REVIEW ARTICLE

Cell cycle regulation in *Aspergillus* by two protein kinases

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Great progress has recently been made in our understanding of the regulation of the eukaryotic cell cycle, and the central role of cyclin-dependent kinases is now clear. In *Aspergillus nidulans* it has been established that a second class of cell-cycle-regulated protein kinases, typified by NIMA (encoded by the *nimA* gene), is also required for cell cycle progression into mitosis. Indeed, both p34^{cdc2}/cyclin B and NIMA have to be correctly activated before mitosis can be initiated in this species, and p34^{cdc2}/cyclin B plays a role in the mitosis-specific activation of NIMA. In addition, both kinases have to be proteolytically destroyed before mitosis can be completed. NIMA-related kinases may also regulate the cell cycle in other eukaryotes, as expression of NIMA can promote mitotic events in yeast, frog or human cells.

Moreover, dominant-negative versions of NIMA can adversely affect the progression of human cells into mitosis, as they do in *A. nidulans*. The ability of NIMA to influence mitotic regulation in human and frog cells strongly suggests the existence of a NIMA pathway of mitotic regulation in higher eukaryotes. A growing number of NIMA-related kinases have been isolated from organisms ranging from fungi to humans, and some of these kinases are also cell-cycle-regulated. How NIMA-related kinases and cyclin-dependent kinases act in concert to promote cell cycle transitions is just beginning to be understood. This understanding is the key to a full knowledge of cell cycle regulation.

INTRODUCTION

Dividing cells traverse the cell cycle in order to duplicate their constituents and then undergo division [1]. During the cell cycle, nuclear DNA is replicated during S phase and segregated equally into two daughter nuclei during mitosis. Cytokinesis then segregates the divided nuclei, along with cytoplasmic components, into two separate cells. Cells therefore have to make a continuum of decisions about when to start and when to stop cell-cycle-specific functions. They also have to accurately detect that nuclear DNA has been replicated exactly once in each turn of the cell cycle, and that DNA has passed quality controls, before division, to avoid transmission of defective genetic information. Finally, multicellular organisms need to restrict the number and pattern of divisions that their cells are allowed to complete, to ensure normal growth and morphogenesis. How cells orchestrate these different levels of regulation over the cell cycle is just beginning to be unravelled, and will continue to come under intensive study due to the implications for human diseases such as cancer.

Great insight into the regulation of the cell cycle has been obtained from the study of conditional cell-cycle-specific mutants isolated in model genetic systems [2–5]. By utilizing molecular genetics it has been possible to clone such genes and then analyse their products biochemically. The combined approaches of classical genetics, molecular genetics and biochemistry have proven extremely powerful for unravelling how cells regulate their cell cycles biochemically. The realization has also been made that cell cycle regulatory mechanisms are highly conserved from lower genetic systems, such as fungi, through evolution to humans. In this review we will cover recent contributions made utilizing the experimentally amenable fungus *Aspergillus nidulans* and try to integrate this information with that derived from other systems. For an earlier review, see Doonan [6].

Analysis of cell cycle regulation utilizing *A. nidulans* was

pioneered by N. Ronald Morris, who isolated a collection of temperature-sensitive mutations affecting a range of biological processes including cell cycle progression, septation and nuclear movement [2,7] (Figure 1). Of the 1000 temperature-sensitive strains analysed by Morris (Figure 1), 23 were characterized as being required for interphase progression (*nim*, for **never in mitosis** mutants) and six for progression through mitosis (*bim*, for **blocked in mitosis** mutants). Notably, multiple alleles of only one *nim* gene were isolated (four alleles of *nimA*), indicating that the screen for interphase-specific functions was not saturating. In addition to mutations specifically affecting cell cycle progression, four mutations affecting the deposition of septa (*sep* mutants) and two affecting nuclear migration (*nud* mutants) were identified. Several of these genes have been isolated by complementation, and their analysis has provided unique insights into the regulation of the eukaryotic cell cycle [8–19].

THE NIMA KINASE

In the field of cell cycle research much attention has focused on cyclin-dependent kinases, as these kinases play key roles in cell cycle regulation, during initiation of both DNA replication and mitosis, in organisms ranging from fungi to humans [20,21]. However, there is accumulating evidence that other levels of regulation do exist [22–26]. In *A. nidulans* much attention has focused on the role of *nimA* in the initiation and completion of mitosis. Early studies demonstrated that *nimA* function is required specifically at the G₂–M transition [8,27,28]. This was most dramatically demonstrated by temperature shift experiments. At the restrictive temperature for *nimA5* the function of NIMA (the protein product of *nimA*; see below) is destroyed and cells accumulate, within one cell cycle, in late G₂ but continue to grow to a considerable size (Figure 1; see *nim* mutants at 42 °C).

