The EMBO Journal vol.10 no.9 pp.2669 - 2679, 1991

Activation of the *nim*A protein kinase plays a unique role during mitosis that cannot be bypassed by absence of the *bim*E checkpoint

Aysha H.Osmani, Kerry O'Donnell[1], Robert T.Pu and Stephen A.Osmani

Department of Cell Biology, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030 and [1]National Center for Agricultural Utilization Research, Agricultural Research Service, USDA, 1815 N.University, Peoria, IL 61604, USA

Communicated by V.Pirrotta

Mutation of *nim*A reversibly arrests cells in late G_2 and *nim*A overexpression promotes premature mitosis. Here we demonstrate that the product of *nim*A (designated NIMA) has protein kinase activity that can phosphorylate β-casein but not histone proteins. NIMA kinase activity is cell cycle regulated being 20-fold higher at mitosis when compared to S-phase arrested cells. NIMA activation is normally required in G_2 to initiate chromosome condensation, to nucleate spindle pole body microtubules, and to allow an MPM-2 specific mitotic phosphorylation. All three of these mitotic events can occur in the absence of activated NIMA when the *bim*E gene is mutated (*bim*E7). However, the *bim*E7 mutation cannot completely bypass the requirement for *nim*A during mitosis as entry into mitosis in the absence of NIMA activation results in major mitotic defects that affect both the organization of the nuclear envelope and mitotic spindle. Thus, although *nim*A plays an essential but limited role during mitosis, mutation of *nim*A arrests all of mitosis. We therefore propose that mutation of *nim*A prevents mitotic initiation due to a checkpoint arrest that is negatively mediated by *bim*E. The checkpoint ensures that mitosis is not initiated until NIMA is mitotically activated.

Key words: Aspergillus nidulans/cell cycle regulation/checkpoints/*nim*A and *bim*E/protein kinase

Introduction

We are using the filamentous fungus *Aspergillus nidulans* as a model system to help elucidate the regulation of mitosis. Numerous conditional mutations in cell cycle specific genes have been isolated (Morris, 1976). Some of these mutations arrest in interphase at the restrictive temperature (*nim* mutants = *n*ever *i*n *m*itosis) and some arrest in mitosis (*bim* mutants = *b*locked *i*n *m*itosis) (Morris, 1976). The *nim* and *bim* mutants have been used to clone several cell cycle specific genes (Osmani *et al.*, 1987; Doonan and Morris, 1989; Enos and Morris, 1989; Engle *et al.*, 1990) to help understand their role in cell cycle progression. These studies have helped to formulate the hypothesis that mitosis is regulated, at least in part, by a protein phosphorylation/dephosphorylation cascade (Murray and Kirschner, 1989; Nurse, 1990) as *nim*A encodes a potential protein kinase (Osmani *et al.*, 1988b) and *bim*G encodes a potential phosphoprotein phosphatase (Doonan and Morris, 1989). In addition, the *bim*E7 mutation has been used to establish that mitosis is under negative regulation during interphase in *A.nidulans* (Osmani *et al.*, 1988a).

During interphase mitotic protein phosphorylation must be held in check to prevent mitotic events from occurring prematurely. At mitosis the level of some protein phosphorylation is modulated during progression into and out of mitosis. These shifts in net protein phosphorylation could be achieved by modulation of the level of kinase activity, modulation of phosphatase activity, modulation of substrate availability or perhaps a combination of all three.

The *nim*A gene encodes a protein that contains the catalytic motifs typical of serine/threonine protein kinases (Osmani *et al.*, 1988b). Temperature sensitive mutations in *nim*A cause a G_2 arrest of the cell cycle at the restrictive temperature (Morris, 1976; Bergen *et al.*, 1984). Cells blocked in such a manner have uncondensed nuclear chromatin and a stable array of cytoplasmic microtubules. Returning *nim*A5 arrested cells to permissive temperature allows a rapid and synchronous entry into mitosis (Oakley and Morris, 1984). Thus, when mutated, the *nim*A gene product becomes limiting for mitotic initiation at a point late in G_2. The mRNA of *nim*A is under cell cycle specific regulation being highest at mitosis (Osmani *et al.*, 1987) and artificial induction of *nim*A has been shown to induce premature mitosis (Osmani *et al.*, 1988b). The putative protein kinase encoded by *nim*A could therefore be involved in the protein kinase cascade of mitotic initiation. NIMA could also play a role in shifting the balance of protein phosphorylation during G_2 if its activity were under appropriate cell cycle regulation. The *bim*E gene encodes a large protein (250 kDa) that contains several potential membrane spanning domains (Engle *et al.*, 1990).

By raising peptide-specific antibodies we have developed an assay for the *nim*A protein kinase (NIMA). We show that the activity of this kinase is indeed under cell cycle specific regulation in a manner indicating that NIMA is involved in changing the balance of protein phosphorylation during the G_2-M transition. The role of the activated NIMA protein kinase during mitosis has been investigated using the ability of the *bim*E7 mutation to bypass the requirement for NIMA in mitotic initiation. These studies reveal a limited role for the NIMA kinase during mitosis. If the NIMA kinase plays a limited role during mitosis why do mutations in this gene arrest cells in G_2? We discuss the existence of a checkpoint at G_2 that prevents initiation of mitosis until NIMA is fully activated. As the *bim*E7 mutation circumvents this checkpoint the *bim*E gene must encode a function that mediates the G_2 checkpoint.

Results

Synthesis of NIMA and antibody production

The protein kinase reading frame of *nim*A was placed into an expression vector for the production of mRNA *in vitro* which was then used to direct protein synthesis in an *in vitro*

transalation system (Figure 1A). The mRNA directed the synthesis of a protein (M_r 84 kDa) the size of which is in reasonable agreement with the theoretical size predicted (79 kDa) from the cDNA sequence of *nim*A (Osmani *et al.*, 1988b).

Polyclonal antibodies were raised against two peptides corresponding to parts of the predicted amino acid sequence of NIMA and tested for their ability to immunoprecipitate *in vitro* synthesized NIMA. Pre-immune sera of injected animals failed to precipitate NIMA whereas the different immune sera did precipitate this protein (Figure 1B). As serum raised against the ANYRED peptide (ANYRED serum) appeared to be more effective in these precipitation experiments it was used for the rest of this study. ANYRED serum was tested on Western blots against part of NIMA synthesized in *Escherichia coli* and proved positive against this overexpressed protein (Figure 1C). However, using *Aspergillus* protein extracts we were unable to detect NIMA by Western blot analysis even when *nim*A mRNA was overexpressed from a strong inducible promoter. The inability to detect NIMA in *Aspergillus* protein by Western blot is presumably due to its low abundance compared to that artificially expressed in bacteria (Figure 1C).

NIMA protein kinase activity

As NIMA was predicted to be a protein kinase (Osmani *et al.*, 1988b) ANYRED immunoprecipitates derived from *Aspergillus* extracts were assayed for potential protein kinase activity using partially dephosphorylated mixed casein as exogenous substrate. The precipitates contained a protein kinase activity that could phosphorylate this artificial substrate (Figure 2, lane 1). The specificity of the kinase activity in the immune precipitates was tested by pre-incubation of the antiserum with carrier buffer, Affigel coupled with ethanolamine or Affigel coupled to antigenic peptide (Figure 2, lanes 1, 2 and 3). Subsequently the three sera were used to precipitate proteins from an *Aspergillus* extract and kinase reactions run on the washed precipitates in the presence of mixed casein. Pre-clearing the immune serum with Affigel coupled to peptide removed the activity that is able to phosphorylate the smaller casein species (Figure 2, lane 3). NIMA kinase activity is therefore specific for the lower casein species (β-casein). The activity that phosphorylates the larger 26 kDa casein species (α-casein) is not affected by any of the preincubations and is therefore not specific to NIMA. By pre-clearing protein extracts with pre-immune sera and protein A−Sepharose this non-specific kinase can be dramatically reduced with little effect on the specific β-casein kinase activity. All protein extracts were subsequently pre-cleared in this manner prior to assaying NIMA.

Two additional immune-specific protein species are phosphorylated in the ANYRED immune precipitates. These are endogenous substrates, one of which may be NIMA. Future experiments will address this issue.

To establish further that we were detecting NIMA specific kinase activity in the ANYRED immune precipitates we followed this activity in extracts derived from a strain of *Aspergillus* that can inducibly express high levels of *nim*A mRNA. By transferring this strain (GR5-2, see Materials and methods) from media containing acetate to media containing ethanol it is possible to induce mRNA transcription from several extra copies of *nim*A that are integrated into the genome of GR5-2 and are under control of the *alc*A promoter of *Aspergillus* (Waring *et al.*, 1989). This induction has the effect of promoting spindle formation and chromosome condensation and leads to a mitotic block (Osmani *et al.*, 1988b).

