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Expression of the noncatalytic domain of the NIMA kinase causes a G_2 arrest in *Aspergillus nidulans*

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Temperature-sensitive mutation of the *nim*A gene of *Aspergillus nidulans* causes a reversible G_2 arrest, whereas overexpression of *nim*A causes premature entry into mitosis from which the cells cannot exit. The *nim*A gene encodes a Ser/Thr-specific protein kinase (NIMA) which contains an extended COOH-terminal noncatalytic domain. To evaluate the role of this enzyme in nuclear division control, we introduced various mutant *nim*A cDNAs under the control of the inducible alcohol dehydrogenase gene promoter into a strain of *Aspergillus nidulans* containing a temperature-sensitive *nim*A mutation (*nim*A5). While expression of the wild type NIMA complemented the *nim*A5 mutation and induced a premature mitotic arrest when overexpressed, expression of a kinase-negative NIMA containing a single amino acid mutation in the putative ATP-binding site could not rescue the *nim*A5 mutation but resulted in a specific G_2 arrest when overexpressed. An identical phenotype was observed with cells expressing only the noncatalytic COOH-terminal domain of NIMA, whereas overexpression of the inactive kinase domain was without effect. The G_2 arrest produced by overexpression of the full-length inactive or COOH-terminal NIMA molecules did not prevent activation of the endogenous NIMA or H1 kinase activity precipitable by p13 beads. We suggest that this dominant-negative phenotype results from competitive inhibition of the association of active NIMA with a cellular target(s) and that appropriate targeting is essential for the mitotic function of the NIMA kinase.
Key words: Aspergillus nidulans/cell cycle/NIMA kinase

Introduction

Progression through the cell cycle is regulated by protein kinases and phosphatases that have been shown to be functionally conserved during evolution. The protein kinase ($p34^{cdc2}$) encoded by the *cdc2* gene of *Schizosaccharomyces pombe* and its homologs in other species is the most widely studied of the mitotic protein kinases (Beach *et al.*, 1982; Lee and Nurse, 1987; for reviews see Murray and Kirschner, 1989; Nurse, 1990; Pines and Hunter, 1990; Lu and Means, 1993a). The $p34^{cdc2}$ protein kinase is the catalytic subunit of maturation promotion factor (MPF), a multi-protein complex that includes $p34^{cdc2}$ and cyclin B. Although the level of $p34^{cdc2}$ protein is constant during the cell cycle, its activity fluctuates due to phosphorylation/dephosphorylation of specific Tyr and Thr residues as well as interaction with its regulatory cyclin subunits. The cyclin B–$p34^{cdc2}$ complex has been shown to be involved in regulation of mitosis and meiosis in all eukaryotes including *Aspergillus nidulans* (Osmani *et al.*, 1991a). However, whereas activation of $p34^{cdc2}$ kinase is required, it is not sufficient to trigger mitosis in *A.nidulans* if the NIMA protein kinase is not activated (Osmani *et al.*, 1991a).

The NIMA kinase is the product of the *nim*A gene, which was isolated by genetic complementation of a temperature-sensitive mutation in the *A.nidulans nim*A gene (Osmani *et al.*, 1988). Cells carrying temperature-sensitive mutations in the *nim*A gene were specifically arrested in G_2 at the restrictive temperature, but rapidly and synchronously entered mitosis when shifted to the permissive temperature. In contrast, overexpression of the *nim*A gene product induced premature mitotic arrest (Osmani *et al.*, 1988). These results indicated that NIMA played a critical role in the progression of cells into mitosis. Although NIMA kinase activity seems required for mitotic progression, the *nim*A gene has not yet been shown to be essential and no information is available to address the specific role of NIMA. It is known that NIMA kinase activity fluctuates during the nuclear division cycle peaking in late G_2 and mitosis (Osmani *et al.*, 1991b). This increase in activity does not occur when either calcium or calmodulin levels are held low (Lu *et al.*, 1993b). We (Lu *et al.*, 1993a) have shown NIMA to be a Ser/Thr-specific protein kinase whose activity is also regulated by phosphorylation/dephosphorylation of Ser/Thr residues. These observations suggest that NIMA activity is regulated both by when the protein is expressed during the nuclear division cycle and by a protein kinase(s) that phosphorylates the enzyme in G_2.

In addition to a proper kinase activity, the function of cell cycle-regulated protein kinases also depends on the appropriate intracellular localization through protein–protein interaction. NIMA contains a kinase domain located at its NH_2-terminus and an extended noncatalytic COOH-terminal domain. To investigate the roles of these different domains in the regulation of NIMA function *in vivo*, we have created strains of *A.nidulans* in which NIMA, an inactive NIMA, an inactive NIMA catalytic domain or the noncatalytic domain can be expressed by manipulation of the activity of the *alc*A promoter. Surprisingly, we find that either the inactive enzyme or its noncatalytic COOH-terminal domain acts as a dominant-negative repressor that specifically arrests cells in G_2. Both proteins arrested cells in a concentration-dependent manner with similar potency, and the arrest was independent of the activity of the endogenous NIMA or p13-associated H1 kinase. The results suggest that NIMA is essential and demonstrate a critical role for the

noncatalytic COOH-terminal domain in modulating the mitotic function of the enzyme.

Results

Expression of a kinase-negative mutant NIMA results in a lethal phenotype in a concentration-dependent manner

We have previously demonstrated that mutation of a lysine residue (K40) in the ATP-binding loop of NIMA to methionine (M), results in an inactive protein kinase (Lu *et al.*, 1993b). Since *nim*A plays a critical role for entry into mitosis in *A.nidulans*, we wanted to determine whether expression of this kinase-negative mutant *nim*A (K40M) could serve as a dominant-negative repressor. The wild type and K40M mutant *nim*A cDNAs were ligated into the *A.nidulans* expression vector pAL5 in both sense and antisense orientations, resulting in plasmids pAL-A and pAL-A-R as well as pAL-K40M and pAL-K40M-R, respectively (Figure 1A). The pAL5 vector was chosen because it contains (i) the *pyr*4 gene from *Neurospora crassa* which is a selectable marker, complementing the *pyr*G89 mutation present in SO6, (ii) the alcohol dehydrogenase gene promoter (*alc*A), the activity of which is regulated by the carbon source present in the growth medium, and (iii) a 3.8 kb 3′ untranslated sequence of the *A.nidulans* histone H3A gene which allows a high frequency of homologous recombination at the non-essential H3A gene 3′ locus (Doonan *et al.*, 1991). These recombinant plasmids were used to transform the SO6 strain, which contains not only the *pyr*G89 mutation but also a temperature-sensitive *nim*A5 mutation which results in G_2 arrest at the restrictive temperature. The resulting transformants were grown in *alc*A-repressing medium (glucose as the carbon source) at the permissive temperature (32°C). Conidia (dominant spores) from 44 random transformant colonies out of each transformation were then inoculated onto repressing or inducing (ethanol as the carbon source) medium for 2−3 days at the permissive temperature. All of the colonies derived from transformants containing pAL-A-R and pAL-K40M-R plasmids which contain *nim*A cDNAs in the antisense orientation were able to grow on both media. In contrast, the colonies containing either the wild type (PL11) or K40M mutant (PL12) *nim*A in the sense orientation were significantly or completely growth inhibited on inducing medium but grew normally on repressing medium at the permissive temperature (Figure 2 and data not shown). Similar phenotypes were also obtained when these constructs were introduced into the GR5 strain which is wild type for the *nim*A gene (data not shown). Since it is possible to turn off endogenous NIMA function in the SO6 genetic background by raising the temperature which allows us to test the function of the exogenous *nim*A constructs, all subsequent experiments were carried out in the SO6 genetic background.