Figure 1 Terminal phenotypes of *nim*, *bim* and *nud* mutants of *Aspergillus nidulans*

The growth characteristics and nuclear divisions are shown for conidial spores inoculated into media at either a restrictive temperature (42 °C) or a permissive temperature (32 °C).

Figure 2 Potential functional domains and important residues in NIMA

Such cells show no cytological signs of mitosis, as microtubules are present in stable interphase arrays and nuclear DNA is not condensed. Upon return to a permissive temperature, NIMA

function is restored, cytoplasmic microtubules disassemble, the mitotic spindle is formed and chromosomes condense as nuclei rapidly and synchronously enter mitosis. The function of *nimA* is therefore specifically required to initiate all cytological aspects of mitosis [8,27,28].

Molecular cloning of *nimA* by complementation of the *nimA5* temperature-sensitive phenotype [6] and sequence analysis indicated that it encodes a protein (699 amino acids) containing the hallmarks of serine/threonine protein kinases [9,29], which was designated NIMA (Figure 2). NIMA is the founding member of a growing family of protein kinases with members isolated from *Saccharomyces cerevisiae*, *Neurospora crassa*, *Trypanosoma*, mice and humans [30–34]. All contain their catalytic domains in the N-terminus and all have a very high isoelectric point (> 10) (Figure 2).

Biochemical analysis of NIMA, either isolated by immunoprecipitation from *A. nidulans* or expressed in *Escherichia coli*, has shown it to contain serine/threonine protein kinase activity which is independent of second messengers [35,36]. Of the commonly used artificial protein kinase substrates tested, NIMA has a clear preference for β -casein, and it has a pH optimum ranging from 7.5 to 9.5 [35,36]. Screening of a peptide library for potential artificial substrates identified a good peptide substrate for NIMA [PLM-(58–72): GTFRSSIRRLSTRRR] which is phosphorylated at Ser-6 [37]. Mutational analysis of residues

around the phosphorylation site of the peptide indicated that a key determinant of NIMA specificity is the Phe residue at position -3 N-terminal to the site of phosphorylation.

CELL-CYCLE-SPECIFIC REGULATION OF NIMA

The level of NIMA protein kinase activity is regulated throughout the cell cycle, being low during G_1 and S phase and then increasing during G_2 to reach a maximum during late G_2 and early mitosis [35]. If cells are arrested in mitosis, e.g. by depolymerizing microtubules with nocodazole, then NIMA kinase activity remains at an elevated level. When cells are allowed to progress through mitosis, however, NIMA kinase activity is abolished [38]. Therefore progression through interphase and into early mitosis correlates with accumulation of active NIMA, but progression through mitosis inactivates NIMA.

If normal regulation of NIMA is overcome by induction from an inducible promoter then mitotic events are promoted, even if cells are first arrested in S phase, leading to death by untimely mitosis [9]. This appears to be a universal property of NIMA, as expression of *A. nidulans* NIMA in the fission yeast *Schizosaccharomyces pombe* [39], *Xenopus* oocytes or human cells [40] promotes mitotic events in these heterologous systems. This suggests that the NIMA specific substrates that mediate mitotic events are conserved from fungi through to humans, and there is mounting evidence that NIMA-like protein kinases are involved in cell cycle regulation in many, if not all, eukaryotes (see below).

ROLE OF NIMA ACCUMULATION DURING THE CELL CYCLE

Like the NIMA kinase activity, the level of NIMA protein also increases during interphase [38], peaking during mitosis, as does the level of *nimA* mRNA [8]. However, a clear understanding of the cell-cycle-specific expression of *nimA* is not available. For instance, changes in the level of *nimA* mRNA could be due to increased rates of transcription or decreased rates of mRNA degradation during cell cycle progression. Similarly, the accumulation of NIMA protein may be influenced by increased rates of translation or by a decreased rate of NIMA proteolysis. However, some information regarding the role of other cell cycle regulators in the expression of NIMA protein is available. In particular, the relationship between *nimA* and $p34^{cdc2}$ has been under study. First isolated from *S. cerevisiae* [41] and subsequently from *S. pombe* [42], the $p34^{cdc2}$ protein kinase is the founding member of the cyclin-dependent family of protein kinases [43], and is a key regulator of mitosis [20,44].

It was initially concluded that $p34^{cdc2}$ plays no role in the expression of *nimA*, as inactivation of $p34^{cdc2}$ to arrest cells in G_2 did not prevent accumulation of apparently fully active NIMA [22]. This analysis was performed using the *nimT23^{cdc25}* mutation, as a mutation in the $p34^{cdc2}$ homologue of *A. nidulans* was not isolated in the original screen by Morris. *nimT^{cdc25}* encodes the Cdc25 type tyrosine phosphatase of *A. nidulans* and is required to dephosphorylate and activate $p34^{cdc2}$ [10]. Consequently, mutations in *nimT^{cdc25}* arrest at G_2 because they impair the mitosis-promoting function of $p34^{cdc2}$ [22,45–48]. At the G_2 arrest point of *nimT23^{cdc25}*, active NIMA protein accumulates, thus demonstrating that activation of $p34^{cdc2}$ by tyrosine dephosphorylation is not required for expression of active NIMA kinase [22].