Inducing *nim*A mRNA expression in strain GR5-2 caused a rapid and persistent increase in the level of kinase activity in ANYRED immune complexes toward β-casein (Figure 3). The increase in kinase activity correlated with induction of nuclear chromatin condensation. At 30 min of induction NIMA activity toward β-casein increased by 100-fold over

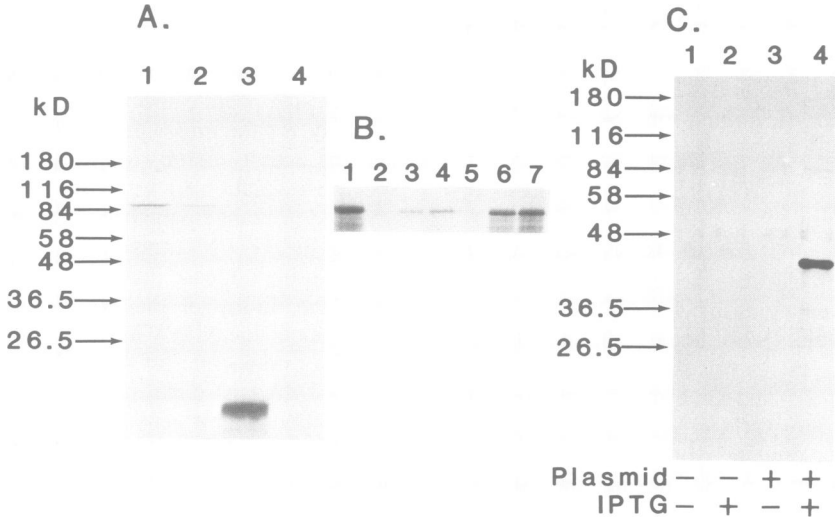

Fig. 1. Synthesis of NIMA and antibody analysis. **A.** A cDNA encoding NIMA was used to generate mRNA *in vitro* which was then translated in the presence of [^{35}S]methionine. *nim*A mRNA (lanes 1 and 2), control mRNA (Stratagene, encoding 16 kDa protein, lane 3), no added mRNA (lane 4). Synthesized protein was visualized by SDS−PAGE and autoradiography. **B.** NIMA made *in vitro* (lane 1) was precipitated with preimmune serum (lanes 2 and 5) or FASTY serum (lanes 3 and 4, different bleeds) or ANYRED serum (lanes 6 and 7, different bleeds). Protein was visualized by SDS−PAGE and autoradiography. **C.** A portion of NIMA cDNA was cloned into a T7 expression vector and transformed into *E.coli*. Expression from the plasmid is dependent on addition of IPTG. Bacterial extracts were separated by SDS−PAGE and Western blotted using ANYRED serum.

steady state levels and induced a significant increase in the chromosome mitotic index. Within 1 h of the induction the chromosome mitotic index increased to >90% and the level of kinase activity in the ANYRED immune complexes increased 180-fold (Figure 3). This level of kinase activity was maintained for several hours if cells were left in the inducing medium and the chromosome mitotic index remained elevated. No such dramatic increases in mitotic index or NIMA activity were observed in the control strain that was treated in an identical manner (Figure 3).

The β-casein kinase activity in the ANYRED precipitates derived from *nim*A-induced extracts was specifically removed by preincubation of the ANYRED serum with antigenic peptide coupled to Affigel (Figure 3), showing the induced kinase activity to be immune specific.

These results demonstrate that NIMA has protein kinase activity that can be assayed in immune precipitates using β-casein as substrate. Under the conditions employed for this study the assay is linear with respect to both protein concentration and time (data not shown).

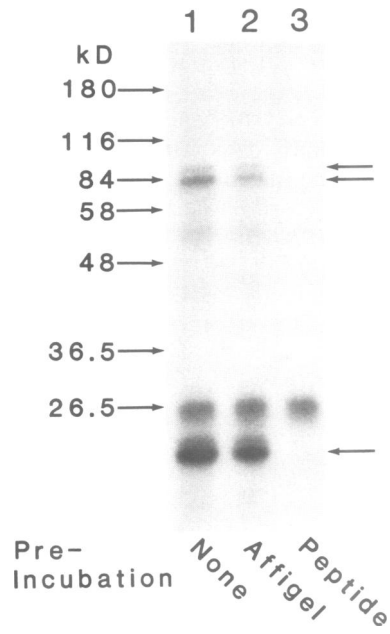

Fig. 2. Kinase activity of NIMA. Anti-peptide serum (ANYRED serum) immunoprecipitates from *Aspergillus* protein were used in a kinase reaction with mixed casein added as exogenous substrate. ANYRED serum were pretreated by incubation with Affigel or Affigel linked to ANYRED peptide as indicated. Phosphoproteins were visualized by SDS–PAGE and subsequent autoradiography. Arrows to the right indicate two endogenous phosphoproteins and a lower band corresponding to β-casein, all of which are immune specific.

Artificial substrates of NIMA protein kinase

In order to characterize the protein kinase activity of NIMA several commonly used artificial protein kinase substrates were tested for their ability to act as phosphate acceptor in NIMA-specific immune precipitates. The proteins tested included phosvitin, α-casein, β-casein, and histones H1, H2A, H2B, H3 and H4. Of the proteins tested β-casein was the only substrate that was readily phosphorylated by NIMA (data not shown). The H1 protein tested is readily phosphorylated by an H1 kinase activity isolated from *A.nidulans* (Osmani,A.H., McGuire,S.L.M. and Osmani,S.A., unpublished) demonstrating that the histone tested is a *bona fide* protein kinase substrate.

Cell cycle regulation of NIMA kinase activity

We have previously shown that the level of *nim*A mRNA fluctuates through the nuclear division cycle of *Aspergillus* indicating that perhaps the activity of NIMA is also regulated through the cell cycle (Osmani *et al.*, 1987). We tested this possibility directly using several different approaches to obtain synchronous cultures.

We first asked if the level of NIMA activity was responsive to cell cycle blocks, either in S-phase by using the specific DNA synthesis inhibitor hydroxyurea, or in M-phase, using the anti-microtubule drug benomyl. A culture of *Aspergillus* was grown to early log phase at which time the culture was

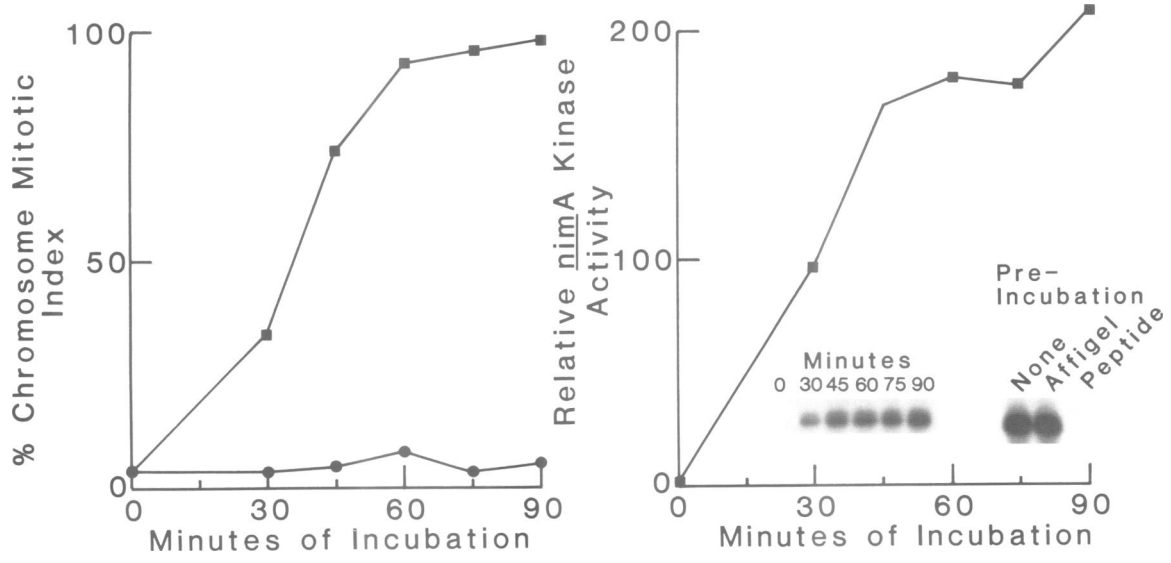

Fig. 3. Overexpression of NIMA. A strain of *Aspergillus* (GR5-2) containing multiple copies of *nim*A under the control of an inducible promoter (■) and a wild-type (●) were transferred to inducing media at time zero. Samples were monitored for chromosome condensation (left panel) and NIMA activity (right panel). Insert shows β-casein.

split into three. To one no addition was made, to the second benomyl was added, and to the third hydroxyurea was added. Samples taken at 15 min intervals were assayed for NIMA activity (see above and Materials and methods) and for the percentage of cells in mitosis. If cells were arrested at mitosis (addition of benomyl) they blocked with increased levels of NIMA activity. In contrast, if arrested during DNA replication (addition of hydroxyurea), cells showed a much reduced level of NIMA activity (Figure 4). Comparison of NIMA activity in mitotic cells to the activity of cells blocked in S-phase indicates a 20-fold variation in the kinase level through the cell cycle.