Although the growth of transformants containing either the wild type or K40M mutant *nim*A was inhibited on inducing medium, there were some variations among colonies. Since the transgene could integrate into a locus of the *Aspergillus* genome at various copy numbers of the plasmid during the transformation, it was possible that the differential effects observed were copy number dependent. To explore this possibility, we isolated several pure clones by streaking representative transformant colonies from each

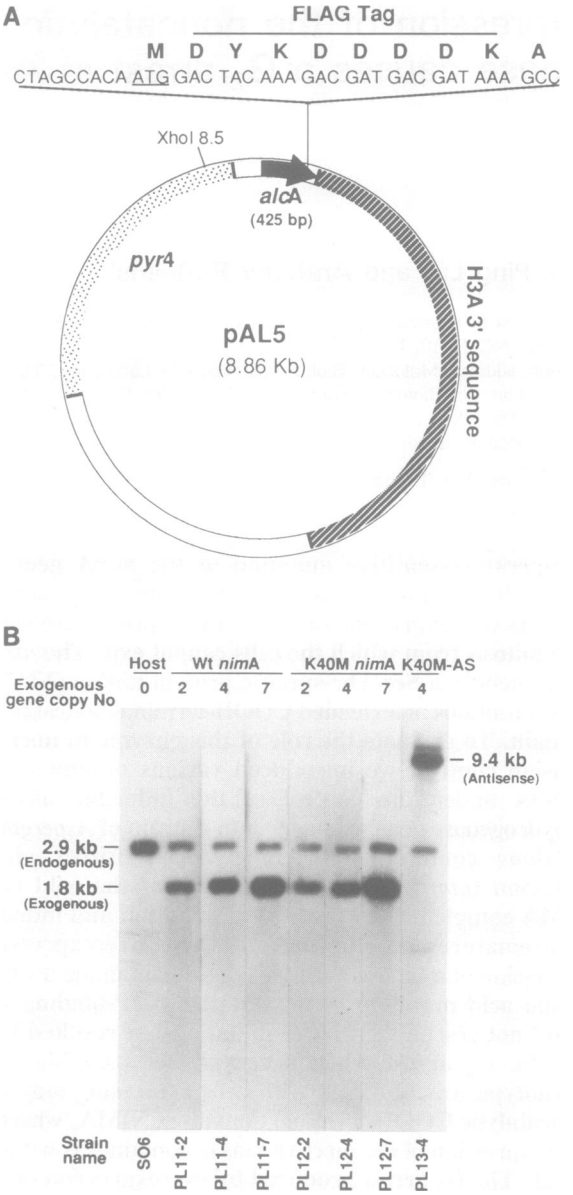

Fig. 1. Expression vector and Southern analysis of *Aspergillus* transformants. (**A**) pAL5 vector and FLAG tag. pAL5 contains the inducible *alc*A promoter, the 3′ untranslated sequence of the *A.nidulans* histone H3A gene and a selectable nutritional marker, the *pyr*4 gene. The *nim*A cDNAs were subcloned into the *Sma*I site located between the promoter and 3′ sequence. When the FLAG was used, a double-stranded oligonucleotide, the sequence of which is shown, was inserted at the translation initiation site of the *nim*A cDNA before it was subcloned into the pAL5 vector. (**B**) Southern analysis. SO6 strain cells were transformed with either pAL-A (Wt *nim*A), pAL-K40M (K40M *nim*A) or pAL-K40M-R (K40M-AS) and the transformants selected in the absence of uradine and uracil. Conidia from the transformants and the parent strain (Host) were grown in YG medium for 2 days and genomic DNA was isolated. After digesting with *Xho*I, the DNA samples were subjected to electrophoresis and transferred to a membrane. The filter was probed with a ^{32}P-labeled *Nco*I−*Xho*I fragment of the *nim*A cDNA.

transformation to single colonies three times. The genomic DNA was isolated from these strains and digested with *Xho*I, followed by Southern analysis using the 1.0 kb *Nco*I−*Xho*I fragment of *nim*A cDNA as a probe. Under these conditions, the endogenous *nim*A gene produces a 1.8 kb hybridizing fragment that can be used as a single copy standard

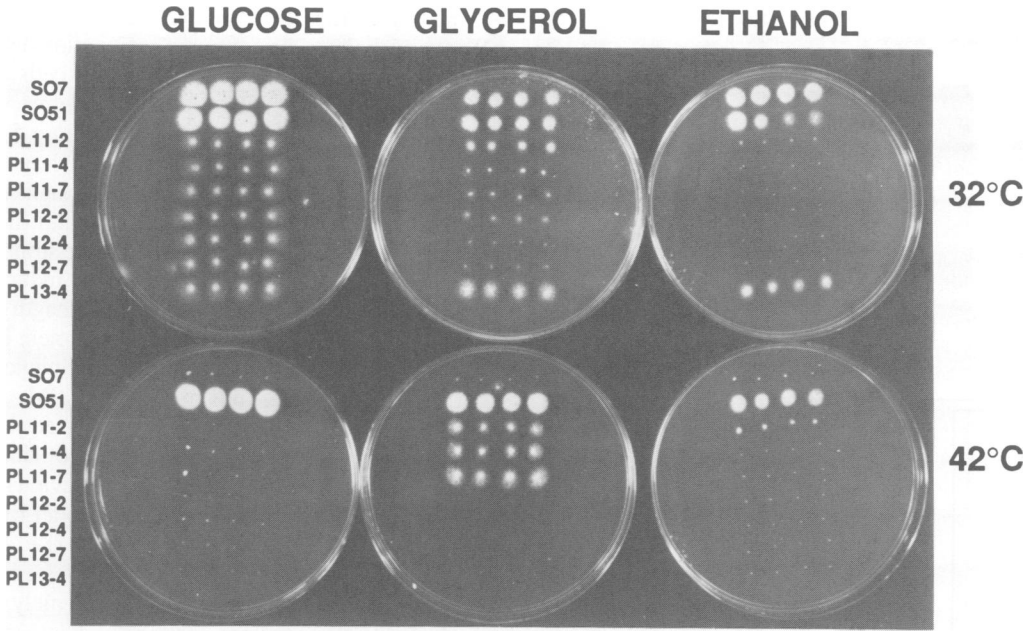

Fig. 2. Effect of expressing wild type and kinase-negative mutant NIMAs on cell growth. Conidia from pAL-A (PL11), pAL-K40M (PL12) or pAL-K40M-R (PL13) transformants and the temperature-sensitive *nim*A5-containing control strain (SO7) as well as wild type strain (SO51) were transferred onto repressing (glucose), derepressing (glycerol) or inducing (ethanol) plates and cell growth was assessed after 3 days of incubation at 32°C (permissive temperature) or 42°C (restrictive temperature).

(Figure 1B, 'Host' lane). On the other hand, similar digestion of the transformant strains produces a 2.9 kb *nim*A fragment from the sense construct and a 9.4 kb fragment from the antisense orientation (Figure 1B). Since the growth of the transformants containing a single copy of the transgenes was markedly slowed but not completely inhibited on inducing medium, it was difficult to characterize the phenotype. We therefore selected transformants containing more than one copy of the transgene for further analysis. As shown in Figure 1B, pure strains containing approximately two, four or seven copies of either the wild type or mutant *nim*A were obtained as was a strain containing four copies of the mutant *nim*A in the antisense orientation. Based on the copy number, they were named PL11-2, PL11-4 and PL11-7 for the wild type *nim*A, PL12-2, PL12-4 and PL12-7 for the mutant *nim*A and PL13-4 for the mutant *nim*A in the antisense orientation (Figure 1B). Southern analysis of the genomic DNA from pure strains showed a single transgene integration site at the histone H3A 3′ locus (Figure 1B and data not shown).

