using the PCR product amplified from cosmid W15D10 as a probe and 13 positive plaques were identified. Preliminary sequence analysis indicated that all cDNAs were derived from the same gene. The sequence of the largest cDNA was obtained on both strands (Fig. 1). Hypothetical translation identified an open reading frame encoding a protein with the highest similarity to the cdc2 protein kinase of *S. pombe* (Fig. 2). A genomic copy of the gene was sequenced and three introns identified, the positions of which are indicated in Fig. 1.

By comparing the *Aspergillus* sequence to that of other p34^{cdc2}-related sequences it was clear that it had an insert in the region between amino acid 95 (M) and 111 (V) of cdc2. In a sample of nine different p34^{cdc2} type protein kinases (Elledge and Spottswood, 1991) the distance between these two residues was found to average 13 amino acids with the range being between 15 (*S. pombe*) and 11 amino acids (*Drosophila* 2C). In *Aspergillus* this region is significantly larger at 30 amino acids, making this immediate member of the p34^{cdc2} family unique. We considered the possibility that the cDNA that we had sequenced could contain an unspliced intron, as introns have been observed in other cDNAs isolated from the library used (Osmani et al., 1990). However, sequence analysis of the 13 cDNAs isolated gave no indication of an unspliced intron in any. The finding that the original PCR reactions using total cDNA generated a product of identical size to that generated by PCR reactions using genomic DNA also indicates that there are no introns present in this region.

As the *Aspergillus* gene product contains an additional 15 amino acids in this region when compared to *S. pombe cdc2* we asked if it could complement a mutation in the *cdc2* gene. To do this we placed a copy of the gene under the control of the *S. pombe* thiamine-repressible *nmt1* promoter in a modified version of the pREP1 vector (see Materials and Methods) to generate plasmid pREP1*cdc2^{asp}*. pREP1*cdc2^{asp}* and a control plasmid were introduced into *cdc2-33*-containing *S. pombe* cells and *leu*$^+$ transformants were selected at 25°C on minimal media plates containing 2 μM thiamine. Cells were tested for complementation by plating on minimal medium in the absence of thiamine and incubating at 25°C and 37°C (Fig. 3). The control strain could grow at 25°C but not at 37°C and the cells expressing the *A. nidulans* gene were able to grow at both temperatures. The *A. nidulans* gene is therefore able to functionally complement the *cdc2-33* mutation. High level expression was apparently not required for function in *S. pombe*, as we could also observe complementation in the presence of 2 μM thiamine (data not shown), when expression of the gene would be expected to be very low (Maundrell, 1990; Gould et al., 1991). However, when the expression of this gene was derepressed to high levels the cells were semi-wee, as is seen in *S. pombe* with the endogenous gene expressed from the same promoter (Fleig and Nurse, 1991).

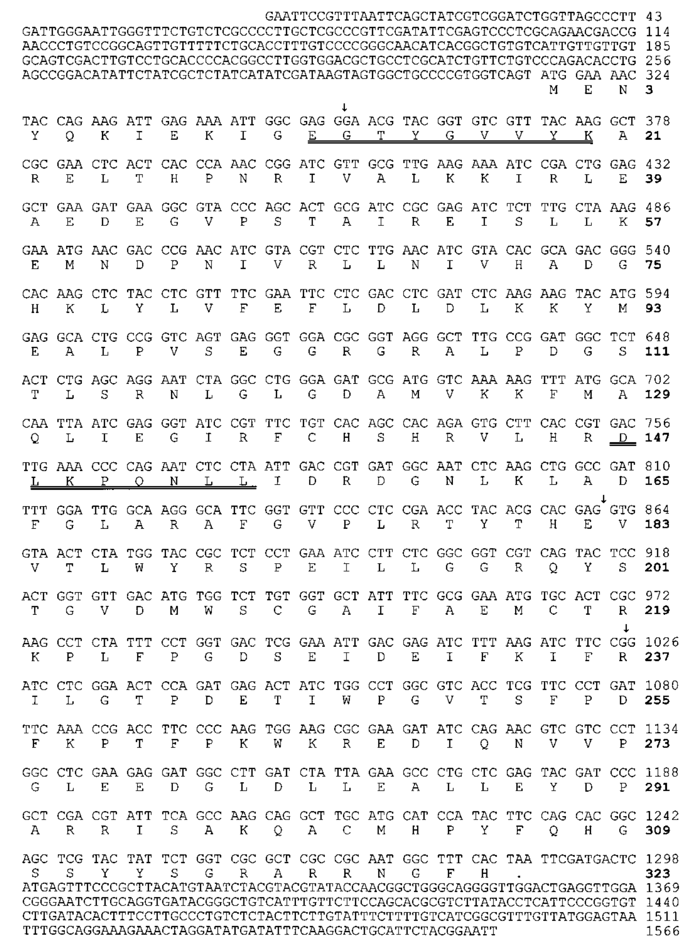

Fig. 1. cDNA sequence of *nimX*^{cdc2} and derived translation product. Nucleotide number is given above the amino acid number, which is in bold. Double underlines indicate the regions used to design degenerate PCR primers. Arrows indicate the position of three introns. Accession no., U07169.