The subsequent isolation of a functional homologue of *cdc2*, called *nimX^{cdc2}*, from *A. nidulans* [49] enabled the consequences of direct inactivation of $p34^{cdc2}$ on *nimA* expression to be addressed. Direct inactivation of $p34^{cdc2}$ caused arrest of the cell

cycle in both G_1 and G_2 and also prevented the accumulation of NIMA protein [38,49]. Therefore, although inactivation of the mitotic form of $p34^{cdc2}$ does not prevent accumulation of NIMA protein, direct inactivation of $p34^{cdc2}$ to arrest cells in G_1 or G_2 does prevent the accumulation of NIMA.

This situation arises because $p34^{cdc2}$ is the catalytic subunit of more than one protein kinase complex [50–52], and different $p34^{cdc2}$ -containing complexes are thought to promote the various stages of the cell cycle in fungi [53–56]. For example, in *S. cerevisiae*, Cdc28 (the $p34^{cdc2}$ homologue of budding yeast) binds to G_1 cyclins (Clns) to promote G_1 progression and then binds to B-type cyclins (Clbs) to promote S phase and mitosis [52,53,57–60]. In *A. nidulans*, $p34^{cdc2}$ function is also required at least twice during the cell cycle, to promote both the G_1 -S and the G_2 -M transitions [49]. At present only one cyclin homologue, *nimE^{cyclinB}* [10], has been isolated from *A. nidulans*, but it is likely that NIMX^{cdc2} associates with different partners to promote the G_1 -S and G_2 -M transitions in this species also, or at least exists in two distinct forms during G_1 and G_2 . By inactivating *nimT^{cdc25}* only one particular function of $p34^{cdc2}$ is therefore impaired. However, direct inactivation of $p34^{cdc2}$ is likely to impair all of its functions, both in G_1 -S and in G_2 -M. It can therefore be concluded that the mitotic form of $p34^{cdc2}$ /cyclin B is not required for expression of *nimA* but that some other, non-mitotic, form of $p34^{cdc2}$ is essential for *nimA* expression, presumably a form that is required for progression through G_1 -S- G_2 . The level at which $p34^{cdc2}$ is required for expression of active NIMA is currently not known.

ROLE OF PHOSPHORYLATION IN NIMA REGULATION

The kinase activity of NIMA is regulated not only through the cell cycle by accumulation and degradation of NIMA protein, but also by phosphorylation [38]. Both recombinant NIMA and that isolated from *A. nidulans* are inactivated by enzymic dephosphorylation. This suggests that NIMA may need to be activated by a NIMA-activating kinase. A potential activating phosphorylation site, analogous to the autophosphorylation site of cAMP-dependent protein kinase, has been defined by mutation in NIMA [61]. This site is required for normal activation of the cAMP-dependent kinases [62], and mutation of the analogous site (Thr-199) in NIMA to a non-phosphorylatable residue also renders NIMA non-functional and inactive as a casein kinase [61]. It is noteworthy that the proposed activating phosphorylation site in NIMA is conserved in all NIMA-related kinases and that this site conforms to a minimal NIMA phosphorylation site [37] (FXXT/S) in NIMA and some NIMA-related kinases. This suggests that this residue may be a site for autophosphorylation. Indeed, when expressed in *E. coli*, NIMA undergoes autophosphorylation and so activates itself. However, if dephosphorylated *in vitro* and then subjected to autophosphorylation, NIMA is unable to activate itself as a kinase [36]. This suggests that NIMA has a limited capacity to activate itself by autophosphorylation, leaving open the possibility that there is a NIMA-activating kinase required for its efficient activation in *A. nidulans*.

Biochemical analyses of NIMA thus indicate that it could be regulated by phosphorylation, and recent evidence suggest that this is indeed the case. The level of NIMA phosphorylation has been followed during the cell cycle of *A. nidulans* [38] by observing mobility shifts on SDS/PAGE and by probing with the MPM-2 monoclonal antibody, which detects certain mitosis-specific phosphoproteins [63]. Two major changes in the phosphorylation state of NIMA occur during the cell cycle (Figure 3). NIMA is synthesized early in interphase in an apparently

Figure 3 Degradation and phosphorylation of NIMA through the cell cycle

During G_1 and S phase, NIMA is in low abundance and may not be phosphorylated. During G_2 , NIMA becomes phosphorylated and its level increases. During the G_2 -M transition NIMA is hyperphosphorylated and becomes reactive to the MPM-2 monoclonal antibody that detects mitotic phosphoproteins. As cells exit from mitosis, NIMA is proteolytically destroyed.