To examine the effect of gene dosage on growth, each transformant strain was inoculated onto media containing different carbon sources and incubated at either the permissive or restrictive temperature (42°C, Figure 2). In order to control for the different media and temperatures used, we included strains SO51 and SO7. SO51 cells should grow in all three media and at both temperatures, while SO7 should grow in all three media but only at the permissive temperature. At the restrictive temperature, which does not allow the endogenous NIMA to function normally, none of the transformant strains could grow on any of the three media except those containing the wild type *nim*A (PL11 series). The PL11 series, like the parent SO7 strain, grew on basal medium (glycerol, which is non-inducing and non-repressing for the *alc*A promoter and therefore allows a basal level expression of the *alc*A-driven gene), but grew very poorly or did not grow at all on inducing medium depending on the transgene copy number. These data confirm prevous results of Osmani *et al.* (1988) and demonstrate that the wild type *nim*A transgene could complement the *nim*A5 temperature-sensitive mutation and that the temperature-sensitive mutation of *nim*A was a loss-of-function mutation. These results extend previous observations by showing that neither the K40M *nim*A mutant nor antisense *nim*A exhibits *nim*A function in the cell. At the permissive temperature, all strains grew on repressing medium. The growth differences on the repressing medium between control strains and transformants (Figure 2) were probably due to different genotypes, since the growth of the transformants was similar to that of the parent strain SO6 when both strains were grown on the same medium supplemented with uradine and uracil (data not shown). On basal medium, the growth of strains containing either wild type or the K40M mutant *nim*A was inhibited in a copy number dependent manner, whereas wild type (SO51), the *nim*A5 strain (SO7) and the strain (PL13-4) containing the mutant *nim*A in the antisense orientation grew normally. On inducing medium, growth inhibitory effects of expressing wild type or mutant *nim*A became even more severe. Colonies were completely inhibited for all strains except PL11-2 which contained only two extra copies of the wild type *nim*A and grew a little. These results demonstrate that introduction of either wild type or kinase-negative *nim*A cDNA into cells resulted in a lethal phenotype in a gene dosage-dependent manner.

To ensure that the various *nim*A cDNAs expressed the predicted proteins in cells, we inserted an oligomer encoding an 8 amino acid FLAG epitope tag preceded by a translation initiation sequence into the normal translation initiation site of the *nim*A cDNAs. These tag-containing cDNAs were then subcloned into the pAL5 vector (Figure 1A), resulting in pAL-A-P1 and pAL-K40M-P1 which were transformed into the SO6 strain, as described above. Transformants were then

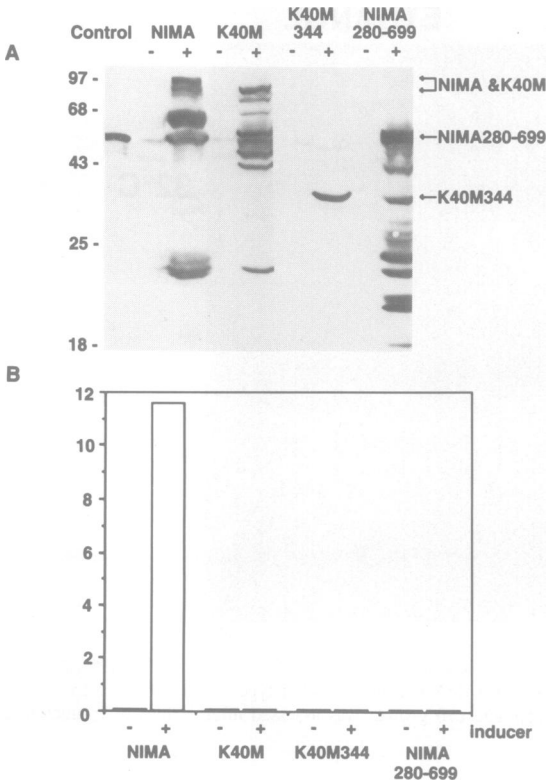

Fig. 3. Determination of expression levels of the various NIMA constructs. (**A**) Protein levels. Conidia from FLAG-NIMA (PL14-7), FLAG-K40M (PL15-6), FLAG-K40M344 (PL17-4) and FLAG-NIMA280−699 (PL18-4)-containing strains were germinated in either repressing (−) or inducing (+) medium for 14 h. Cells were harvested and ground in EB buffer and 200 μg of soluble proteins were separated by PAGE, followed by transfer to membranes. The filters were stained with Ponceau S to ensure equal loading of proteins among lanes (data not shown), before probing with the monoclonal M2 antibody. The expected products are indicated by arrows. Control lane was a purified FLAG-containing protein provided by Kodak. The prestained molecular weight markers were from BRL. (**B**) Kinase levels. 50 μg of soluble proteins prepared as described in A were incubated with the M2 antibodies for 2 h and then with protein A beads for 0.5 h. After washing with EB buffer containing 0.2% NP40, samples were assayed for NIMA protein kinase activity using the PLM(58−72) peptide as a substrate, as described in Materials and methods.

grown on media containing one of the three different carbon sources at either the permissive or restrictive temperature. In all cases the phenotypes observed were identical to those produced by the equivalent cDNAs without the FLAG tag (data not shown). Thus cells containing the FLAG-NIMA could not grow on inducing medium at the permissive temperature, but did grow on basal medium at the restrictive temperature, while cells transformed with the FLAG-K40M NIMA could not grow on either medium. These results show that the presence of the FLAG epitope did not alter the effect of overproduction of the NIMA proteins in the cell. Several colonies were further purified and two pure clones were obtained which contained seven copies of the the FLAG-NIMA (PL14-7) and six copies of the FLAG-K40M NIMA (PL15-6), as indicated by Southern analysis of genomic DNA (data not shown). To determine the level of protein expression from the transgene, spores from these pure strains were germinated on repressing or inducing medium overnight, and germlings were harvested and ground in an extraction buffer. The soluble extracts were subjected to Western analysis or immunoprecipitation using the anti-FLAG monoclonal M2 antibody. Since cells carrying wild type *nim*A cDNA could not form germ tubes when directly germinated on inducing medium (see Figure 5A), they were germinated on repressing medium for 10 h, and then shifted to inducing medium in order to obtain enough cellular protein for a Western blot. On repressing medium, neither protein nor kinase activity were detected in these strains (Figure 3A and B). In contrast, when grown on inducing medium, 80−85 kDa proteins expected for full-length NIMA, were detected for both strains (Figure 3A). Protein kinase activity was also present in the immunoprecipitates from cells carrying wild type, but not the K40M *nim*A cDNA (Figure 3B). The difference in the apparent M_r of the wild type and K40M NIMAs might be due to NIMA autophosphorylation, since we have previously shown that NIMA is a phosphoprotein and undergoes autophosphorylation when expressed in bacteria (Lu *et al.*, 1993a). It also appears from analysis of the Western blot that the full-length NIMA proteins may be extremely susceptible to proteolysis. Nevertheless, these results demonstrate that the transgenes express the expected proteins and that the substitution of a single amino acid, K40M, inactivated NIMA as a kinase in cells just as previously shown for the bacterially produced mutant protein (Lu *et al.*, 1993a).

Expression of the kinase-negative NIMA causes a G_2 arrest

Overexpression of wild type *nim*A has been previously shown to induce premature entry into mitosis from which cells cannot exit (Osmani *et al.*, 1988). We determined the terminal phenotype of strain PL11-7, which contains seven copies of the *nim*A transgene when grown on different media at the permissive temperature. Cells were fixed and stained with the DNA fluorochrome DAPI, followed by examination of nuclear number and morphology (Lu *et al.*, 1992, 1993b). When spores were germinated on repressing medium, they formed a germ tube and underwent the normal nuclear division cycle, as did wild type cells (data not shown). However, when repressing medium was removed and replaced with inducing medium, the mitotic index increased with time and reached >95% by 1.5 h (data not shown). When spores were directly germinated on inducing medium, although the size of spores became bigger, >95% of spores failed to form germ tubes and contained only a single highly condensed nucleus per cell (Figure 5A). Under various inducing conditions, we could not detect any significant differences between strains PL11-7 and 18D which contains five extra copies of the *nim*A genomic fragment under the control of the same *alc*A promoter (Osmani *et al.*, 1988). These results indicate that the cells were arrested in mitosis. Quantification of DNA synthesis, measured by [^{3}H]adenine incorporation, revealed that cells of strain PL11-7 failed to undergo a round of DNA synthesis when grown on inducing medium (Figure 4, solid squares), indicating that these cells entered mitosis prematurely. These results are entirely consistent with the previous observations of Osmani *et al.* (1988).