Conidia (asexual spores) of *Aspergillus* are naturally arrested in a dormant stage and upon germination enter the cell cycle at G_1 (Bergen and Morris, 1983). It is possible to generate a cell cycle synchrony in *Aspergillus* by germinating conidia in the presence of hydroxyurea and then releasing the hydroxyurea block. We monitored such a synchronous culture for nuclear division and NIMA activity (Figure 5). After release of the hydroxyurea block, cells progressed through S-phase (which takes 30–40 min, Bergen and Morris, 1983) during which time the NIMA kinase activity remained low. As G_2 was entered an increase in NIMA activity was detected that reached a peak (6-fold increase) prior to nuclear division. The appearance of the activity correlated with late G_2 and mitosis. As nuclei proceeded through division the level of NIMA activity dropped.

The cell cycle variation in NIMA activity observed following the pattern of accumulation of *nim*A mRNA through the nuclear division cycle of *Aspergillus* previously observed (Osmani *et al.*, 1987).

Relationship between bimE and NIMA kinase activity

Previous studies have demonstrated that loss of *bim*E function leads to the initiation of mitosis and an arrest of nuclei having condensed chromatin and a mitotic spindle (Osmani *et al.*, 1988a). To investigate the potential interaction between the negative mitotic regulator *bim*E and the positive element *nim*A we have determined the level of

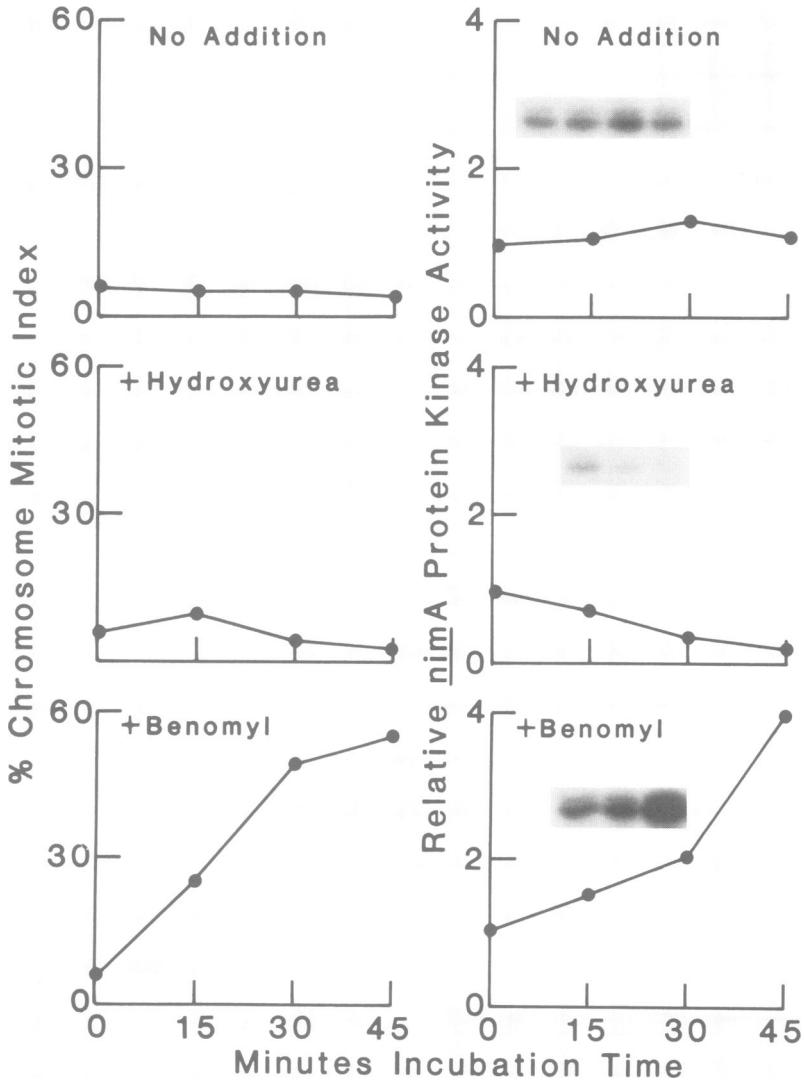

Fig. 4. Effects of cell cycle blocks on NIMA activity. A wild-type strain of *Aspergillus* was grown to early log phase (time zero). To a third of the culture no addition was made. To the remainder either benomyl or hydroxyurea was added. Samples were monitored for chromosome condensation (left panels) and NIMA activity (right). Insert shows phospho β-casein at time zero, 15, 30 and 45 min for no addition and 15, 30 and 45 min for the hydroxyurea and benomyl cultures.

NIMA kinase activity in a mitotically wild-type strain, strains containing the single *bim*E7 or *nim*A5 mutations and a double mutant *bim*E7 + *nim*A5 strain, when shifted from permissive to restrictive temperature (Figure 6). The wild-type control strain maintained a relatively constant mitotic index and had slightly elevated NIMA kinase activity after the shift to restrictive temperature which then returned to basal levels at 90 min. The *nim*A5 strain had a lower steady state level of NIMA kinase at the permissive temperature and this level rose during the temperature-induced G_2 arrest by a factor of 2.8 (Figure 6). If such G_2-blocked cells are returned to permissive temperature the cells enter mitosis and there is another doubling of NIMA kinase activity (data not shown). The *bim*E7 strain became blocked in mitosis and sustained an elevated level of kinase activity after the shift to the restrictive temperature. The *nim*A5 + *bim*E7 double mutant strain was able to enter a mitotic state in the absence of any increase in the activity of NIMA kinase (Figure 6). Thus, when the *bim*E7 mutation is imposed chromatin condensation can occur in the absence of an increase in NIMA kinase activity. These data indicate that the *bim*E7 mutation does not cause initiation of mitosis by artificially increasing the level of NIMA activity as we had previously suggested (Osmani *et al.*, 1988a). Instead, *bim*E7 appears to circumvent the requirement for NIMA activation in the process of mitotic initiation.

Mitotic defects caused by absence of NIMA activation at mitosis

Antitubulin immunofluorescent studies on the *bim*E7 + *nim*A5 mutant strain have shown that an abnormal mitotic block occurs at the restrictive temperature in this strain (Osmani *et al.*, 1988a). As these mitotic events occur in the absence of increased NIMA kinase activity (Figure 6) and are not due to artificially high levels of the NIMA kinase, we were particularly interested to know what specific mitotic defects occur in this strain. This information would indicate what aspects of mitosis require elevated NIMA kinase activity for them to be executed properly. To this end we carried out a study of the phenotype of the mutant strains at the restrictive temperature using freeze substitution and electron microscopy (Figures 7 and 8).

Electron microscopy showed that wild-type nuclei were primarily in interphase (Figure 7A) as demonstrated by the absence of condensed chromatin and spindle microtubules. The spindle pole body (SPB) at interphase is attached to the outer surface of the nuclear envelope and, in its duplicated form, is composed of two identical SPB discs connected by a bridge.

At the restrictive temperature, mitotic spindle organization was normal in the *bim*E7 strain (Figure 7B). The *bim*E7 strain arrested uniformly at medial nuclear division. Each layered SPB disc occupied the poles of an intranuclear spindle composed of a central bundle of non-kinetochore microtubules which were in near parallel arrangement. Condensed chromosomes, attached to either pole by a single continuous kinetochore microtubule, were spread around and along the spindle (Figure 7B).