unphosphorylated form during S phase. It then becomes phosphorylated during G_2 , which can be visualized as a small retardation of NIMA on SDS/PAGE, and this phosphorylation allows partial activation of NIMA as a protein kinase. This initial site of phosphorylation is likely to be Thr-199. Upon the G_2 -M transition, and only after activation of p34^{cdc2}/cyclin B, NIMA becomes hyperphosphorylated and shows marked retardation on SDS/PAGE. The hyperphosphorylation of NIMA further activates its level of activity in G_2 and also generates the MPM-2 epitope on NIMA. Soon after its hyperphosphorylation during mitosis, NIMA is proteolytically destroyed (Figure 3).

The phosphorylation of NIMA during mitosis, which generates the MPM-2 antigen, is not only dependent upon activation of p34^{cdc2}/cyclin B but may be carried out directly by the mitotic form of the p34^{cdc2} kinase. This is suggested because, *in vitro*, phosphorylation of a kinase-negative version of NIMA by p34^{cdc2}/cyclin B causes the molecular mass shift of NIMA detected on SDS/PAGE and also generates the MPM-2 antigen [38]. Thus, using purified p34^{cdc2}, it is possible to reconstruct *in vitro* the characteristics of mitotic NIMA phosphorylation promoted after activation of p34^{cdc2} H1 kinase *in vivo*. This role for p34^{cdc2} in the function of NIMA was initially not observed [22] because NIMA is very sensitive to inactivation by dephosphorylation during isolation procedures. However, incorporation of okadaic acid into isolation buffers was found to

markedly stabilize the activity of NIMA during its isolation [38]. This suggests the existence of an okadaic acid-sensitive phosphatase that has high activity towards NIMA, perhaps at the proposed Thr-199 phosphorylation site.

NIMA DEGRADATION DURING MITOTIC EXIT

The level of NIMA protein falls dramatically during progression through mitosis and is stabilized if cells are arrested in mitosis [38]. Mitotic destruction of NIMA may therefore be important for mitotic exit, and data have been presented to support this notion. A C-terminal truncated form of NIMA has a considerably longer half-life than full-length NIMA when expressed in *A. nidulans*. The truncated protein retains the ability to promote mitosis but is resistant to degradation during mitotic progression and is therefore highly toxic [39,64]. The importance of the mitotic degradation of NIMA has been investigated by expression of the truncated NIMA in cells arrested at G_2 [64]. The level of expression was kept low so that the truncated NIMA did not promote mitotic events. Then, by releasing the G_2 arrest, the ability of cells to traverse mitosis in the presence of non-degradable NIMA was determined. The cells were able to enter mitosis but were unable to complete it in the normal fashion. This indicates that mitotic proteolysis of NIMA is required for mitotic exit [64].

NIMA is not the first protein to be identified that is specifically destroyed during mitosis and whose destruction is required for mitotic exit. The first such protein identified was cyclin B [65–70]. The role of cyclin B in mitotic regulation has been well documented in many systems, including *A. nidulans*, both biochemically and genetically [10,53,59,60,71–87]. It functions as an essential subunit of the p34^{cdc2}/cyclin B H1 kinase, acting to first activate the p34^{cdc2} H1 kinase during interphase and then to inactivate this kinase during mitosis by being proteolytically destroyed. The N-terminus of cyclin B contains a conserved motif, called the cyclin destruction box, which acts to target cyclin B for ubiquitin-mediated proteolysis during mitosis. The cyclin destruction box acts to target lysine residues for poly-ubiquitination specifically during mitosis. The poly-ubiquitinated cyclin B is then thought to be proteolysed by a 26 S proteasome complex [88–90]. Thus, as with NIMA, truncation of cyclin B to remove the cyclin destruction box renders the protein mitotically stable (due to the lack of ubiquitination during mitosis) which then prevents normal mitotic exit.

The relationship between the mitotic destruction of NIMA and cyclin B remains to be elucidated. However, these two proteins, although both degraded during mitosis, are proteolysed with different kinetics, with cyclin B being degraded earlier than NIMA, perhaps suggesting different mechanisms of degradation [64]. The C-terminal domain of NIMA that contains determinants of instability does not contain a cyclin B-type destruction box, so NIMA is unlikely to be degraded by a mechanism involving this type of motif. However, the C-terminal portion of NIMA that is involved in specifying rapid degradation is rich in PEST sequences. Such motifs are rich in proline (P), glutamate (E), serine (S) and threonine (T) residues, and are involved in targeting proteins for destruction [91].