To determine the terminal phenotype resulting from expression of the kinase-negative mutant NIMA, spores containing two or seven copies of the K40M *nim*A (PL12-2 and -7) or four copies of the antisense *nim*A (PL13-4) were

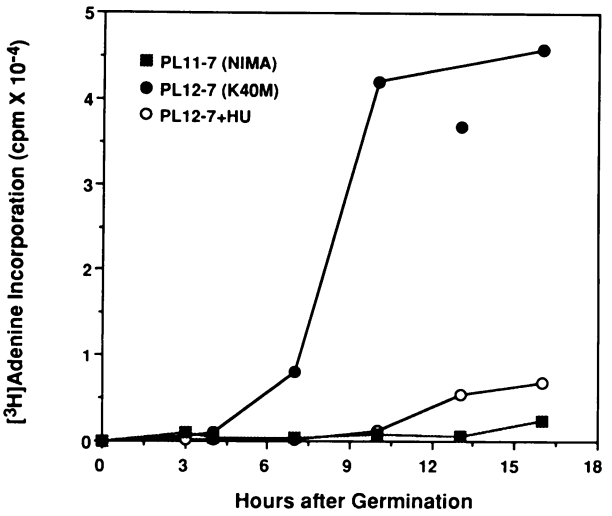

Fig. 4. Effect of expressing wild type and kinase-negative mutant NIMAs on DNA synthesis. Conidia from PL11-7 and PL12-7 strains were germinated in inducing medium in the absence or presence (+HU) of 100 mM hydroxyurea at 32°C. At various times samples were taken and incorporation of [^{3}H]adenine was determined as described previously (Lu and Means, 1993b).

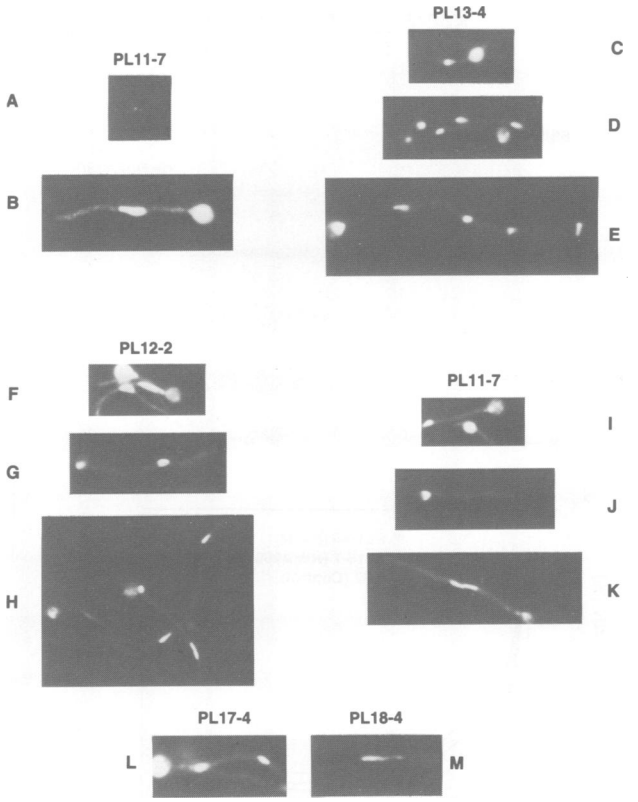

Fig. 5. Effect of expressing various mutant NIMAs on progression through the nuclear division cycle. Spores from various pure strains were germinated in inducing medium at the permissive temperature for various times except in B where spores were germinated in repressing medium at the restrictive temperature. Cells were fixed and stained with DAPI for assessing cell growth. **A**, PL11-7, 14 h; **B**, PL11-7, 10 h; **C**, PL13-4, 12 h; **D**, PL13-4, 14 h; **E**, PL13-4, 16 h; **F**, PL12-2, 12 h; **G**, PL12-2, 14 h; **H**, PL12-2, 16 h; **I**, PL12-7, 12 h; **J**, PL12-7, 14 h; **K**, PL12-7, 16 h; **L**, PL17-4, 14 h; **M**, PL18-4, 14 h. All photographs are 400× magnification.

germinated on inducing medium at the permissive temperature for various times. All strains formed germ tubes and grew normally for at least 14−16 h, except PL12-7 which exhibited slower growth after 14 h (Figure 5C−K). In contrast, there were dramatic differences in nuclear division cycle progression. While the nuclei in the PL13-4 strain divided normally (Figure 5C−E), the nuclear division cycle was significantly slowed in PL12-2 (Figure 5F−H) and completely inhibited in the PL12-7 strain in which cells contained a single nucleus even after 20 h of incubation (Figure 5I−K and data not shown). The nuclei of these division-arrested cells were larger than those present in conidia of the other strains and contained clearly identifiable nucleoli. These nuclear morphologies were indistinguishable from those of cells arrested in G_2 by incubating *nim*A5-containing spores at the restrictive temperature (Figure 5B), indicating that these cells were probably arrested in G_2. Unlike the temperature-sensitive G_2 arrest which can be rapidly reversed upon return to the permissive temperature, it was difficult to reverse the arrest induced by overexpressing the kinase-negative mutant *nim*A by shifting to repressing medium (data not shown). To confirm that these cells were blocked in G_2, rather than in S phase, [^{3}H]adenine was added at the beginning of germination and incorporation into DNA was measured as a function of time. [^{3}H]adenine incorporation began at 7 h and reached a plateau by 10 h of germination (Figure 4, solid circles). The rate of [^{3}H]adenine incorporation in PL12-7 was similar to that in the PL13-4 strain up to 13 h. However, the latter strain underwent a second round of [^{3}H]adenine incorporation at later times since it contained four copies of K40M *nim*A in the antisense orientation and could continue to grow in inducing medium (Figure 2). The DNA synthesis inhibitor hydroxyurea completely inhibited [^{3}H]adenine incorporation in the PL12-7 strain (Figure 4, open circles). Since cells should have finished the first round of DNA synthesis by 12 h, these results indicate that expression of K40M NIMA did not affect synthesis of DNA.

We also examined the effect of expressing K40M on the nuclear division cycle in cycling cells. Spores containing FLAG-K40M (PL15-6) were germinated in repressing medium for 12 h and shifted to inducing medium for various times. Cells were harvested and examined for K40M expression and nuclear division status. K40M began to be expressed by 0.5 h, reached the maximal level by 3 h and remained at a high level for at least 9 h (Figure 6A). The mitotic index of these cells was ~4% in repressing medium and decreased after 3 h in inducing medium. By 9 h, growth was arrested and >90% of cells contained nuclei which were enlarged and had clearly identifiable nucleoli, characteristic of G_2-arrested nuclei. Collectively, these results suggest that the kinase-negative NIMA acted in a dominant-negative manner which resulted in the arrest of cells in G_2.