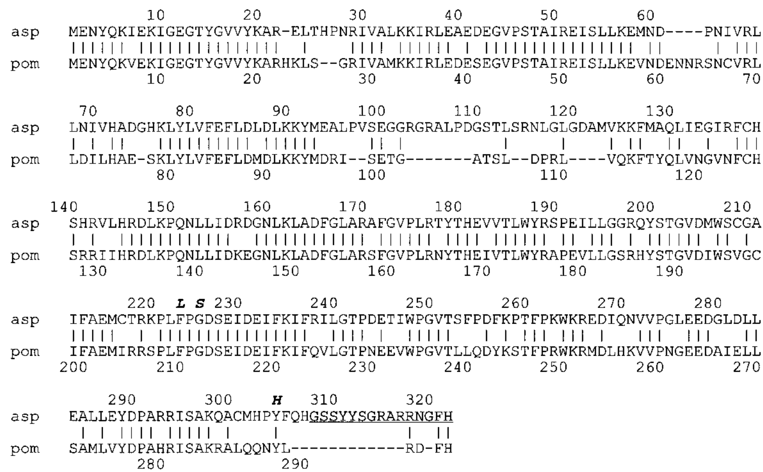

Fig. 2. Comparison of *S. pombe* cdc2 (pom) and *A. nidulans* *nimX*^{cdc2} (asp). Identities are indicated by lines and gaps by dashes. The three amino acids changed to generate temperature-sensitive strains are indicated in bold italics above the wild-type sequence. The C-terminal region used to generate peptide-specific antiserum is underlined.

Deletion of the p34^{cdc2}-related gene generates the nim phenotype

To determine if the p34^{cdc2}-related gene is required in the cell cycle of *A. nidulans* we deleted it using the heterokaryon rescue technique (Osmani et al., 1988). This was achieved by removing a portion of the gene (nucleotides 153-1027) and replacing it with a copy of the *A. nidulans pyrG* gene (Oakley et al., 1987). This construct was used as a linear fragment to transform a *pyrG*89 mutant strain to uridine/uracil prototrophy.

Homologous recombination would replace the wild-type allele with the deleted allele. If the gene is essential, then the deleted allele could only be carried in a heterokaryotic state along with nuclei containing the wild-type allele. Vegetative *A. nidulans* cells (mycelia) are multinucleate. The disrupted allele is, however, selected for on the basis of the *pyrG* marker. To identify heterokaryotic colonies, conidia (uninucleate asexual spores) were streaked from colonies on the transformation plates onto selective media (Fig. 4A1). Of 81 colonies tested eight were shown to produce conidia that were unable to grow into colonies on selective medium, even though the parental colony could grow as a heterokaryon on this medium. When the cells were observed by phase-contrast microscopy, two classes of conidia were observed (Fig. 4A2). Those that were unable to germinate on selective medium (*pyrG*$^{-}$ but *cdc2*-like gene^{+}) and those that could germinate and produce a germtube but then arrest as long germlings (*pyrG*$^{+}$ but *cdc2*-like gene^{-}). By Southern blot analysis (data not shown) we identified a heterokaryon that contained a single copy of both the deleted and wild-type alleles. This heterokaryon was designated H8.

Heterokaryon H8 contains nuclei of two different genotypes (see above) that are segregated into uninucleate spores (conidia) during conidiation. It was therefore possible to look at the germination phenotype of conidia derived from heterokaryon H8 by observing nuclei after staining with DAPI. On both selective and non-selective media a class of cell was apparent (*pyrG*$^{+}$ but *cdc2* gene^{-}) that was able to grow to a considerable length but not undergo mitosis (Fig. 4B5). Analysis by indirect immunofluorescence using anti-tubulin antibody showed a stable interphase array of microtubules with no mitotic spindles present (data not shown). Molecular deletion of this gene is therefore lethal and leads to a cell cycle-specific defect in interphase but does not prevent germination or short-term growth. This is the nim (***n***ever-***i***n-***m***itosis) phenotype seen for mutations in *A. nidulans* that arrest the cell cycle in interphase. The gene was designated *nimX*^{cdc2} as the *nimA* to *nimW* designations have already been used (Morris, 1976) and genetic analysis indicated that none of the other chromosome II-specific *nim* genes are allelic with *nimX*^{cdc2} (data not shown, and Dr Steve James, personal communication).