Ultrastructural analysis of the mitotic defects in the *nim*A5 + *bim*E7 double mutant strain at the restrictive temperature (Figures 7D and 8) revealed aberrant pleiotropic phenotypes which differed significantly from those observed in wild-type (Figure 7C) and *bim*E7 (Figure 7B) mitotic cells. Astral and spindle microtubules were assembled at the SPBs and chromosome condensation occurred in the double mutant;

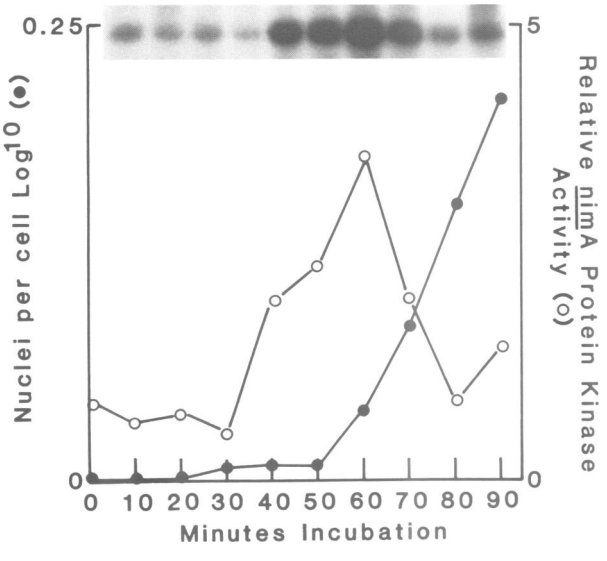

Fig. 5. Cell cycle fluctuations in NIMA activity. A synchronous culture of *Aspergillus* was generated by germinating spores in hydroxyurea. The drug was removed (time zero) to allow a partially synchronous nuclear division cycle to occur. The number of nuclei per cell and the level of NIMA activity were monitored as cells progressed through $S-G_2-M-G_1$. Insert shows phospho β-casein.

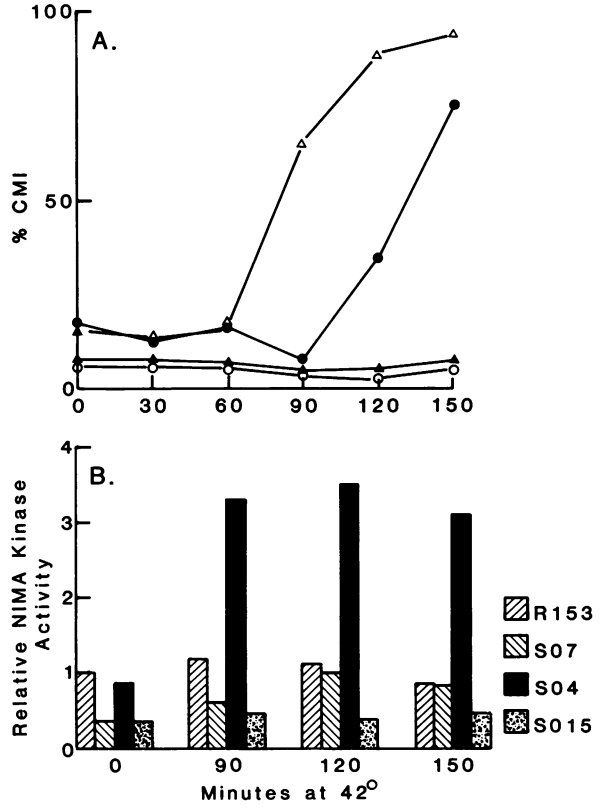

Fig. 6. Effect of *bim*E arrest on NIMA kinase activity. Four strains of *A.nidulans* were grown for a period at 25°C before shifting to 42°C (time 0). Samples were taken at the times indicated and analyzed for chromosome mitotic index (panel **A**) and NIMA kinase activity (panel **B**). Strains were: R153 (wild-type); SO7 (*nim*A5); SO4 (*bim*E7); SO15 (*nim*A5 + *bim*E7).

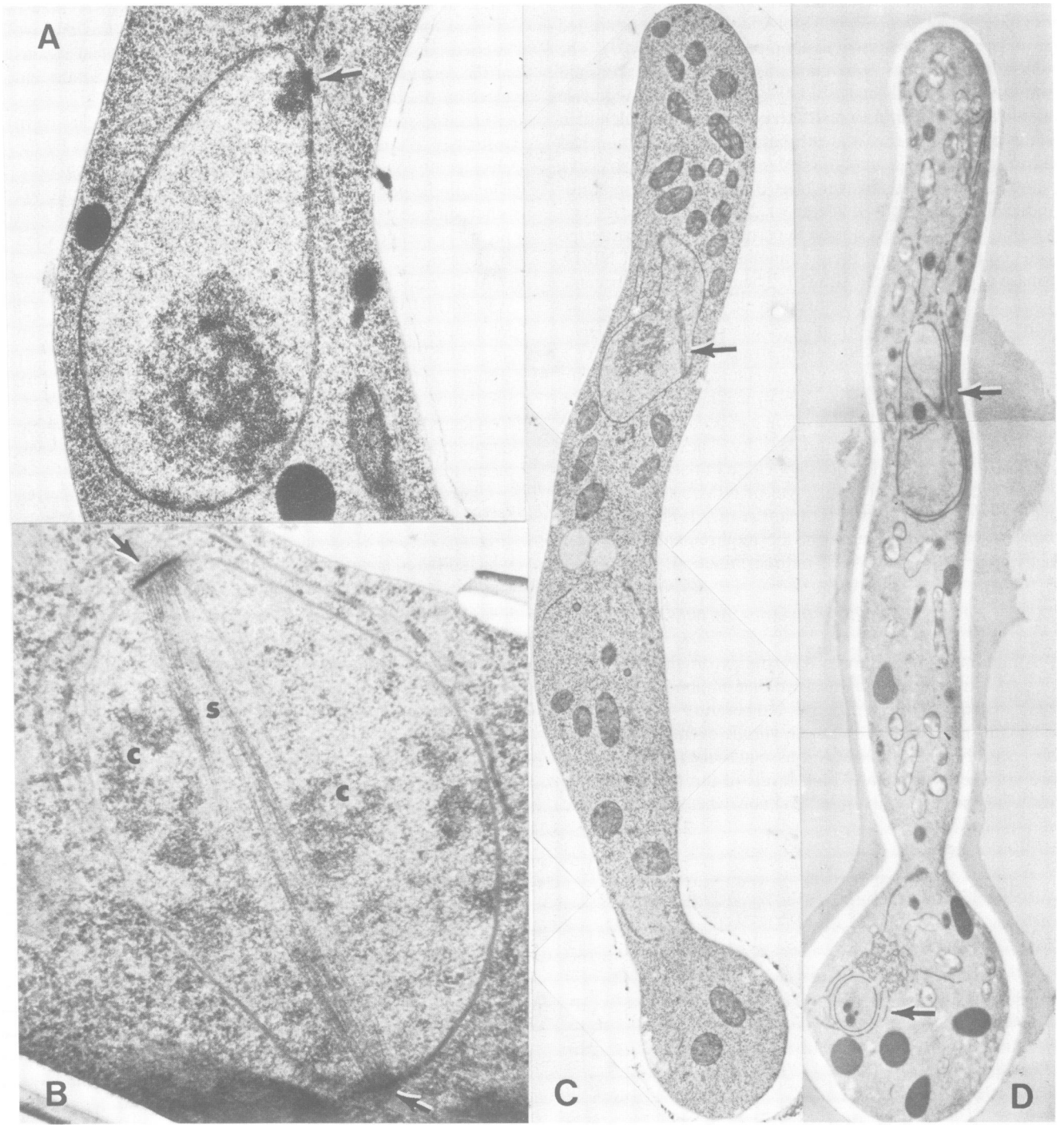

Fig. 7. Electron micrographs of wild-type and cell cycle mutants of *A.nidulans* at the restrictive temperature. Conidia were grown at 25°C to the 2–4 nucleate stage, then shifted to 42°C for 2.5–3 h at which time they were processed for electron microscopy. Nuclei of wild-type germlings (**A**) exhibited normal interphase nuclear morphology (>95%) while the *bim*E7 germlings were uniformly blocked in medial nuclear division (**B**) with spindles (s) and condensed chromsomes (c) identical to those observed in the wild-type at the same stage of mitosis. The arrow designates the duplicated SPB in (A) the SPBs in (B) and an obliquely sectioned mitotic spindle in (**C**). Germlings carrying *nim*A5 + *bim*E7 (**D**) exhibited aberrant nuclear morphologies characterized by the proliferation of endomembranes around and within nuclei (arrows). Magnifications, A = ×27 400; B = ×35 000; C = ×8000; D = ×7500.

however, spindles were grossly aberrant in size and shape and they were associated with multidirectional, apparently unorganized, mitotic microtubules (Figure 8A). Several instances of multiple SPBs in close proximity were observed (Figure 8D) as were SPBs bearing rudimentary half-spindles detached from the nucleus (Figure 8E,F).