The noncatalytic domain of NIMA is both necessary and sufficient for the dominant-negative phenotype

In addition to the kinase domain located in the NH_2-terminal portion of NIMA, the protein contains a long noncatalytic COOH-terminal domain (>350 amino acids). To determine which domain of the kinase-negative NIMA molecule was responsible for the dominant-negative phenotype, we examined the effects of expressing each separate domain on cell growth. One construct would express

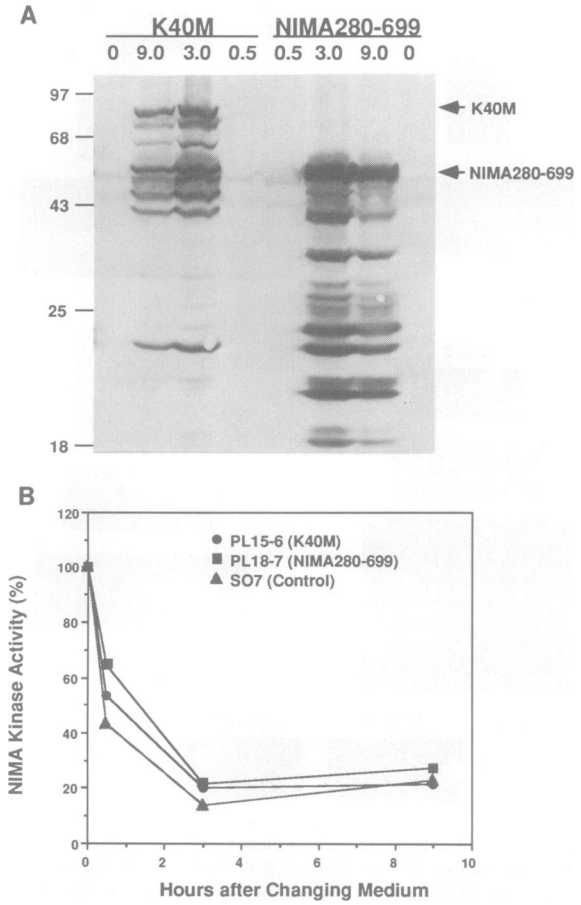

Fig. 6. Effect of expressing mutant NIMAs on the endogenous NIMA kinase activity. (**A**) The time course of expression. Conidia from FLAG-K40M- (PL15-6), and FLAG-NIMA280−699 (PL18-7)-containing strains were germinated in repressing medium for 12 h (0). Cells were washed free of this medium and inducing medium was then added for various times as indicated. Cells were harvested and soluble proteins were subjected to Western analysis using the M2 antibody. (**B**) Effect on kinase activity. Conidia from FLAG-K40M- (PL15-6), and FLAG-NIMA280−699 (PL18-7)-containing strains as well as SO7 control strain were germinated in repressing medium for 12 h (0). After being washed free of repressing medium, cells were incubated in inducing medium for various times as indicated. Cells were harvested and the endogenous NIMA was immunoprecipitated with an excess of NIMA-specific peptide antibody, followed by a kinase assay using the PLM(58−72) peptide as a substrate, as described in Materials and methods.

the NH_2-terminal 344 amino acids (1−344 with the K40M mutation, K40M344) and the other would produce the COOH-terminal 420 amino acids (NIMA280−699). In addition, both constructs were engineered to contain the FLAG tag at the NH_2-terminus, then ligated into the pAL5 vector and used to transform the SO6 strain, as described before. To determine expression of the transgenes, pure strains were obtained from several FLAG tag-containing transformants and the copy number of the transgenes integrated was determined by Southern analysis of genomic DNA as described previously (data not shown). When spores from the pure strains containing four copies of either K40M344 (PL17-4) or NIMA280−699 (PL18-4) were germinated on inducing medium, there were similar amounts of proteins with the expected M_r detected by the M2 antibody, which were not detectable when grown on repressing medium (Figure 3A). The Western blot indicates that the kinase domain seemed to be more stable than the noncatalytic domain, raising the possibility that the considerable proteolysis observed in the full-length proteins might occur in the noncatalytic region. These results reveal that both NH_2- and COOH-terminally truncated NIMAs were expressed in an *alc*A-inducible manner.

To determine the effects of expressing the truncated NIMAs on cell growth, at least 20 transformants from each transformation were examined for their ability to grow on media containing different carbon sources at the permissive or restrictive temperature (Figure 7). At the restrictive temperature, none of the transformants were able to grow on basal, repressing or inducing medium, indicating that neither portion of NIMA could rescue the temperature-sensitive mutation of the *nim*A gene, as expected, since neither could the full-length inactive molecule. At the permissive temperature, K40M344 transformants were able to grow on all three media. In contrast, the growth of NIMA280−699 transformants was significantly or completely inhibited on basal or inducing medium, respectively, although they grew normally on repressing medium. To determine whether these growth inhibitory effects were copy number dependent, pure strains containing either two, four, seven or 14 copies of NIMA280−699 were obtained and grown on different media (Figure 8). Although all strains grew similarly on repressing medium, inhibition was copy number dependent on basal medium. As shown in the middle panel of Figure 8, they grew differently on basal or inducing medium, especially on basal medium. Two copies of NIMA280−699 allowed cells to grow reasonably well, four copies resulted in nearly complete suppression and more than four copies completely inhibited growth. In contrast, none of the strains could grow in inducing medium. These results show that expression of the COOH-, but not NH_2-terminal domain of NIMA inhibited cell growth with a similar potency to the full-length kinase-negative molecule.

To determine the terminal phenotype resulting from expression of the noncatalytic domain of NIMA, spores were germinated on inducing medium at the permissive temperature for various times, followed by quantification of nuclear number and examination of nuclear morphology. Spores (PL17-4) containing four copies of K40M344 formed germ tubes and underwent the normal nuclear division cycle (Figure 5L), containing more than eight nuclei (the detection limit) after 20 h of incubation under the conditions used. However, >95% of spores (PL18-4) containing four copies of FLAG-NIMA280−699 were unable to undergo nuclear division, although they were able to form germ tubes normally (Figure 5M). Even after 20 h of incubation there was only a single condensed nucleus per cell which contained a prominent nucleolus (Figure 5M). Similar nuclear morphologies were obtained when NIMA280−699 was induced to express in exponentially growing cells (data not shown) and were the same as those observed due to expression of the full-length kinase-negative NIMA (K40M). Thus the noncatalytic domain of the NIMA molecule was both necessary and sufficient to act as a dominant-negative repressor and arrest cells in G_2.

Effect of expressing inactive NIMAs on endogenous NIMA kinase and histone H1 kinase activities

It seemed possible that the G_2 arrest produced by expression of either full-length kinase-negative NIMA or the noncatalytic domain of NIMA was due to inactivation of

nimA Constructs	Cell Growth					
	32°C			42°C		
	Repressed for *alcA*	Derepressed for *alcA*	Induced for *alcA*	Repressed for *alcA*	Derepressed for *alcA*	Induced for *alcA*
NIMA (1 Kinase Domain 699)	+++	+	-	-	+++	+
FLAG-NIMA	+++	+	-	-	+++	+
K40M (K40M)	+++	+	-	-	-	-
FLAG-K40M (K40M)	+++	+	-	-	-	-
K40M344 (K40M, 344)	+++	+++	+++	-	-	-
FLAG-K40M344 (K40M)	+++	+++	+++	-	-	-
FLAG-NIMA280-699 (280)	+++	+	-	-	-	-
NIMA-AS ("699" (antisense) "1", K40M)	+++	+++	+++	-	-	-

Fig. 7. Effect of expressing truncated NIMAs on cell growth. Different *nim*A cDNAs (depicted schematically on the left; thick black bar represents the FLAG epitope tag) were subcloned into the pAL5 expression vector and transformed into the SO6 strain. The resulting transformants were transferred onto repressing, derepressing or inducing plates and incubated at 32°C or 42°C for 3 days. Cell growth was categorized as normal (+++), partially inhibited (+) or completely inhibited (−).