To analyze further the phenotype caused by the deletion of *nimX*^{cdc2} we took samples after germination on selective media for a period of nine hours and then after a further period of growth overnight. During the early time points of the germination (4 to 7 hours) a high percentage of the cells contained many DAPI staining mitochondria (Oakley and Rinehart, 1985) that tended to obscure the fainter staining nucleus (Fig. 4B1 and 2, also see Fig. 5C and D for similar phenotype caused by a temperature-sensitive mutation in *nimX*^{cdc2}). Some of the mitochondria became very bright and persisted as the cell continued to grow but no nuclear division was seen to have occurred at any time point (Fig. 4B3 and 4). At later time points it became easier to observe the nucleus as mitochondrial DNA replication apparently does not continue indefinitely, and as the cell grows the mitochondria became more disperse, thus revealing the nucleus (Fig. 4B5). We observed very few nuclei in a typical interphase configuration, with nuclear DNA

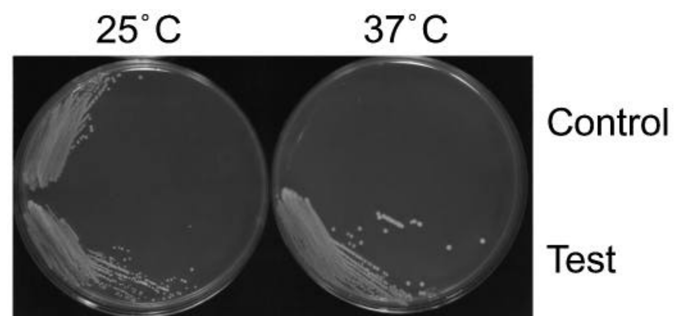

Fig. 3. Complementation of *S. pombe cdc2-33* by *nimX*^{cdc2}. A temperature-sensitive *cdc2*-33 mutant strain was transformed by a control plasmid and a plasmid directing transcription of *nimX*^{cdc2}. Resulting transformants were streaked on plates and incubated at permissive and restrictive temperature to allow colonies to grow.

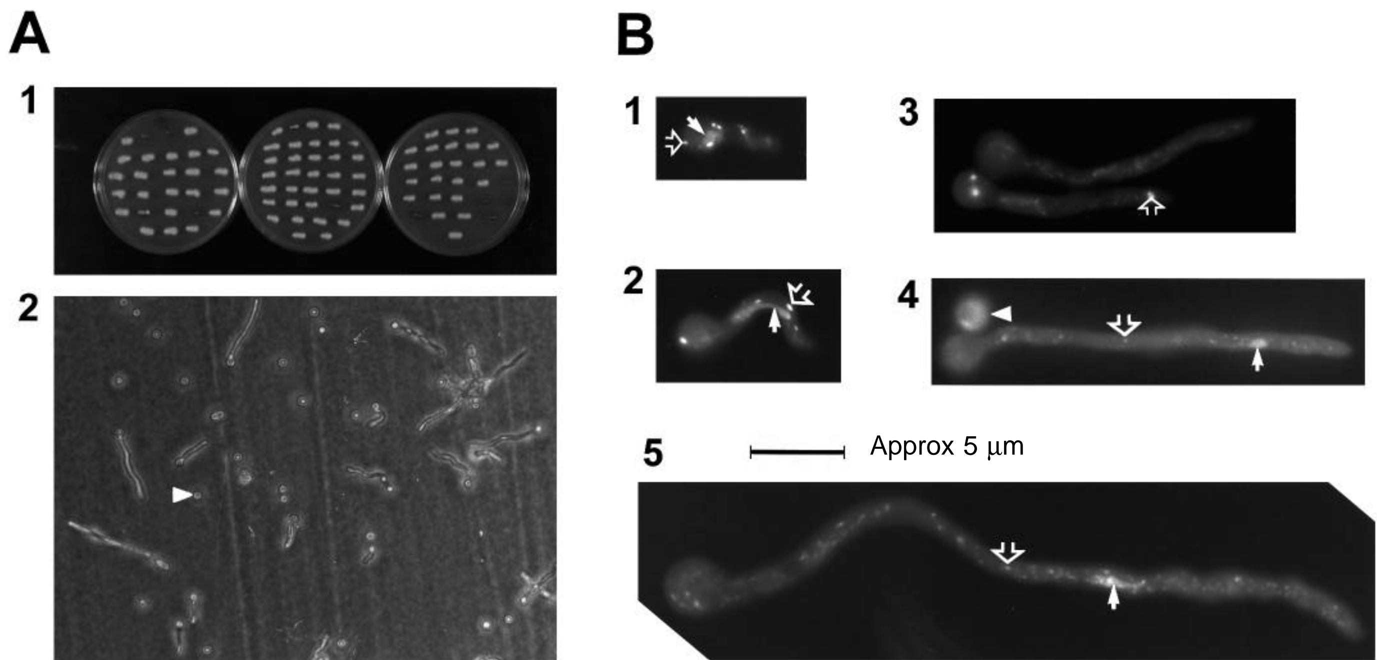

Fig. 4. Deletion of *nimX*cdc2 by heterokaryon rescue. (A1) Strain GR5 was transformed with *nimX*cdc2 linear deletion construct and conidia plated from the primary transformants onto selective media to identify heterokaryons that produce conidia unable to form colonies on selective media. (A2) Conidia from a confirmed heterokaryon were viewed by phase-contrast after germination on selective media. The white arrowhead indicates a *pyrG*– *nimX*+ conidium that is unable to germinate on selective medium. The conidia able to germinate and arrest as germlings are *pyrG*+ but *nimX*–. B1-5 show representative spores at various stages of germination from the heterokaryon stained with DAPI to reveal mitochondria and nuclei. Open arrows indicate mitochondria and white arrows nuclei. The arrowhead in B4 indicates a *pyrG*– *nimX*+ conidium that is unable to germinate on selective medium. All other cells shown are *pyrG*+ *nimX*– germlings that are unable to undergo mitosis and therefore arrest with a single nucleus. The typical terminal phenotype is shown in B5.

forming a crescent around a non-staining nucleolus typical of the wild-type nucleus (e.g. see Fig. 5A and B), suggesting that *nimX* cdc2 function is required to establish correct nuclear structure, although this could be an indirect result of an extended interphase arrest.