Recent work investigating the stability of the *S. cerevisiae* G_1 cyclin Cln3, and the transcription factor Gcn4, suggests that PEST sequences may be another type of signal that targets proteins for ubiquitin-mediated proteolysis [92]. Cln3 is very unstable and is essential for progression through Start in the G_1 phase of *S. cerevisiae*. It is also rich in PEST sequences in its C-terminus, and mutations that remove the PEST sequences stabilize Cln3 and also shorten G_1 [93,94].

It has been shown that the PEST-containing region of Cln3 can confer instability to a reporter protein, such as β -galactosidase, and that degradation occurs through the ubiquitin-mediated pathway of degradation [92]. Similarly, the transcription factor Gcn4 is degraded via the ubiquitin-mediated pathway and also contains PEST sequences important to its instability [95]. NIMA may therefore be degraded by the ubiquitin pathway of proteolysis during mitosis, or potentially G_1 , by a mechanism directed by PEST sequences. What makes this possibility even more interesting is that it has been proposed that PEST sequences may act to couple phosphorylation with ubiquitination, as Cln3 protein is stable if the Cdc28 kinase is inactivated. Furthermore, if a Cdc28 consensus phosphorylation site is mutated within the PEST domain of the Cln3- β -galactosidase fusion protein, this protein is stabilized. Therefore Cdc28-mediated phosphorylation may target Cln3 for ubiquitination and subsequent degradation [92]. This scenario has obvious similarities to the situation with NIMA degradation. NIMA also contains PEST sequences which have been implicated in its mitotic degradation, and NIMA is also phosphorylated by a Cdc2(28)-dependent mechanism just prior to its degradation (Figure 3). NIMA also contains numerous Cdc2 phosphorylation sites in the region rich in PEST sequences. Thus phosphorylation of NIMA during mitosis may mark it for ubiquitin-dependent proteolysis in a manner analogous to the PEST-directed degradation of Cln3 during G_1 .

Two lines of evidence suggest that proteins other than cyclin B need to be degraded in order for cells to complete anaphase. Using mitotic extracts derived from *Xenopus* oocytes it has been shown that the addition of non-degradable cyclin B does not prevent initiation of anaphase [67]; nor does stable cyclin B prevent anaphase progression in *S. cerevisiae* [96]. However, if ubiquitin-mediated proteolysis is inhibited then anaphase cannot be initiated in *Xenopus* extracts [67]. This indicates that proteins other than cyclin B need to be degraded during mitosis by the ubiquitin pathway of degradation in order for anaphase to be initiated.

Secondly, mutation of either *CDC16* or *CDC23* stabilizes the Clb2 B-type cyclin in *S. cerevisiae* during anaphase and G_1 , suggesting that they are involved in the degradation pathway leading to destruction of Clb2 [97]. Both Cdc16 and Cdc23 are found in a complex with Cdc27 in *S. cerevisiae* [98], and each is a member of a family of proteins termed the TPR [99,100] proteins. The TPR (tetratricopeptide repeat) motif is thought to be involved in protein-protein interactions. Inactivation of any one of these three TPR proteins prevents the onset of anaphase in organisms ranging from fungi to humans [11,97,101,102]. In addition, both Cdc27 and Cdc16 have been found by biochemical analysis to be components of a large multi-protein complex which acts to ligate ubiquitin to cyclin B during mitosis and has been called the anaphase-promoting complex (APC) [103]. However, although the APC can function as a mitosis-specific cyclin B ubiquitin ligase, this complex may target other proteins for mitotic degradation and so promote anaphase, as cyclin B degradation is not required to initiate anaphase [67,96]. In addition, a mutation of *CDC27* in *S. cerevisiae* does not stabilize cyclin B but does arrest cells prior to anaphase [97], further implicating the proteolysis of non-cyclin proteins in the initiation of anaphase.

It has been proposed that some proteins active in binding sister chromatids together during early mitosis are degraded during the metaphase-to-anaphase transition [67]. Alternatively a regulatory protein, such as NIMA, may function to promote sister chromatid attachment, and destruction of NIMA may then consequently allow sister chromatid separation during anaphase. Further study will reveal the relationship between NIMA degra-

dation and the ubiquitin pathway of proteolysis and the onset of anaphase.

There is another relationship evident between cyclin B and NIMA, as activation of each is apparently required for the degradation of the other. For instance, if cells are arrested in G_2 by inactivation of NIMA, then cyclin B is stable, even though the p34^{cdc2}/cyclin B H1 kinase activity is at an elevated mitotic level. Only when NIMA is activated do cells enter mitosis and trigger cyclin B degradation. This suggests that activation of the degradation pathway leading to the destruction of cyclin B requires mitotic NIMA kinase activity. Conversely, if cyclin B is inactivated then NIMA protein accumulates and is stable, and only becomes unstable after activation of p34^{cdc2} H1 kinase activity. Using the same logic, it is clear that p34^{cdc2}/cyclin B activation helps to trigger cyclin B degradation and that NIMA activation helps to trigger NIMA degradation. It appears that some common triggering mechanism, which becomes activated only after mitotic activation of both NIMA and p34^{cdc2}/cyclin B protein kinases, may be responsible for the degradation of both cyclin B and NIMA. The ubiquitin ligase for cyclin B (APC [103] or cyclosome [104]; see above) is regulated by p34^{cdc2}/cyclin B kinase [78,103,105] and, given that NIMA activation is required for cyclin B degradation, it is possible that NIMA could also play a role in regulating the APC.