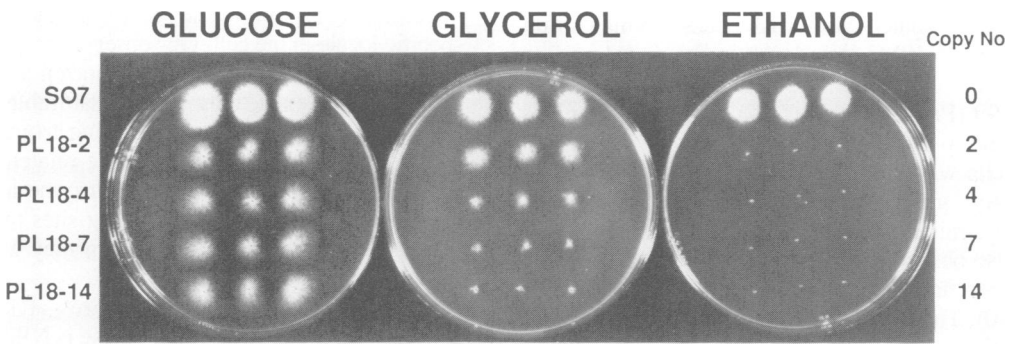

Fig. 8. Effect of copy number of the noncatalytic NIMA on cell growth. Conidia from pure strains containing zero, two, four, seven or 14 copies of FLAG-NIMA280−699 were transferred onto repressing, derepressing or inducing plates and incubated for 3 days at 32°C.

endogenous NIMA. To examine this possibility, we first determined the effect of the noncatalytic domain of NIMA on NIMA kinase activity *in vitro*. NIMA280−699 was expressed in bacteria as a glutathione S-transferase fusion protein and purified on a glutathione affinity column as described previously (Lu *et al.*, 1993a). Different amounts of the purified NIMA280−699 were incubated with the purified wild type NIMA kinase for 5 min, followed by determination of NIMA kinase activity. As shown in Figure 9, the noncatalytic protein had no inhibitory effect on NIMA kinase activity even at 500-fold excess. The small but reproducible increase in kinase activity might be due to stabilization of the enzyme by the noncatalytic NIMA domain. We further asked if expression of the full-length or noncatalytic NIMA affected endogenous NIMA kinase activity *in vivo* (Figure 6B). Following growth of the strains (PL15-6 and PL18-7) containing FLAG-K40M or FLAG-NIMA280−699 in repressing medium for 12 h, the medium was replaced with inducing medium for various times and cells were harvested to measure expressed protein levels by Western analysis using M2 monclonal antibody and to assay NIMA kinase activity after immunoprecipitation using NIMA-specific peptide antibodies. K40M and NIMA280−699 proteins were detectable within 0.5 h after induction, reached maximal levels by 3 h and remained at high levels for at least 9 h (Figure 6A). Upon the change to inducing medium, endogenous NIMA kinase activity

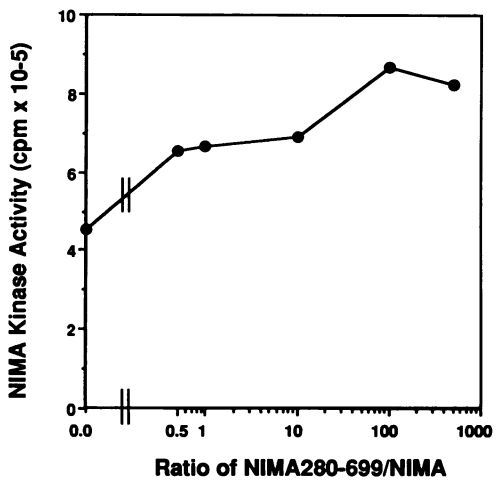

Fig. 9. Effect of the noncatalytic NIMAs on NIMA kinase activity. NIMA280−699 was expressed in bacteria as a glutathione S-transferase fusion protein and purified as described previously (Lu *et al.*, 1993a). Different amounts of the purified noncatalytic domain were incubated with the purified wild type NIMA kinase for 5 min at 37°C at the ratios indicated, followed by assaying NIMA kinase activity using the PLM(58−72) peptide as a substrate.

decreased within the first 0.5 h and remained constant afterwards (Figure 6B). Since the decrease in NIMA kinase activity occurred prior to the time the exogenous NIMA

began to be expressed and since a similar decrease in NIMA kinase activity was observed in both the non-transformed *nim*A5 strain (SO7) (Figure 6B) and strain (PL13-4) containing K40M in the antisense orientation (data not shown), we surmise that the decrease in NIMA kinase activity in all strains was due to the change of medium from glucose to ethanol. Collectively, these results showed that neither the kinase-negative full-length NIMA nor the noncatalytic NIMA domain inhibited the kinase activity of endogenous NIMA.

It has been shown that whereas $p34^{cdc2}$ kinase activity is also required for cells to enter mitosis in *A.nidulans*, when *nim*A5-containing cells were arrested in G_2 at the restrictive temperature, this enzyme was fully active as determined by analysis of p13-associated H1 kinase and was ~3-fold higher than the activity present in asynchronous cells (Osmani *et al.*, 1991a). Therefore, we questioned whether expression of the inactive NIMAs inhibited activation of $p34^{cdc2}$. Spores containing different mutant NIMA constructs were germinated in inducing medium for 12 h and cells were harvested to measure both mitotic index and histone H1 kinase activity associated with p13 beads. While strain PL17-4 containing K40M344 underwent the normal nuclear division cycle, cells containing either K40M or NIMA280–699 (PL15-6 and PL18-7) were arrested in G_2 (Figure 5). The p13-precipitable H1 kinase activity in G_2-arrested cells was ~3-fold higher than that present in asynchronously growing K40M344-containing cells (Figure 10). A similar increase in p13-associated H1 kinase activity was also observed in cells that were induced for 9 h after having been first germinated in repressing medium for 12 h (Figure 10). The levels of the H1 kinase activity in these G_2-arrested cells were similar to those present in mitotic cells (Osmani *et al.*, 1991a and data not shown). Therefore we conclude that expression of neither the full-length kinase-negative NIMA, nor the noncatalytic domain of NIMA affected activation of the p13-associated H1 kinase, although in both cases cells were arrested in G_2.

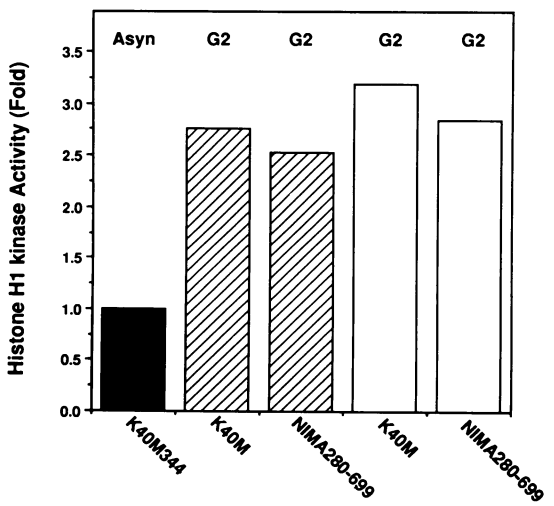

Fig. 10. Effect of expressing mutant NIMAs on histone H1 kinase activity. Conidia from FLAG-K40M344-, FLAG-K40M- and FLAG-NIMA280–699-containing strains were germinated directly in inducing medium for 12 h (left three bars) or first in repressing medium for 12 h and then in inducing medium for 9 h. Cells were harvested and ground in HK buffer. The p13-binding proteins were precipitated with p13 beads and assayed for H1 kinase activity.