Temperature-sensitive mutations in *nimX* arrest at G_1 and G_2

In order to generate temperature-sensitive mutations in *nimX*cdc2 the gene was mutated site-specifically at three codons known to generate temperature-sensitive *cdc2* alleles in *S. pombe* (MacNeill et al., 1991). The mutated genes were used to replace the wild-type allele using a modified (see Materials and Methods) two-step gene replacement technique (Miller et al., 1985) and strains were screened for temperature sensitivity. Three different temperature-sensitive strains were isolated after backcrossing to wild type, which carry the following mutations in *nimX*cdc2 : SO58 = *nimX*cdc2G225S; SO64 = *nimX*cdc2F223L; and SO65 = *nimX*cdc2Y306H.

To determine the effect of the *nimX*cdc2 mutations, conidia were incubated for 10 hours at the restrictive temperature of 42°C, during which wild-type cells would normally undergo several rounds of nuclear division. At this temperature none of the mutations prevented germination or short-term growth but all inhibited nuclear division. For the *nimX*cdc2Y306H mutation no nuclear division occurred, for *nimX*cdc2F223L only 1.3% of nuclei had divided and for *nimX*cdc2G225S 35.7% of nuclei had divided. Increasing the restrictive temperature to 42.5°C prevented virtually all nuclear division from occurring in any of the strains but did not affect wild type and so this temperature was employed as the restrictive temperature.

The temperature-sensitive alleles of *nimX*cdc2 also caused an increase in DAPI staining of mitochondria at the restrictive temperature as was seen with deletion of *nimX*cdc2. This is shown for strain SO65 (*nimX*cdc2Y306H) after 7 hours growth at the restrictive temperature (Fig. 5). When compared to a wild-type cell grown under identical conditions (Fig. 5A and B), it is clear that DNA replication is continuing to a greater extent in the mitochondria of the *nimX*ts– cells (Fig. 5C and D), and based on DAPI staining, nuclear DNA replication appears to be slower compared to the wild type over the same growth period. As with the deletion mutation, the strong DAPI staining of mitochondria made visualization of the poorly defined nucleus difficult at the early phases of germination but the nucleus became more apparent as cells elongated (data not shown).

To determine the point(s) at which *nimX*cdc2 is required in the cell cycle we employed the reciprocal shift method previously used to establish the arrest points of other *nim* mutants (Bergen et al., 1984). Conidia were first arrested in S-phase using hydroxyurea (HU) and were then washed free of the HU and shifted to the restrictive temperature (HU then 42.5°C). The percentage of cells able to undergo nuclear division was then determined by counting the number of cells containing more than a single nucleus, as *A. nidulans* mycelia grow as a multinucleated syncytium. In a parallel experiment conidia

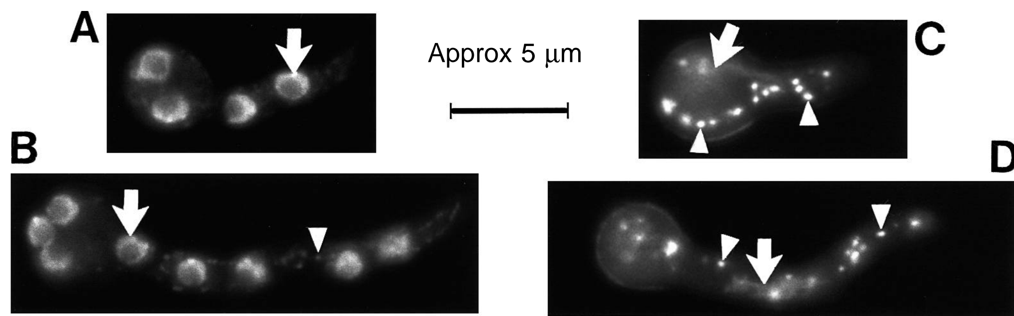

Fig. 5. Effect of temperature-sensitive mutation in *nimX* on nuclei and mitochondria. Conidia of a wild-type strain (A and B) and a strain (SO65) containing a temperature-sensitive *nimX* mutation (C and D) were germinated for 7 hours, fixed and stained with DAPI. Arrows indicate nuclei and arrowheads mitochondria.

were first arrested at the restrictive temperature and then HU was added before shifting to the permissive temperature (42.5°C then HU). In this way the arrest point of the mutation was defined in relationship to the arrest point of HU, i.e. S-phase. A mutation that arrests in G_1 will not divide when tested with the 42.5°C then HU regime but will for the HU then 42.5°C regime. For S-phase mutations both regimes will prevent nuclear division. For G_2 mutations, HU then 42.5°C will arrest division but 42.5°C then HU will not (Bergen et al., 1984). The reciprocal shift analysis was carried out for each of the *nimX*^{cdc2} mutations, and a ts$^-$ mutation in *nimA* that arrests in G_2, *nimA*5, was used as a control.