In addition to defects in mitotic microtubule organization, the double mutant displayed dramatic peculiarities in nuclear

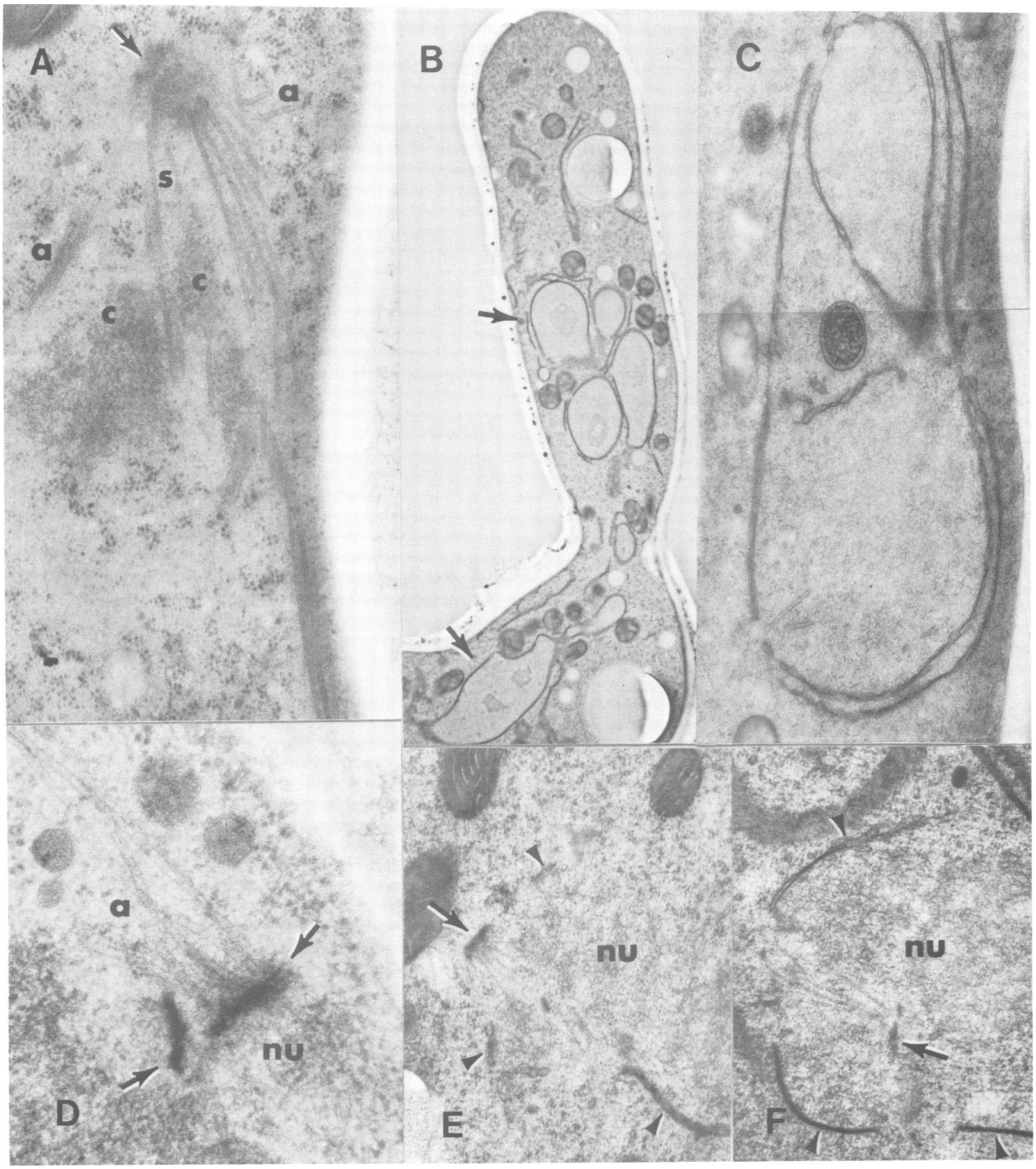

Fig. 8. Electron microscopy of *nim*A5 + *bim*E7 at restrictive temperature. Samples were prepared as described in Figure 7 using the double *nim*A5 + *bim*E7 mutant strain SO15. Telophase-like spindles (**A**) with atypical spindle pole bodies (arrow) bearing abnormal spindle (s) and astral (a) microtubule arrays and condensed chromsomes (c) were observed in many cells. Nuclei appeared to coalesce (**B**) and form irregular shaped and enlarged nuclei (arrows) with multiple nucleoli (B). A series of sections through this germling (B) revealed that the upper nuclear cluster was composed of two, or possibly only one nucleus, as the nucleoplasm was contiguous between the two 'pairs' of 'nuclei'. Endomembranes proliferated around and within nuclei and organelles invaded the nucleoplasm (**C**). Multiple spindle pole bodies were discernible on some nuclei. For example, the spindle pole depicted in (**D**) is in a telophase state with numerous astral microtubules (a) emanating upwards from two adjacent spindle pole bodies (arrows) something never seen in wild-type or *bim*E7 mitotic figures (nu = nucleus). Discontinuities in the nuclear envelope were frequently observed (**E**, **F**), and in these nuclei spindle pole bodies (arrows) bearing rudimentary half-spindles were observed detached from the nuclear envelope (arrowheads). Magnifications, A = ×44 000; B = ×6450; C = ×28 000; D = ×68 000; E = ×28 000; F = ×28 000.

envelope structure. Nuclei were frequently enlarged with multiple nucleoli (Figure 8B), suggesting that perhaps mitosis had been completed without proper segregation of nuclei or that nuclei had coalesced to form irregularly shaped and enlarged nuclei with multiple nucleoli. The nuclear envelope was typically discontinuous and often fragmented (Figure 7D, Figure 8B,C,E,F) and endomembranes proliferated around and within nuclei and organelles invaded the nucleoplasm (Figure 7D, enlarged in Figure 8C).

These studies demonstrate that if mitosis is initiated without activation of *nim*A, chromosome condensation and rudimentary spindle formation can occur. However, lack of *nim*A causes major organizational problems for both the nuclear envelope and mitotic spindle. Whether the primary defect is associated with the organization of the nuclear envelope or the mitotic spindle we cannot say.

The bimE7 mutation changes the requirement for NIMA in mitotic specific phosphorylation

It is possible to follow some mitotic specific protein phosphorylation by using the MPM-2 monoclonal antibody. Originally raised against mitotic HeLa cells the MPM-2 monoclonal antibody has been shown to react with mitotic specific phosphoproteins in numerous systems (Davis *et al.*, 1983; Vadre *et al.*, 1986) including (using immunofluorescence) *A.nidulans* (Engle *et al.*, 1988).

We have used Western blot analysis to reveal phosphorylated proteins detected by MPM-2 during mitosis. One particular MPM-2 reactive protein(s) appeared as a doublet, with a mobility just below that of the 58 kDa marker (designated p58). The phosphorylation of p58 was consistently observed to be specific to mitotic cells. In asynchronous cells or cells arrested in G_2 by the *nim*A5 mutation p58 was not detected. Upon release from the *nim*A5 arrest p58 was detected within 3 min of the release as cells entered mitosis (Figure 9, lanes 1–5). When the *bim*E7 mutation was imposed cells arrested in mitosis and p58 was detected only in extracts from the mitotic arrested culture (Figure 9, lanes 6–10). MPM-2 specific phosphorylation of p58 is thus specific to mitosis and this phosphorylation normally requires NIMA activation.

We next followed the appearance of p58 phosphorylation in proteins derived from wild-type, *nim*A5 or *bim*E7 or *nim*A5 + *bim*E7 strains when shifted from permissive to restrictive temperature (Figure 10). As expected, p58 was not observed at permissive or restrictive temperature in either the wild-type or *nim*A5 containing strains (Figure 10A and B). However, p58 was readily detected in the *bim*E7 and *bim*E7 + *nim*A5 mutant strains (Figure 10B and C) when they were arrested in mitosis. In the single *bim*E7 mutant a good correlation between p58 appearance and mitotic index was observed. It was noticeable that p58 could be detected in the double mutant *bim*E7 + *nim*A5 strain before an increase in chromosome mitotic index was detected.

These data demonstrate that MPM-2 specific p58 phosphorylation is dependent on the NIMA protein kinase only when *bim*E function is active.