Further insight into the mode of degradation of NIMA may be obtained utilizing mutations of *bimA*, a functional homologue of *CDC27*. Temperature-sensitive mutations of *bimA* cause arrest in metaphase at the restrictive temperature [2,11]. Affinity-purified antibodies directed against BIMA^{CDC27} (the protein product of *bimA*) detect both BIMA^{CDC27} and a post-translationally modified form of BIMA^{CDC27} on Western blotting [106]. A similar situation has been reported for the *Xenopus* counterpart of BIMA^{CDC27} (*Xenopus* CDC27), with the higher-molecular-mass form corresponding to phosphorylated *Xenopus* CDC27 [103]. The phosphorylation of *Xenopus* CDC27 occurs during mitosis and may function to activate its ubiquitin ligase activity. It will be interesting to determine if BIMA^{CDC27} is also phosphorylated during mitosis and to ascertain the potential role of NIMA in this phosphorylation.

BIMA^{CDC27} was found to be localized to the spindle pole body in *A. nidulans* [106], and human CDC27 has a similar subcellular localization, being localized to the centrosome and also the mitotic spindle [102]. These proteins are localized to the spindle pole body or centrosome throughout the cell cycle, further suggesting that their mitotic function may be regulated by post-translational modification after activation of both p34^{cdc2} and NIMA.

The kinase activity of NIMA is thus subjected to several levels of regulation which ensure that it is activated at the appropriate time during transition through interphase and that it is then irreversibly inactivated during mitosis, presumably to guarantee that cells do not attempt a second mitosis before DNA has been replicated (Figure 3). There are obvious parallels that can be drawn between the regulation of mitotic p34^{cdc2} and NIMA activities. The levels of both kinase activities fluctuate through the cell cycle in very similar ways, peaking during G_2 and early mitosis and then being inactivated during mitotic exit. The kinase activity of mitotic p34^{cdc2} depends upon binding to cyclin B, but no essential partner protein is known for NIMA; however, it may need to form a homo-oligomer for activity. Both kinases are regulated by cell-cycle-specific phosphorylation and both are also irreversibly inactivated during mitosis by proteolysis. For the mitotic p34^{cdc2}/cyclin B complex, inactivation by proteolysis functions through destruction of the activating cyclin subunit, but for NIMA the whole kinase is destroyed. These two kinases

are therefore regulated by very similar mechanisms to achieve similar patterns of activity through the cell cycle.

ROLE OF NIMA DURING MITOSIS

By studying the conditional loss of function alleles of *nimA*, it has been shown that inactivation of NIMA still allows p34^{cdc2} to become fully activated as an H1 kinase, but this activated form of p34^{cdc2} cannot induce any cytological aspects of mitosis, such as chromosome condensation or spindle formation [22]. This demonstrates that NIMA plays no role in the activation of p34^{cdc2} as an H1 protein kinase. It does not, however, preclude the possibility that NIMA function is required to stimulate the mitosis-promoting functions of activated p34^{cdc2} H1 kinase. For instance, active p34^{cdc2} may not be able to get to its mitotic substrates in the nucleus in the absence of NIMA function. Alternatively, key mitotic substrates of p34^{cdc2} may have to be phosphorylated not only by p34^{cdc2} but also by NIMA in order to manifest mitotic events. If NIMA has no role in stimulating the mitosis-promoting activity of p34^{cdc2} (which seems unlikely), this would place NIMA function downstream of p34^{cdc2}, making NIMA the kinase responsible for the phosphorylation of all proteins required to promote mitosis, a role currently thought to be played directly by p34^{cdc2}.

As NIMA expression is dependent on p34^{cdc2}, and NIMA is phosphorylated during mitosis after activation of mitotic p34^{cdc2}, it is likely that p34^{cdc2} promotes mitosis, at least in part, by activating NIMA, which may then promote some specific aspects of mitosis (Figure 4). This favours the view that p34^{cdc2} promotes mitosis by regulating at least one other mitotic regulator rather than by directly causing mitotic events such as chromosome condensation and spindle formation. Another kinase shown to act downstream of p34^{cdc2} during mitosis is the Plo1 kinase of *S. pombe* [107]. This kinase, which is related to the Polo kinase of *Drosophila* [108] and Cdc5 kinase of *S. cerevisiae* [109], plays a role in spindle formation and septum formation. When overexpressed in the absence of p34^{cdc2} function Plo1 is able to promote septum formation, suggesting that, like NIMA, the Plo1 kinase is responsible for a particular part of the transition through G₂-M-G₁ which is normally only executed after activation of mitotic p34^{cdc2}. However, lack of Plo1 (or Polo or Cdc5) does not prevent initiation of mitosis; it leads to an

abnormal mitosis. In contrast, lack of NIMA actually prevents the initiation of mitosis.