The results of the reciprocal shift analysis are shown in Table 1. None of the mutations caused an arrest pattern expected for an arrest in G_1 (42.5°C then HU, no division; HU then 42.5°C, division OK). Two of the mutations, the control *nimA5* (strain SO54) and *nimX*^{cdc2}G225S (strain SO58), gave an arrest pattern suggesting an arrest in G_2, as a significant number of their nuclei could divide in the first experiment (42.5°C then HU) but very few could divide in the reciprocal shift (HU then 42.5°C) experiment. The *nimX*^{cdc2}F223L mutation (strain SO64) allowed some division in the first experiment but none in the second experiment, which made interpretation difficult but suggests that at least some cells are arrested in G_2 by this mutation. Finally, the *nimX*^{cdc2}Y306H mutation (strain SO65) gave the cleanest result as no nuclei could divide when challenged with either the HU then 42.5°C or by the 42.5°C then HU reciprocal shifts. If it is assumed that this mutation arrests at a single point in the cell cycle the result indicates that this mutation causes an arrest in S-phase.

However, the experiments described above would not distinguish a mutation causing an S-phase arrest from one causing cell cycle arrest in both G_1 and G_2. As Cdc2/28 is required for cell cycle progression in G_1/S and G_2/M in fission and budding yeast, we decided to extend our analysis of the mutation to see whether *nimX* was required in a similar fashion in *Aspergillus*. Cells were first arrested in S-phase by growth for 7 hours in medium containing HU. The HU-imposed S-phase arrest was then released by washing into fresh medium, and growth was continued for a further 3 hours at either 30°C or 42.5°C. At 30°C, 94% of cells contained two or more nuclei, showing they had been released from the HU arrest point into mitosis. In contrast, no cells containing more than a single nucleus were seen in cultures grown at the restrictive temperature of 42.5°C. In order to determine whether these cells had progressed from the HU arrest point into G_2, or had remained arrested in S-phase, these cultures were subsequently moved back to the permissive temperature of 30°C for a further 3 hours in either the presence or absence of HU. In the absence of HU, 85% of cells were seen with two or more nuclei, showing that they had recovered from the double block of HU then *nimX*^{cdc2}Y306H at restrictive temperature. When released in the presence of HU, 57% of cells had two nuclei, which corresponds to 67% of those surviving the experimental regime in the control (Table 2). These cells with two nuclei had first been arrested in S-phase by HU, progressed into the subsequent G_2 phase where they were then arrested by the *nimX*^{cdc2}Y306H mutation, and then, upon shift back to the permissive temperature, had undergone mitosis and progressed to the subsequent S-phase, where they were once again arrested by HU. This demonstrates that the *nimX*^{cdc2}Y306H mutation arrested cells reversibly in G_2 after the first HU arrest. As *nimX*^{cdc2}Y306H also arrested at restrictive temperature before the HU arrest point (Table 1), this mutation results in two cell cycle arrests points: one prior to HU in G_1, and one after HU in G_2.

We attempted to confirm the reciprocal shift experiments with determination of DNA content by FACS. In our hands, such analyses in *Aspergillus* are difficult to perform as cell wall material of young germlings stains with propidium iodide, resulting in an extremely broad signal that does not always correspond to the DNA content of the cell. In the *nimX*^{cdc2}Y306H mutant, this situation worsened, to the extent of making this analysis uninformative, as a result of the fluorescence from the mitochondrial DNA, which proliferates to high levels (Fig. 5).

Table 1. Reciprocal shift experiments

	A	B	C	D	E	F
SO54	5.5	98.5	64.6	0.5	84	0
SO58	0.4	98.5	46.7	0	93.3	3.7
SO64	0	100	25.2	7.7	93.5	0
SO65	0	89	0	13.3	96.5	0

Conidia of the indicated strains (SO54 = *nimA5*; SO58 = *nimX*^{cdc2}G225S; SO64 = *nimX*^{cdc2}F223L; and SO65 = *nimX*^{cdc2}Y306H) were grown as detailed below and the percentage of cells in which nuclear division had occurred determined after staining fixed cells with DAPI. At least 200 cells were counted for each determination. (A) 9 hours at 42.5°C; (B) 6 hours at 42.5°C then 3 hours at 30°C; (C) 6 hours at 42.5°C then 3 hours at 30°C with HU; (D) 9 hours at 30°C with HU; (E) 6 hours at 30°C with HU then 3 hours at 30°C without HU; (F) 6 hours at 30°C with HU then 3 hours at 42.5°C without HU.