Discussion

Overexpression of the NIMA protein Ser/Thr kinase in *A.nidulans* results in premature mitosis from which the cells cannot recover (Osmani *et al.*, 1988). This observation suggests that the action of NIMA is a dominant signal for entry into mitosis but that either degradation of the enzyme and/or dephosphorylation of its putative substrates must be required for cells to exit mitosis. On the other hand, conditional mutations in the *nim*A gene cause a specific cell cycle block in G_2 at the restrictive temperature that is readily reversible upon return to permissive temperature (Bergen *et al.*, 1984; Osmani *et al.*, 1987). Our data reveal that overexpression of a kinase minus mutant of NIMA also results in a specific G_2 arrest. The finding that overexpression of the noncatalytic COOH-terminal fragment but not the kinase containing NH_2-terminal fragment of NIMA recapitulates the G_2 arrest produced by the full-length kinase-minus protein argues that this phenotype must be due to some role of NIMA that is not directly related to its enzyme activity. The most obvious such role of this noncatalytic region would be to target the enzyme to a specific locale in the cell. Our observation that the dominant-negative effect is copy number dependent implies that if such targeting sites exist, they may be saturable. If this is true, then the mitotic catastrophe resulting from overexpression of wild type NIMA might be more related to the aberrant timing of the appearance of the enzyme and unscheduled occupancy of the putative targeting sites than to an excess of enzymatically active molecules if they were produced at the appropriate time in G_2.

Transition from G_2 to M requires at least two kinase activities in *A.nidulans*. One of these is NIMA and the other resembles the $p34^{cdc2}$ kinase component of MPF. Studies have shown that neither an active NIMA nor an active $p34^{cdc2}$ kinase is sufficient to allow cells to enter mitosis in the absence of an active form of the other (Osmani *et al.*, 1991a). There are remarkable similarities in the cell cycle dependent regulation of these two critical kinases to ensure maximum activity in G_2 but the mechanisms used to express activity at the appropriate time and place vary considerably. The novel finding of the present study is that the COOH-terminal noncatalytic fragment of NIMA is both necessary and sufficient to produce specific arrest in G_2. Localization of the $p34^{cdc2}$-related protein kinases may also be important for regulation of the G_2/M transition. Whereas mitotic cyclins serve as nonenzymatic regulatory subunits for $p34^{cdc2}$, proteins of the Cks family, of which the *suc*1 gene product is a prime example, apparently serve to target or dock the $p34^{cdc2}$–cyclin complex. Genetically it had been shown that *suc*1 is a suppressor of temperature-sensitive *cdc2* mutations yet the sequence of the Suc1 protein did not suggest any enzymatic function (Hayles *et al.*, 1986; Hindley *et al.*, 1987; Hadwiger *et al.*, 1989). Parge *et al.* (1993) have now determined the three-dimensional structure of *suc*1 protein (called Cks Hs2) and shown it to be a hexamer of three interlocked dimers. These authors suggest that each assembled hexamer could bind six kinase subunits and thus appropriately position the enzyme for its participation in cell cycle control. A second similarity is that the activity of NIMA and $p34^{cdc2}$ are both maximal at G_2/M. NIMA is not present in spores and upon germination only becomes detectable at about the time of the first nuclear division

(Osmani *et al.*, 1987). A precipitous decrease of both NIMA mRNA and protein occurs as cells exit mitosis and is required for this process. These changes in gene expression and protein level are similar to those that govern the concentration of mitotic cyclins which are required to activate $p34^{cdc2}$ that is present in similar amounts throughout the cell cycle (Murray and Kirschner, 1989; Nurse, 1990). In addition to the regulatory mechanisms that ensure that NIMA is present at the appropriate time and place to exercise its role in mitotic progression, the activity of this kinase is regulated post-translationally. We have shown that NIMA activity at G_2 requires the presence of Ca^{2+} and calmodulin (Lu *et al.*, 1993b). In addition, NIMA is phosphorylated on multiple Ser/Thr residues either by itself or by yet to be defined protein kinases and this phosphorylation is required for activity (Lu *et al.*, 1993a). Similarly, $p34^{cdc2}$ is regulated by phosphorylation/dephosphorylation of specific Tyr and Thr residues even when bound to mitotic cyclins. Therefore, whereas the regulatory mechanisms that govern the abundance, localization and activity of NIMA and $p34^{cdc2}$ during the cell cycle achieve the same end, they are largely intermolecular in the $p34^{cdc2}$ pathway but intramolecular for NIMA. The reagents and strains generated in the present study should considerably enhance the quest to determine physiologically relevant NIMA interacting proteins.

Targeting of enzymes by proteins that do not themselves possess enzymatic activity is a recurring theme in signal transduction cascades. Most frequently these interactions are intermolecular and involve the association of a nonenzymatic regulatory subunit of an enzyme or enzyme complex with a targeting molecule. For example a series of anchoring proteins have recently been characterized that serve to target the cAMP dependent protein kinase holoenzyme to specific intracellular organelles (Scott and McCartney, 1994). The anchoring proteins appear to be integral components of the organelle and specifically bind to the noncatalytic RII regulatory subunit of the holoenzyme. Cyclic AMP binding to the anchor-associated regulatory subunit allows release of the catalytic subunit in an enzymatically active form. A second example of targeting subunits is those known to be associated with protein phosphatase 1. These subunits are noncatalytic but appear to serve the dual purpose of localization and regulation of enzyme substrate specificity (Hubbard and Cohen, 1993). In the case of some such subunits, post-translational modifications have also been suggested to influence the activity of their associated catalytic partners. Examples of targeting proteins that directly impinge upon signalling pathways influencing mitogenesis are the src homology 3 (SH3) domain containing proteins. The SH3 domains are found in many critical intracellular signalling molecules and serve to mediate specific protein–protein interactions (Musacchio *et al.*, 1992). The growth factor-receptor bound protein (GRB-2) via its SH2 domain recognizes tyrosine phosphorylated domains in a variety of proteins, and utilizes its SH3 domains to link other proteins such as SOS to the signalling complex (Lowenstein *et al.*, 1992). Analyses of such interactions have led to the discovery of a variety of upstream regulators of Ras and have revealed that GRB-2 localization to cellular organelles is independent of its interaction with tyrosine phosphorylated molecules (Bar-Sagi *et al.*, 1993). Similar approaches designed to identify downstream targets of Ras resulted in the demonstration that an NH_2-terminal fragment of Raf kinase served such a role (Vojtek *et al.*, 1993). However, *ras* mutants that cannot bind GTP do not bind Raf but do result in a dominant-negative response when introduced in cells presumably by competitively binding upstream regulatory molecules such as the Son-of-sevenless protein (Baltensperger *et al.*, 1993; Skolnik *et al.*, 1993). Finally the proper subcellular localization of the *Drosophila* Abl protein is required for its role in axonal pathfinding, but is independent of its tyrosine kinase activity (Henkemeyer *et al.*, 1990). Yet, like our findings for NIMA, both functions reside in discrete regions of the same polypeptide.