Discussion

Cell cycle regulation of the NIMA protein kinase

We demonstrate that the product of the G_2-specific *nim*A gene (NIMA) has protein kinase activity. The NIMA protein kinase can utilize β-casein as an artificial phosphate acceptor but cannot phosphorylate histones H1, H2A, H2B, H3 or H4. The substrate specificity of NIMA indicates that it falls into the category of protein kinases known as casein kinases. These are operationally classified as messenger-independent kinases that preferentially utilize acidic proteins such as casein over histones as artificial substrates. NIMA appears from present data (mol. wt and sequence comparison) to be a new member of this class of enzyme (Edelman *et al.*, 1987).

The kinase activity of NIMA is readily increased by overexpression of *nim*A and this increase in activity correlates with the induction of mitotic events. Data derived from cell cycle staged extracts indicate that the NIMA kinase is under cell cycle specific regulation. If cells are blocked

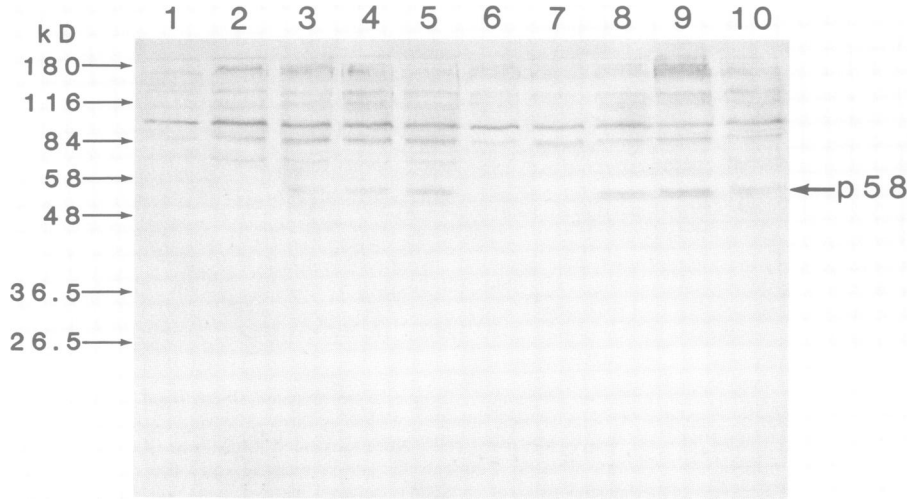

Fig. 9. MPM-2 staining in *nim*A5 and *bim*E7 generated mitotic cells. A strain (SO7) containing the *nim*A5 mutation was grown at 35°C overnight (lane 1), shifted to 42°C for 3 h (G_2 arrest, lane 2) and returned to 25°C for 3, 5 or 7 min (entry into mitosis, lanes 3, 4 and 5). Strain SO4 (*bim*E7) was grown overnight at 25°C (lane 6) and shifted to 42°C for 1, 2, 3 or 4 h (mitotic arrest, lanes 7, 8, 9 and 10). Protein was extracted from each culture, separated by SDS–PAGE, transferred to nitrocellulose and processed for MPM-2 antibody staining. The mitotic specific MPM-2 reactive protein is indicated (p58).

in mitosis NIMA kinase activity is held at an elevated level but if cells are blocked in S-phase NIMA kinase levels fall. During a partially synchronous cell cycle the NIMA kinase activity is held low during S-phase, increases during G_2 and is high during mitosis. As nuclear division is completed NIMA kinase activity is reduced. Thus *nim*A encodes only the second protein kinase that is required for G_2 progression shown biochemically to have G_2-M regulated activity. The data indicate that *nim*A plays a regulatory role during the cell cycle by mediating protein phosphorylation during the G_2-M transition that leads to the initiation of mitosis. The histone H1 protein kinase activity associated with $p34^{cdc2}$ is the only other G_2 specific kinase demonstrated to have G_2-M regulated activity (Arion *et al.*, 1988; Draetta and Beach, 1988; Booher *et al.*, 1989; Brizuela *et al.*, 1989; Labbe *et al.*, 1989; Meijer *et al.*, 1989; Moreno *et al.*, 1989).

What is elevated NIMA activity required for?

Imposition of the *bim*E7 mutation induces an increase in the level of NIMA protein kinase activity and this leads to nuclei arresting in mitosis with condensed chromatin and a normal metaphase spindle. At the restrictive temperature a *nim*A5 + *bim*E7 double mutant strain did not undergo an increase in NIMA protein kinase activity and some, but not all, mitotic events occurred. This strain, therefore, allowed us to determine which aspects of mitosis require elevated NIMA kinase activity for them to be executed correctly.

Two hallmarks of mitosis in *A.nidulans* could occur in the double mutant in the absence of elevated NIMA kinase activity. Both chromatin condensation and SPB nucleation of mitotic microtubules could take place in the double mutant at the restrictive temperature.

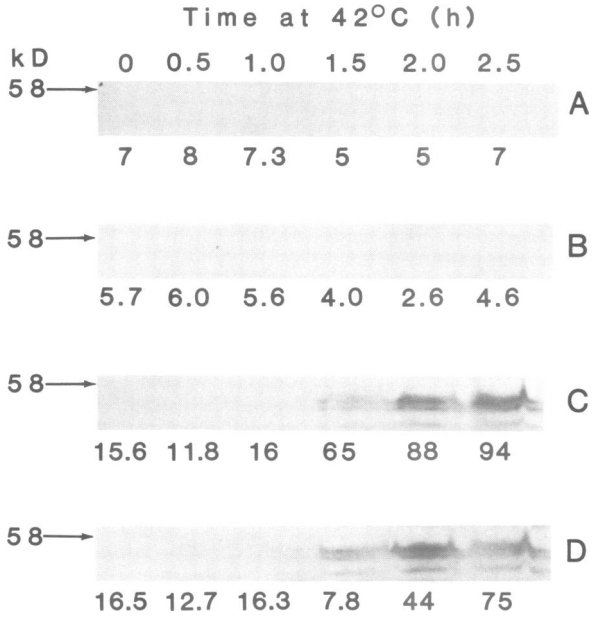

Fig. 10. MPM-2 specific p58 detection in cell cycle blocked mutant strains. Four strains of *A.nidulans*, R153 (wild-type, panel **A**), SO7 (*nim*A5, panel **B**), SO4 (*bim*E7, panel **C**), SO15 (*nim*A5 + *bim*E7, panel **D**), were grown for a period at 25°C (time 0) before shifting to 42°C. Samples were taken at the time indicated and analyzed for chromosome mitotic index (indicated under each panel) and reactivity of protein to MPM-2 antibody by Western blot analysis. Only the region of p58 staining is shown.

Conversely, two other aspects of mitosis were significantly modified in the absence of increased NIMA activity at mitosis. Spindle organization was dramatically affected by the absence of increased NIMA activity and no normal spindle formation was observed in the double mutant strain. Although the SPBs became competent to nucleate microtubules in the double mutant, these microtubules were not organized in a manner seen in the wild-type or single *bim*E7 mutant strain. The second defect caused by lack of NIMA during mitosis was the pronounced disruption of the integrity of the nuclear envelope. Normally, the nuclear envelope maintains its integrity in *A.nidulans* during mitosis. However, it is likely that significant re-organization of the nuclear envelope occurs in order to complete successful nuclear division. The defects in nuclear envelope integrity we have observed in the double mutant indicate that NIMA protein kinase activity is involved in maintaining the order of the nuclear envelope during its re-organization at mitosis. These observations strongly suggest that the NIMA protein kinase plays a limited role during mitosis. Increased NIMA activity is not required for chromosome condensation or SPB activation but it is involved in the organization of both the mitotic spindle and the nuclear envelope during mitosis.

Does NIMA carry out MPM-2 detected protein phosphorylation?