Table 2. Double reciprocal shift experiments

	D	E	F	–HU REL.	+HU REL.
SO65	1	94	0	85	57

Conidia of strain SO65 (*nimX*^{cdc2}Y306H) were grown as described and cells were analyzed as for Table 1. (D) 7 hours 30°C with HU; (E) 7 hours 30°C with HU then 3 hours at 30°C without HU; (F) 7 hours 30°C with HU then 3 hours at 42.5°C without HU. Cells were then returned to 30°C for 3 hours in the absence of HU (–HU REL.) or after addition of 80 mM HU in the final 15 minutes at 42°C and then for 3 hours at 30°C (+HU REL.).

Production of antibodies against NIMX^{cdc2} and NIME^{cyclinB} and assay of H1 kinase activity

NIME^{cyclinB} was expressed and HIS-tagged, purified from *E. coli*, and four rabbits were injected with the purified NIME^{cyclinB} (see Materials and Methods). A peptide-specific antibody was generated in sheep against the C terminus of NIMX^{cdc2} and affinity-purified against the antigenic peptide (see Materials and Methods). The immune serum and pre-immune serum for each immunogen were tested on western blots of total soluble *A. nidulans* protein. All NIME^{cyclinB} immune sera reacted with a protein of the size expected for NIME^{cyclinB} and no pre-immune sera reacted with this band, indicating that each anti-NIME^{cyclinB} serum reacted specifically with NIME^{cyclinB} (Fig. 6A). Using standard western blot analysis and the NIMX^{cdc2} peptide-specific antibodies we failed to detect NIMX^{cdc2} in total protein extracts or when p34^{cdc2} type proteins were affinity-purified using p13 beads (data not shown). The anti-NIMX^{cdc2} peptide-specific antibodies did however work well in immunoprecipitation experiments (see below).

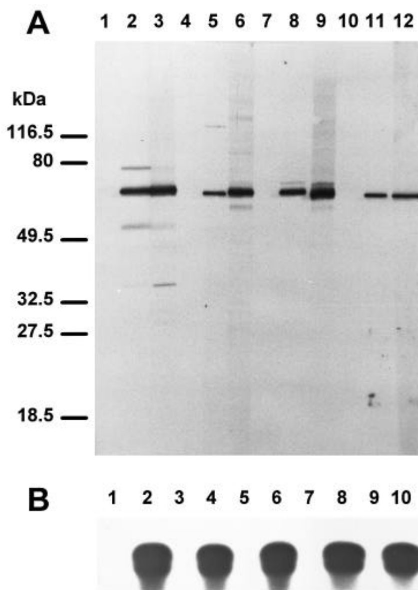

Fig. 6. Western blot and H1 kinase of NIMX/NIME complexes (A) Pre-immune sera (lanes 1, 4, 7, 10) immune sera one (lanes 2,5,8,11) and second bleed immune sera (lanes 3,6,9,12) were generated against bacterially expressed NIME^{cyclinB} in four rabbits and used in a western blot of 100 μg of total *A. nidulans* protein. (B) Pre-immune (lanes 1,3,5,7,) and immune sera (2,4,6,8,) of the NIME^{cyclinB}-immunized rabbits were used to precipitate and assay H1 kinase activity from *A. nidulans* extract. Lane 9 is pre-immune sera from sheep and lane 10 affinity-purified antibodies generated against the C terminus of NIMX^{cdc2} assayed in the same manner. Autoradiogragh of ^{32}P-labelled H1 is shown.

To test the ability of the antibodies to precipitate a histone H1 kinase activity, immunoprecipitates were prepared using pre-immune serum and immune-serum, and assayed for histone H1 kinase activity. The results (Fig. 6B) indicate that immune-specific serum against either NIME^{cyclinB} or NIMX^{cdc2} was able to precipitate an equal amount of histone H1 kinase activity, and that the respective pre-immune precipitates were negative for H1 kinase activity.

To determine what p34^{cdc2} proteins were precipitated by our antibodies, protein was precipitated using anti-NIME^{cyclinB}, anti-NIMX^{cdc2} or p13 beads and prepared for western blotting after SDS-PAGE. The blot was probed with PSTAIR-specific antisera to detect PSTAIR-containing proteins of the p34^{cdc2} family. Both the anti-NIMX^{cdc2} antibodies and p13 beads were able to precipitate two proteins that were detected by the PSTAIR-specific serum, one of around 34 kDa and the other around 37 kDa. However, the anti-NIME^{cyclinB} antiserum only precipitated the 34 kDa PSTAIR-containing protein (Fig. 7A). The same interactions were confirmed using a strain in which the wild-type *nimE* had been replaced with a hemagglutinin (HA)-tagged version of NIME^{cyclinB} (data not shown). This suggests that NIMX^{cdc2} exists in two forms, a 37 kDa form that is not associated with NIME^{cyclinB} and a 34 kDa form that is associated with NIME^{cyclinB}.