Table I. *Aspergillus nidulans* strains used in this study

Strain	Genotype	Source
SO51	*wA2*	S.A.Osmani[a]
SO6	*nim*A5; *wA2*; *pyr*G89; *cnx*E16; *cho*A1; *yA2*; *cha*A1	Osmani *et al.* (1987)[a]
SO7	*nim*A5; *wA2*	Osmani *et al.* (1987)[a]
18D	five copies of *alc*A::*nim*A; *fw*A1; *pyr*G89; *ben*A22; *paba*A1	Osmani *et al.*, (1988)[a]
GR5	A773; *wA2*; *pyr*G89; *pyro*A4	Rasmussen *et al.* (1990)
PL11-2	two copies of *alc*A::*nim*A, *nim*A5; *wA2*; *pyr*G89; *cnx*E16; *cho*A1; *yA2*; *cha*A1	this study[b]
PL11-4	four copies of *alc*A::*nim*A, *nim*A5; *wA2*; *pyr*G89; *cnx*E16; *cho*A1; *yA2*; *cha*A1	this study[b]
PL11-7	seven copies of *alc*A::*nim*A, *nim*A5; *wA2*; *pyr*G89; *cnx*E16; *cho*A1; *yA2*; *cha*A1	this study[b]
PL12-2	two copies of *alc*A::K40M *nim*A, *nim*A5; *wA2*; *pyr*G89; *cnx*E16; *cho*A1; *yA2*; *cha*A1	this study[b]
PL12-4	four copies of *alc*A::K40M *nim*A, *nim*A5; *wA2*; *pyr*G89; *cnx*E16; *cho*A1; *yA2*; *cha*A1	this study[b]
PL12-7	seven copies of *alc*A::K40M *nim*A, *nim*A5; *wA2*; *pyr*G89; *cnx*E16; *cho*A1; *yA2*; *cha*A1	this study[b]
PL13-4	four copies of *alc*A::AS K40M *nim*A, *nim*A5; *wA2*; *pyr*G89; *cnx*E16; *cho*A1; *yA2*; *cha*A1	this study[b]
PL14-7	seven copies of *alc*A::FLAG-*nim*A, *nim*A5; *wA2*; *pyr*G89; *cnx*E16; *cho*A1; *yA2*; *cha*A1	this study[b]
PL15-6	six copies of *alc*A::FLAG-K40M *nim*A, *nim*A5; *wA2*; *pyr*G89; *cnx*E16; *cho*A1; *yA2*; *cha*A1	this study[b]
PL17-4	four copies of *alc*A::FLAG-K40M344, *nim*A5; *wA2*; *pyr*G89; *cnx*E16; *cho*A1; *yA2*; *cha*A1	this study[b]
PL18-2	two copies of *alc*A::FLAG-*nim*A280–699, *nim*A5; *wA2*; *pyr*G89; *cnx*E16; *cho*A1; *yA2*; *cha*A1	this study[b]
PL18-4	four copies of *alc*A::FLAG-*nim*A280–699, *nim*A5; *wA2*; *pyr*G89; *cnx*E16; *cho*A1; *yA2*; *cha*A1	this study[b]
PL18-7	seven copies of *alc*A::FLAG-*nim*A280–699, *nim*A5; *wA2*; *pyr*G89; *cnx*E16; *cho*A1; *yA2*; *cha*A1	this study[b]
PL18-14	14 copies of *alc*A::FLAG-*nim*A280–699, *nim*A5; *wA2*; *pyr*G89; *cnx*E16; *cho*A1; *yA2*; *cha*A1	this study[b]

[a]Kindly provided by Stephen A.Osmani
[b]Obtained by transformation of SO6

Our results indicate that downstream molecules in the NIMA pathway will include not only substrates but targeting molecules as well.

Materials and methods

Bacteria and plasmids

Bacterial strains and procedures for subcloning were as described previously (Lu *et al.*, 1992, 1993b). To construct the wild type and kinase-negative NIMA expression vectors, pGEX-A containing the wild type cDNA and pGEX-A(−) containing the mutant in which Lys40 had been changed to Met (K40M) (Lu *et al.*, 1993b) were cut with *Nco*I and *Hin*cII and blunt-ended using the Klenow fragment of DNA polymerase. The blunt-ended DNA fragment was isolated from low melting point agarose and ligated into the *Sma*I site of the pAL5 vector (Doonan *et al.*, 1991) such that either the 5′ ends of the *nim*A cDNAs were adjacent to the *alc*A promoter sequences, resulting in pAL-A and pAL-K40M, respectively, or the 3′ ends of the *nim*A cDNAs were adjacent to the *alc*A promoter sequences, resulting in antisense constructs, pAL-A-R and pAL-K40M-R. To insert the FLAG epitope tag at the NH_2-terminal end of the various NIMAs, a double-stranded oligomer, CTAGCCACAATGGACTACAAAGACGATGACG-ATAAAGCCATGG, which encodes Met-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-Ala (Hopp *et al.*, 1988), was first ligated into the *Sac*II and *Eco*RI sites of an intermediate vector, pUHD10-3 (kindly provided by Prof. Dr Hermann Bujard, Zentrum für Molekulare Biologie der Universität Heidelberg, Germany), resulting in the pUHD-P1 plasmid, followed by cutting this intermediate plasmid with *Eco*RI and blunt-ending it, followed by digestion with *Nco*I. The wild type and K40M *nim*A cDNAs were subcloned into pUHD-P1 as an *Nco*I−*Hin*cII fragment, resulting in NIMA/pUHD-P1 and K40M/pUHD-P1, respectively. Finally, NIMA/pUHD-P1 and K40M/pUHD-P1 were digested with *Nhe*I and *Xba*I, blunt-ended and ligated into the *Sma*I site of the pAL5 vector, resulting in pAL-A-P1 and pAL-K40M-P1, respectively. To generate the COOH-terminal truncation NIMA mutant, pGEX-A(−) was digested with *Nar*I and *Nae*I, blunt-ended and self-ligated, resulting in a mutant *nim*A encoding the NH_2-terminal 344 amino acids of NIMA (NIMA344) plus two unrelated residues. The FLAG epitope tag was inserted at the NH_2-terminal end as described above. To generate the NH_2-terminal truncation, NIMA/pUHD-P1 was digested with *Nco*I and *Bsp*EI, blunt-ended and self-ligated, resulting in a mutant *nim*A encoding the NH_2-terminal 279 amino acids of NIMA (NIMA280−699). These truncation mutant *nim*A cDNAs were also subcloned into the pAL5 vector. All constructs were sequenced to confirm that the open reading frame had not been disrupted.

Aspergillus nidulans strains and general techniques

Strains and their genotypes used in this study are listed in Table I. New strains (from PL11 to PL18) were created from SO6 by transformation with various plasmids containing different *nim*A cDNAs constructed in the pAL5 vector, as described previously (Lu and Means, 1993b). Growth, cell cycle analyses and fluorescence microscopy of *A.nidulans* were as described previously (Lu *et al.*, 1992, 1993b).

Southern analyses

Genomic DNA was isolated as described previously (Lu and Means, 1993b). Two to five micrograms of genomic DNA were cut with *Xho*I overnight and then subjected to Southern blot analysis. Filters were probed with a ^{32}P-labeled *Nco*I−*Xho*I fragment of *A.nidulans nim*A cDNA. After washing, the filters were scanned by a Betascope 603 Blot Analyzer (Betagene, Waltham, MA) to quantify transgene copy number using the single copy endogenous *nim*A gene as a standard.

NIMA protein kinase and histone H1 kinase assays

To assay the endogenous NIMA kinase activity, cells were ground in EB buffer and NIMA was immunoprecipitated with the ANYRED-NIMA specific antibodies (Osmani *et al.*, 1991b; Lu *et al.*, 1993b), followed by a peptide kinase assay in which we used a newly characterized synthetic peptide, PLM(58−72) (GTFRSSIRRLSTRRR), instead of β-casein as a substrate (Lu *et al.*, 1994). Since NIMA phosphorylated this peptide with a V_{max} at least 10-fold higher than β-casein, we required ~10-fold less total cell protein for immunoprecipitation than amounts required previously (Osmani *et al.*, 1991b; Lu *et al.*, 1993b). For assaying the kinase activity of various expressed NIMAs, FLAG-tagged NIMAs were immunoprecipitated with the M2 monoclonal antibody against the FLAG epitope (Kodak International Biotechnologies, Inc.) and the kinase activity was assayed as described above. The H1 kinase was assayed in p13 precipitates using histone H1 as a substrate, as described (Lu *et al.*, 1993b).

Immunoblot analysis

For detecting expressed NIMAs, 200 μg of soluble cell extracts were directly solubilized in SDS buffer. The proteins were separated on 10% acrylamide gels in the presence of SDS and transferred to Immobilon-P membranes. The filters were blocked for 1 h in Tris-buffered saline (TBS), pH 7.4, containing 5% bovine albumin. Subsequently, filters were incubated for 2 h at room temperature with the monoclonal M-2 antibody using the dilution recommended by the manufacturer (Kodak), followed by five washes with TBS:Tween 20 (0.05%). The filters were incubated with alkaline phosphatase-conjugated secondary antibodies (Promega) and binding of the secondary antibodies was visualized as described previously (Lu *et al.*, 1993b).

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