The initiation of S phase in *S. cerevisiae*, as in *S. pombe* [54] and *A. nidulans* [49], is also controlled by a p34^{cdc2} homologue, Cdc28^{cdc2} [57]. Cdc28^{cdc2} promotes S phase by regulating the expression of a range of genes involved in the enzymology of DNA replication [109,110] and also by directly or indirectly activating a second protein kinase, Cdc7 [111–116]. Lack of Cdc7 prevents initiation of S phase after activation of Cdc28^{cdc2} [117]. This suggests that a second commitment point exists, after activation of Cdc28^{cdc2} during G₁, through which yeast cells have to pass in order to initiate DNA replication. This situation is analogous to the requirement for the NIMA kinase to promote mitosis after activation of p34^{cdc2}/cyclin B in *A. nidulans*. This suggests that cyclin-dependent protein kinases can promote different cell-cycle-specific events not only by promoting the expression of different cell-cycle-specific genes but also by activating other cell-cycle-specific protein kinases.

One mitosis-specific role of NIMA may be to promote chromatin condensation, which it is able to do in the absence of p34^{cdc2} when overexpressed [38]. However, it has long been accepted that the way in which p34^{cdc2} mediates chromosome condensation is by direct mitosis-specific phosphorylation of histone H1. This conclusion is based largely upon the close correlation observed between p34^{cdc2} H1 kinase activation and chromosome condensation, and on the observation that the sites of phosphorylation of histone H1 *in vivo* are the same as those phosphorylated by p34^{cdc2} *in vitro* (see Guo et al. [118] for references). The likelihood that p34^{cdc2} directly causes chromosome condensation by phosphorylation of histone H1 has, however, been recently questioned. Removal of histone H1 by immunoprecipitation does not prevent chromosome condensation in *Xenopus* mitotic extracts, suggesting that histone H1 does not play an essential role in chromosome condensation [119]. Secondly, it has been shown that complete inactivation of mitotic p34^{cdc2} does not affect chromosome condensation induced by phosphatase inhibitors [118]. These studies were carried out using the FT210 mouse cell line, which contains temperature-sensitive p34^{cdc2} and arrests in G₂ at the restrictive temperature. Treatment of the G₂-arrested cells with the phosphatase inhibitors fostriecin or okadaic acid causes full chromosome condensation in the absence of p34^{cdc2} activation and in the absence of any detectable histone H1 phosphorylation. Interestingly, chromosome condensation in this system was sensitive to inhibition of protein kinases using the protein kinase inhibitor staurosporine [118]. This indicates that a staurosporine-sensitive kinase, whose activity can be stabilized by okadaic acid, may be responsible for mediating chromosome condensation. One excellent candidate kinase with appropriate characteristics is NIMA. Use of dominant-negative versions of NIMA, as described below, should help to investigate this possibility.

Figure 4 Relationship between p34^{cdc2} and NIMA

A form of p34^{cdc2} that functions during interphase is required for the expression of NIMA. For initiation of mitosis p34^{cdc2} binds to cyclin B to form pre-mitosis promoting factor (pre-MPF). Activation of pre-MPF by tyrosine dephosphorylation to form MPF then leads to the hyperphosphorylation of NIMA. Expression of NIMA from an inducible promoter to a high level can promote chromatin condensation in the absence of active p34^{cdc2} in both *A. nidulans* and human cells, suggesting that this protein normally plays a direct role in chromosome condensation. Induction of NIMA has also been shown to promote spindle formation.

IS THERE A NIMA-LIKE PATHWAY OF MITOTIC REGULATION IN OTHER EUKARYOTES?