Previous experiments indicated that enzymatic de-phosphorylation of p13 precipitates, sufficient to remove tyrosine phosphate groups, did not change the mobility of p34 or p37 (Lu et al., 1993). We have repeated these experiments and found that the kinase activity of the precipitates is not destroyed under these conditions, as would be expected if the phosphothreonine 161 had been de-phosphorylated, as phosphorylation at this site is essential for H1 kinase activity (Gould et al., 1991). To test if there is an inaccessible phosphate group resistant to de-phosphorylation, we denatured p13 precipitates using SDS. These were then diluted in potato

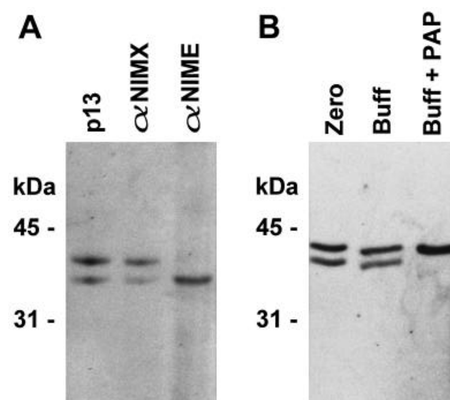

Fig. 7. Co-precipitation of NIMX and NIME. (A) *A. nidulans* extract was precipitated with the reagents indicated and western blotted using PSTAIR-specific antisera. (B) p13 precipitates were either not treated (zero) or diluted in CIP buffer in the absence (Buff) or presence of potato acid phosphatase (+PAP) and western blotted using PSTAIR-specific antisera.

acid phosphatase buffer and phosphatase was added to half of the sample. After incubation the samples were separated by SDS-PAGE, and processed for western blotting using PSTAIRE anti-serum. The untreated and mock-treated samples contained the expected p34 and p37 bands but in the phosphatased sample the p34 band had been completely converted to the p37 form (Fig. 7B).

DISCUSSION

Although reagents are available to biochemically study p34^{cdc2}-related kinases these reagents are not necessarily specific for one member of this family. Analysis of cell cycle regulation in *A. nidulans* has therefore been complicated by the apparent inability to specifically detect p34^{cdc2} biochemically (Lu et al., 1993) and hampered by the inability to genetically manipulate this gene. For this reason we have cloned and analyzed p34^{cdc2} from *A. nidulans*. The gene was able to complement the *cdc2-33* mutation of *S. pombe* but has an insert (15 amino acids) not found in other members of this kinase family. Based on structure determinations for *cdk2* (DeBondt et al., 1993), the insert is located in a linker region (L8) that joins helices α-2 and α-3, which may help to explain why such a large insert can be tolerated without affecting complementation ability.

The *A. nidulans nimX* gene, which encodes a p34^{cdc2} homolog, was deleted and shown to be essential for cell cycle progression. Deletion caused an arrest of nuclear division in interphase, but not short-term growth, which conforms to the nim phenotype *A. nidulans* cell cycle-specific mutations. As we cloned the gene from a chromosome II-specific library it must map to this chromosome. There are four other *nim* genes that genetically map to this chromosome, those being *nimE, nimG, nimR and nimT. nimE* and *nimT* are known to encode cyclinB and a cdc25 type tyrosine phosphatase, respectively, but *nimG* or *nimR* could encode p34^{cdc2}. By genetic experiments, using temperature-sensitive mutations in p34^{cdc2} and *nimG10* and *nimR21* mutant strains, this possibility was eliminated. The gene was therefore designated *nimX*^{cdc2} as the *nimA* to *nimW* designations have already been used (Morris, 1976).

One of the distinctive features of the phenotype caused by deletion of *nimX*^{cdc2} was the continued replication of the mitochondrial genome in the absence of a comparable increase in the nuclear genome. This phenotype was unexpected and we initially doubted if the deletion caused a cell cycle-specific defect as the nuclei were very difficult to distinguish and a similar phenotype had not previously been observed for the other characterized *nim* mutations. This phenotype is therefore currently specific to the *nimX*^{cdc2} mutations. The possibility exists that mutations that generate the phenotype of continued mitochondrial DNA replication, at the expense of nuclear DNA replication, may not have been pursued for further analysis in previous *A. nidulans* mutant screens because they look peculiar in the 'cell cycle' sense. This fact may account for our previous inability to isolate and identify a temperature-sensitive homolog of p34^{cdc2} during classical screens for conditional cell cycle-specific mutants. In *S. pombe* a similar phenotype has been observed for the *cdc10-129* mutation that arrests in G_1 of the cell cycle (Sazer and Sherwood, 1990). As mutation of *nimX*^{cdc2} also arrests cells in G_1 (see below) it is possible that arrest in G_1 allows the mitochondrial genome to continue to replicate in some fungi. It is not, however, a feature of *A. nidulans* mutations that arrest in S, G_2 or M-phase and therefore this phenotype may provide a screen to specifically identify mutations that arrest in G_1-phase of this species.