*nim*A does play an essential role during G_2 as mutation of *nim*A prevents the majority of known mitotic events from occurring, including some mitotic specific protein phosphorylation. For instance, we have shown that phosphorylation of p58, as detected by the MPM-2 monoclonal antibody, is specific to mitosis. A similar sized MPM-2 reactive protein is known to be phosphorylated during *Xenopus* oocyte maturation (Kuang *et al.*, 1989). *A.nidulans* p58 phosphorylation is shown to be dependent on the NIMA protein kinase as *nim*A5 arrests in G_2 and p58 is not phosphorylated at the MPM-2 detectable site. Upon release from the *nim*A5 arrest point into mitosis p58 becomes phosphorylated. However, mutation of *bim*E allows phos-

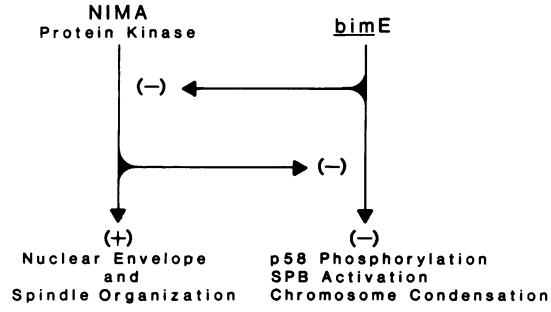

Fig. 11. The checkpoint system between *nim*A and *bim*E. The proposed control mechanism explains the following experimental observations. Increased NIMA activity is normally required for p58 phosphorylation, SPB activation, and chromosome condensation at mitosis. However, NIMA activation is not required for these mitotic events when *bim*E function is impaired. NIMA activation is however always required for correct spindle and nuclear envelope organization during mitosis. We propose that NIMA protein kinase activation during G_2 causes an inhibition of *bim*E. As *bim*E functions in a negative manner (Osmani *et al.*, 1988a), inhibition of *bim*E alleviates negative control on the pathways that initiate mitosis, one of which leads to p58 phosphorylaton, SPB activation and chromosome condensation. The proposed relationship between *bim*E and NIMA explains why mutation of *bim*E allows p58 phosphorylation, chromosome condensation and SPB activation in the absence of increased NIMA. See text for further discussion.

phorylation of p58 independent of increased NIMA activity as a double *nim*A5 + *bim*E7 mutant arrests with similar MPM-2 specific p58 phosphorylation to the single *bim*E7 mutant although NIMA activation does not take place. Therefore, mutation of *bim*E circumvents the requirement for *nim*A in the process of p58 phosphorylation demonstrating that NIMA is not the kinase that phosphorylates p58.

nimA arrests cells in G_2 due to a checkpoint mediated by bimE

As SPB activation, chromosome condensation and p58 phosphorylation are all prevented by mutation of *nim*A, these mitotic events could be on a linear pathway downstream from *nim*A activation. This simple relationship is, however, clearly oversimplified as the *bim*E7 mutation circumvents the requirement for increased NIMA for all of these mitotic events. We propose that the G_2 arrest caused by mutation of *nim*A is the result of a control system mediated by *bim*E.

It is known that *bim*E acts negatively to prevent mitosis during interphase (Osmani *et al.*, 1988a) and when *bim*E is mutated cells enter and arrest in an extended metaphase state. This indicates that all mitotic regulatory systems are affected by *bim*E. This is shown in Figure 11 as *bim*E negatively influencing both *nim*A mediated events and those leading to p58 phosphorylation, SPB activation and chromosome condensation. To reverse the inhibitory effects of *bim*E during late G_2 we propose that increased *nim*A activity leads to inhibition of *bim*E and this allows activation of those pathways under negative *bim*E regulation (Figure 11). Activation of *nim*A is also required separately for correct organization of both the nuclear envelope and mitotic spindle during mitosis as indicated (Figure 11). In the double *nim*A5 + *bim*E7 mutant at restrictive temperature, absence of functional *bim*E leads to p58 phosphorylation, SPB activation and chromosome condensation in the absence of activated *nim*A. This allows these aspects of mitosis to proceed but, due to lack of activated *nim*A, the organization of these mitotic events is defective leading to aberrant mitosis.

The control system described above involving *nim*A and *bim*E constitutes a mechanism by which premature mitosis is prevented until the NIMA protein kinase is mitotically activated. Such a system is required because although NIMA appears to be required during mitosis in a limited capacity, if mitosis proceeds in its absence, major mitotic defects result. Systems that impose order on the cell cycle have been termed checkpoints (Weinart and Hartwell, 1989). A checkpoint is a control mechanism that enforces dependency in the cell cycle and prevents downstream events of the cycle occurring prematurely (Weinart and Hartwell, 1989). There are at present two classic examples of checkpoints. The first is the dependency of mitosis on the completion of DNA replication. A checkpoint exists that can monitor the completion of DNA replication and if this checkpoint is not passed then mitosis is arrested. Mutation of *cdc*25 or *cdc*2 in fission yeast can bypass this checkpoint and allow cells to enter mitosis when their DNA is not replicated indicating that these genes are part of the checkpoint system monitoring DNA synthesis (Enoch and Nurse, 1990). Similarly, if DNA is damaged, mitosis will not proceed until DNA repair is complete and a DNA damage checkpoint is passed. Mutation of the *RAD9* gene in budding yeast bypasses the DNA repair checkpoint and allows mitosis in the presence of damaged DNA. Thus *RAD9* must be able to monitor the completion of DNA repair (Weinert and Hartwell, 1988).

At the present time we do not know if the *nim*A *bim*E checkpoint control mechanism is part of the *cdc*25/*cdc*2 system that ensures the dependency of mitosis on DNA replication or if they are involved with *RAD9* to ensure that mitosis does not occur prior to completion of DNA repair. An alternative possibility is that *nim*A and *bim*E monitor an as yet undefined interphase function required for mitosis.

As a final point, the results presented here further demonstrate the key role that the *bim*E function plays in negative regulation of mitosis in *A.nidulans*. As the *bim*E7 mutation leads to premature MPM-2 specific p58 phosphorylation *bim*E function must act, in part or in total, to prevent mitotic specific phosphorylation occurring inappropriately during interphase.

Materials and methods

Synthesis of NIMA

NIMA was synthesized *in vitro* by first subcloning *nim*A cDNA (Osmani *et al.*, 1988b) as an *Eco*RI fragment into pUC18 to yield p3.3A. A *Hin*dIII–*Kpn*I fragment from p3.3A, containing the complete NIMA open reading frame, was then cloned into *Hin*dIII–*Kpn*I digested pBS M13$^-$ (Stratagene, La Jolla, CA) to yield pBS$^-$A. mRNA was synthesized using T3 RNA polymerase from pBS$^-$A and the mRNA translated in a rabbit reticulocyte system in the presence of [^{35}S]methionine as described by the manufacturer using the undefined RNA encoding a 16 kDa protein as a positive control (Stratagene, La Jolla, CA).

For the production of part of NIMA in *E.coli* a 1.6 kb *Bam*HI portion of the *nim*A cDNA was cloned into the *Bam*HI site of the expression vector pET-3b (Studier and Moffatt, 1986; Rosenberg *et al.*, 1987) downstream of a T7 promoter to yield plasmid pET-3b-nimA. This plasmid was transformed into strain BL21 (DE3) plysS which contains a single copy of the gene for T7 RNA polymerase in the chromosome under control of the IPTG (isopropyl-β-D-thiogalactopyranoside) inducible *lac*UV5 promoter (Studier and Moffatt, 1986) to give strain BL21 (DE3) plysS pET-3b-nimA. In this strain expression of part of NIMA is inducible by the addition of IPTG. For production of NIMA an overnight culture of this strain was diluted 1:50 into 1.5 ml of LB containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol. After 2 h of growth IPTG was added to 0.4 mM and growth continued for 3 h (for controls IPTG was not added). The cells were harvested by centrifugation and total protein isolated by solubilization of the pellet in 100 μl of cracking buffer (6 M urea, 10 mM PO_4, pH 7.2, 1% SDS, 1% β-mercaptoethanol) at 37°C for 1 h. A control strain lacking pET3b-nimA was treated in the same manner. After shearing through a 22G needle, samples were analyzed by SDS–PAGE and Western blotting (Towbin *et al.*, 1979).

Aspergillus strains and general techniques

Strains used in this study were R153 (*w*A3;*pyro*A4), SO7 (*nim*A5;*w*A2), GR5 (*pyr*G89; *w*A3; *pyro*A4), SO4 (*bim*E7; *w*A2; *paba*A1), SO15 (*nim*A5; *bim*E7; *paba*A1; *ribo*B2; *w*A2). Strain GR5-2 was isolated by transformation of GR5 with the plasmid pAL3HK, which has the *nim*A gene under the control of the *alc*A promoter (Osmani *et al.*, 1988b; Waring *et al.*, 1989), and Southern blot analysis to identify a transformant containing several copies of the plasmid integrated into the genome at *nim*A. Growth and transformation of strains and their analysis was as described previously (Morris, 1976; Osmani *et al.*, 1987) as were the staining techniques and microscopy for *Aspergillus* (Osmani *et al.*, 1990) and cell cycle blocks and synchrony (Bergen and Morris, 1983; Osmani *et al.*, 1987).