To genetically study *nimX*^{cdc2} we generated temperature-sensitive alleles by the process of reverse genetics. These mutations all arrested nuclear division at the restrictive temperature and generated a phenotype similar to that seen for deletion of the gene. Each of the mutations introduced into *nimX*^{cdc2} was based on mutations that cause a cell cycle-specific arrest in *S. pombe* when present in *cdc2* (MacNeill et al., 1991). We had originally attempted to make four mutations, two that arrest *S. pombe* cells in both G_1 and G_2 (*cdc2-45* and *cdc2-48*) and two that arrest in G_2 (*cdc2-17/22* and *cdc2-18*; MacNeill et al., 1991). We were unable to generate the equivalent of the *cdc2-18* mutation in *A. nidulans*. In fission yeast a duplication of this mutation is able to revert to *cdc*$^+$ at a high frequency (Carr et al., 1989), indicating a weak effect of this particular mutation on cdc2 function. Therefore, this mutation may not lead to a temperature-sensitive p34^{cdc2} in *A. nidulans*. We were able to generate the equivalent of the other three mutations and used the reciprocal shift method of analysis to determine at which point in the cell cycle these mutations caused arrest. The equivalent (*nimX*^{cdc2}G225S) of the *S. pombe* G_2-specific mutation, *cdc2-17/22*, did not arrest only in G_2 in *A. nidulans* but some cells were arrested in G_2 by this mutation. Neither did the equivalent of the G_1/G_2-arresting *cdc2-45* mutation arrest tightly in G_1 and G_2. Only the equivalent of the *S. pombe cdc2-48* mutation (*nimX*^{cdc2}Y306H) gave a clear result using the reciprocal shift method. The analysis indicated that this mutation arrests the cell cycle of *A. nidulans* at both G_1 and G_2. Thus, although p34^{cdc2} function may be shared between different CDKs in higher eukaryotes, in unicellular and multicellular fungi, one p34^{cdc2} kinase is apparently sufficient for G_1 and G_2 progression. The evolutionary pressure to divert the function of p34^{cdc2} into a G_1 and a G_2 protein is unclear but our data suggest that it is not related to the evolution from unicellular to multicellular microorganisms.

Antibodies were generated against the C-terminal peptide of NIMX^{cdc2} and against NIME^{cyclinB} produced and purified from *E. coli.* Both were able to immunoprecipitate an equivalent histone H1 kinase activity from *A. nidulans* protein extracts. The antiserum directed against the C terminus of NIMX^{cdc2} was shown to react to two forms, p34 and p37, of NIMX^{cdc2}. The antiserum directed against NIME^{cyclinB} only co-precipitated the p34 form. A similar exclusive interaction of a high mobility phosphorylated form of p34^{cdc2} protein with cyclin B protein has been observed in *Xenopus* cell cycle-arrested extracts (Lorca et al., 1992).

It has been suggested that the two proteins detected by p13 precipitation and PSTAIRE-specific antisera could be two different phosphorylated forms of the same protein (O'Connell et al., 1992). However, subsequent enzymatic dephosphorylation experiments indicated that removal of phosphate from native p34 did not change its mobility to the p37 position even though phosphotyrosine was removed from p34. This led to the suggestion that p34 and p37 may be two different gene products (Lu et al., 1993). However, we now show that

affinity-purified anti-peptide antibodies directed against the variable C-terminal region of *nimX*^{cdc2} precipitate both p34 and p37, indicating that they could be two different phosphorylated forms of the same protein. To more thoroughly dephosphorylate NIMX^{cdc2} we denatured it with SDS before phosphatase addition to ensure complete dephosphorylation NIMX^{cdc2}. Under these conditions all of the p34 species were converted to the p37 size, showing conclusively that p34 is a phosphorylated and p37 a non-phosphorylated form of NIMX^{cdc2}. It is most likely that threonine 161 phosphorylation is responsible for the mobility shift, as in other systems a similar shift has been observed (e.g. see Lorca et al., 1992) and this phosphorylation has been shown to be involved in the binding of cyclin proteins to p34^{cdc2} type proteins (Gould et al., 1991; Ducommun et al., 1991; Lorca et al., 1992). As NIME^{cyclinB} exclusively interacts with p34 and overexpression of NIME^{cyclinB} increases the yield of p34 (O'Connell et al., 1992) it is clear that the phosphorylation of p34 is involved in its positive interaction with NIMX^{cdc2} in *A. nidulans*.

During these studies we had significant problems resolving the p34 and p37 forms of NIMX^{cdc2} by SDS-PAGE. We eventually established conditions by which we could separate the two species, and the deciding factor in the resolution appears to be the % crosslinker used in the formulation of the polyacrylamide. At a ratio of 0.8 g bis acrylamide to 29.2 g acrylamide (our standard conditions) separation was poor (O'Connell et al., 1992) or non-existent. But at 1.0 g bis acrylamide to 30 g acrylamide separation could be achieved. This may explain our inability to resolve the p34/p37 doublet in some of our previous experiments (Osmani et al., 1991b).

The mutations and antibodies generated during these studies will now allow further elucidation of the relationship between NIMA and p34^{cdc2} and, in addition, enable us to study p34^{cdc2} function in a genetically tractable multicellular fungus.

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