Protein kinase assays

Aspergillus mycelia were harvested by filtration through Miracloth (Calbiochem-Behring Corp., La Jolla, CA), washed with ice-cold stop buffer (Moreno *et al.*, 1989), briefly pressed dry between paper towels and stored at −80°C before protein extraction. All subsequent procedures were carried out at 4°C. Frozen mycelia were suspended in 2.5 ml per gram weight in either HK buffer (Booher *et al.*, 1989) or EB buffer (Kuang *et al.*, 1989) containing the following protease inhibitors: 10 μg/ml leupeptin, 10 μg/ml trypsin–chymotrypsin inhibitor, 10 μg/ml aprotinin, 10 μg/ml tosyl phenylalanine chloromethyl ketone, 10 μg/ml tosyl arginine methyl ester,

5 mM benzamidine, 0.3 mM phenylmethyl sulfonyl fluoride. The slurry was ground in a mortar and pestle for 5 min and cleared by centrifugation in an Eppendorf for 10 min. The supernatant was frozen in liquid nitrogen and stored at −80°C.

For one NIMA assay 2.5 μl of preimmune sera were added to 0.2 ml 10 mg/ml protein (or 0.2 ml 0.5 mg/ml for NIMA overexpressed extracts) and incubated for 30 min. Protein A−Sepharose (40 μl) was added and incubated for 15 min with rocking followed by centrifugation at 75 000 r.p.m. (Beckman TLA 100.3 rotor) for 30 min and removal of the supernatant. Immune sera (2.5 μl) was added to the supernatant and incubated for 2 h. Then 20 μl protein A−Sepharose was added and left for 30 min with rocking. The immunoprecipitate was collected and washed five times with 1 ml HK buffer and twice with 1 ml KAB buffer (50 mM Tris, pH 7.5, 10 mM MgCl, 1 mM DTT) and the pellet suspended in 20 μl KAB buffer. After equilibration at 22°C the kinase reaction was initiated by the addition of 5 μl of ATP mix containing 5 μl [^{32}P]ATP (Amersham, Pb10218), 1 μl 1 mM ATP, 20 μl 5 mg/ml mixed casein (or 2 mg/ml phosvitin, or 2 mg/ml calf thymus histones H2A, H2B, H3, H4, or 2 mg/ml calf thymus histone H1, or 1 mg/ml α-casein or 1 mg/ml β-casein) and 74 μl H_2O. Reactions were terminated at 10 min by the addition of 25 μl SDS sample buffer followed by boiling for 5 min. Samples were separated by SDS−PAGE, fixed, dried, and exposed to film. Bands corresponding to β-casein were cut out and solubilized overnight at 37°C in Econofluor containing 3% Protosol (NEN Research Products, Boston, MA) before counting.

Immunochemistry

Polyclonal antibodies were elicited in guinea pigs against two peptides, FASTY peptide = CGGLSKLMHSHDFASTY, and ANYRED peptide = CGRNANYREDASLRSSG. The peptides were cross-linked to keyhole limpet hemocyanin using *m*-maleimidobenzyl-*N*-hydroxysulfosuccinimide ester according to the manufacturer's instructions (Pierce). 100 μg of these antigens were used to immunize guinea pigs as previously described (Osmani *et al.*, 1990). Sera were tested for their ability to precipitate *in vitro* synthesized NIMA protein. 50 μl of NET (100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.0), 2 μl of translation mix and 10 μl protein A−Sepharose were mixed and incubated for 30 min followed by a 5 min Eppendorf spin. Preimmune or immune sera (2 μl) were added to the supernatant and incubated for 1 h at 25°C. Immune complexes were collected by addition of 12.5 μl protein A−Sepharose and 40 min incubation at 25°C. The complexes were washed with NET by centrifugation, boiled in SDS sample buffer and subjected to SDS−PAGE followed by autoradiography.

For Western blot analysis proteins were separated by SDS−PAGE, transferred to nitrocellulose and stained with MPM-2 using biotin-conjugated horse anti-mouse antibody then peroxidase-conjugated Streptavidin. The MPM-2 monoclonal antibody used in this study was a kind gift from Dr Potu N.Rao.

Electron microscopy

Conidia were grown on cellophane squares overlaid on solid media at 25°C, shifted to the restrictive temperature (42°C) for 2.5−3 h to impose the cell cycle block, and processed for electron microscopy as previously described (Osmani *et al.*, 1987) except that serial sections were examined in a Philips CM-12 electron microscope.

Acknowledgements

We would like to thank Dr Greg May and Sarah Lea McGuire for their invaluable help during set-up of the Osmani laboratory at Baylor and for constructive discussions during this work. We thank Dr Potu Rao and Dr Jian Kuang for MPM-2 antibody and instruction on Western blot analysis using this reagent. This work was supported by National Institutes of Health grant GM42564 to S.A.O.

References

Arion,D., Meijer,L., Brizuela,L. and Beach,D. (1988) *Cell*, **55**, 371−378.
Bergen,L.G. and Morris,N.R. (1983) *J. Bacteriol.*, **156**, 155−160.
Bergen,L.G., Upshall,A. and Morris,N.R. (1984) *J. Bacteriol.*, **159**, 114−119.
Booher,R.N., Alfa,C.E., Hyams,J.S. and Beach,D.H. (1989) *Cell*, **58**, 485−497.
Brizuela,L., Draetta,G. and Beach,D. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4362−4366.
Davis,F.M., Tsao,T.Y., Fowler,S.K. and Rao,P.N. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 2962−2930.
Doonan,J.H. and Morris,N.R. (1989) *Cell*, **57**, 987−996.
Draetta,G. and Beach,D. (1988) *Cell*, **54**, 17−26.
Edelman,A.M., Blumenthal,D.K. and Krebs,E.G. (1987) *Annu. Rev. Biochem.*, **56**, 567−613.
Engle,D.B., Doonan,J.H. and Morris,N.R. (1988) *Cell Motil. Cytoskeleton.*, **10**, 434−437.
Engle,D.B., Osmani,S.A., Osmani,A.H., Rosbrough,S., Xiang,X. and Morris,N.R. (1990) *J. Biol. Chem.*, **256**, 16132−16137.
Enoch,T. and Nurse,P.(1990) *Cell*, **60**, 665−673.
Enos,A.P. and Morris,N.R. (1990) *Cell*, **60**, 1019−1027.
Kuang,J., Zhao,J., Wright,D.A., Saunders,G.F. and Rao,P.N. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4982−4986.
Labbe,J.C., Picard,A., Peaucellier,G., Cavadore,J.C., Nurse,P. and Doree,M. (1989) *Cell*, **57**, 253−263.
Meijer,L., Arion,D., Golsteyn,R., Pines,J., Brizuela,L., Hunt,T. and Beach,D. (1989) *EMBO J.*, **8**, 2275−2282.
Moreno,S., Hayles,J. and Nurse,P. (1989) *Cell*, **58**, 361−372.
Morris,N.R. (1976) *Genet. Res. Camb.*, **26**, 237−254.
Murray,A.W. and Kirschner,M.W. (1989) *Science*, **246**, 614−621.
Nurse,P. (1990) *Nature*, **334**, 503−508.
Oakley,B.R. and Morris,N.R. (1983) *J. Cell Biol.*, **96**, 1155−1158.
Osmani,S.A., May,G.S. and Morris,N.R. (1987) *J. Cell Biol.*, **104**, 1495−1504.
Osmani,S.A., Engle,D.B., Doonan,J.H. and Morris,N.R. (1988a) *Cell*, **52**, 241−251.
Osmani,A.A., Pu,R.T. and Morris,N.R. (1988b) *Cell*, **53**, 237−244.
Osmani,A.H., Osmani,S.A. and Morris,N.R. (1990) *J. Cell Biol.*, **111**, 543−551.
Rosenberg,A.H., Lade,B.N., Chui,D., Lin,S.W., Dunn,J.J. and Studier,F.W. (1987) *Gene*, **56**, 125−153.
Studier,F.W. and Moffatt,B.A. (1986) *J. Mol. Biol.*, **189**, 113−130.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350−4354.
Vadre,D.D., Davis,F.M., Rao,P.N. and Borisy,G.G. (1986) *Eur. J. Cell Biol.*, **41**, 72−81.
Weinert,T.A. and Hartwell,L.H. (1988) *Science*, **241**, 317−322.
Weinert,T.A. and Hartwell,L.H. (1989) *Science*, **246**, 629−634.
Waring,R.B., May,G.S. and Morris,N.R. (1989) *Gene*, **79**, 119−130.

Received on March 7, 1991; revised on May 16, 1991