Although several NIMA-related protein kinases have been identified in other eukaryotic cells, including humans, only one functional homologue has so far been isolated, that from another filamentous ascomycete, *N. crassa* [61], termed *nim-1*. *N. crassa nim-1* was isolated by hybridization using *nimA* cDNA as a probe, and a single copy of *nim-1* is able to fully complement a temperature-sensitive mutation in *nimA*. Although highly conserved over their catalytic domains, NIMA and NIM-1 are not as highly conserved in their C-terminal domains. This therefore suggests that homologues in higher eukaryotes are

Figure 5 Mutational analysis defining residues ~300–400 of NIMA as functionally important

Potential functional domains are depicted as in Figure 2. Mutation of Lys-40 to Met inactivates the kinase activity of NIMA and generates a dominant-negative version of NIMA that causes G_2 arrest. Deletion of residues 345–395 renders the kinase-negative NIMA unable to affect cell cycle progression. Truncation of NIMA C-terminal to residue 381 generates a dominant-positive version of NIMA that is toxic and causes mitotic arrest. However, further removal of residues 295–381 eliminates the toxicity of the truncated NIMA which is then unable to promote mitosis.

unlikely to be significantly conserved outside their catalytic domains at the primary amino acid sequence level.

The isolation of the functional homologue *nim-1* from *N. crassa* indicates that a *nimA* pathway of regulation exists in other multicellular filamentous fungi. The situation in unicellular fungi is less clear, as the *nimA*-related KIN3 protein [34,120,121] of budding yeast is non-essential. This perhaps indicates a redundant function for KIN3 which would suggest the existence of other *nimA*-related kinases in yeast. The isolation of synthetically lethal mutants in the KIN3-deleted strain may therefore identify other *nimA*-related kinases in yeast, or, perhaps, as with the cyclin genes of *S. cerevisiae*, multiple NIMA-related genes will have to be deleted to observe the phenotype caused by loss of NIMA function.

No complementation of *nimA* function in *A. nidulans* has as yet been reported using either higher eukaryote or yeast *nimA* homologues. These kinases have been termed *nek* (for *nimA*-related kinase) or *nrk* (also for *nimA*-related kinase), due to their protein sequence similarity with *A. nidulans* NIMA [30,31,33,40,122,123]. Two such genes have also been isolated from *Trypanosoma* [32]. As mentioned above, the yeast KIN3 kinase is not essential, but there is little functional information regarding the role of other NIMA-related kinases. Mouse *nek1* is highly expressed in germline cells, suggesting a role in meiosis [30]. The level of human Nek2 protein and activity is regulated through the cell cycle, reaching a maximum during S/ G_2 ,

suggesting that it may play a role earlier in the cell cycle than does NIMA. These data are suggestive of a cell-cycle-specific function for NIMA-related kinases, but none of those so far isolated and studied have the characteristics of a mitosis regulator such as NIMA.

Several lines of indirect evidence indicate that a NIMA-related pathway of mitotic regulation does exist in other cell types. Expression of NIMA in *A. nidulans* from a strong inducible promoter induces several mitosis-specific responses. Cytoplasmic microtubules are depolymerized, abnormal mitotic spindles are transiently formed [8] and chromatin becomes condensed [38]. A more recent study, involving the expression of a truncated form of NIMA, observed similar effects, although spindles were not noted upon induction of truncated NIMA [39]. This may indicate that the C-terminus of NIMA has an essential function in spindle formation. It is clear, however, that induction of NIMA can promote mitotic events in the absence of $p34^{cdc2}$ and can therefore promote chromatin condensation in cells arrested in S phase or in cells lacking $p34^{cdc2}$ function [8,38,39].

Expression of NIMA in *S. pombe*, *Xenopus* oocytes or HeLa cells can also induce mitotic events, most notably chromatin condensation, from any point in the cell cycle [39,40]. The ability of induced NIMA to promote chromatin condensation across such wide species boundaries strongly indicates conservation of NIMA substrates in all eukaryotes. Furthermore, phosphorylation of these substrates by NIMA has the same

Figure 6 Coiled-coil domains found in NIMA and related protein kinases

effect of condensing the chromatin of fungi and human cells, strongly indicating the presence of a NIMA pathway of mitotic regulation in higher eukaryotes [40].

Further support for the existence of a NIMA pathway of mitotic regulation has been obtained by the use of dominant-negative alleles of NIMA (Figure 5). It is possible to generate a dominant-negative form of NIMA by inactivating its protein kinase activity. This has been done by mutating the catalytic site of NIMA and then expressing the kinase-negative form of NIMA from an inducible promoter in *A. nidulans*, which leads to a specific arrest of the cell cycle in G_2 in a manner identical to that induced by the temperature-sensitive alleles of *nimA* [124]. The dominant-negative allele is therefore able to interfere with wild-type NIMA function, leading to G_2 arrest. It has been suggested that the effect of kinase-negative NIMA is not caused by its ability to complex with wild-type NIMA to generate a kinase-inactive complex. Rather, the dominant-negative effects are thought to occur by the binding of the mutant NIMA to a docking site in the cell normally occupied by NIMA, which thus prevents wild-type NIMA from working correctly. The dominant-negative phenotype can also be generated by expression of the C-terminal portion of NIMA which lacks the kinase domain, suggesting that the proposed docking site resides in the C-terminus [124].

The dominant-negative alleles of *nimA* have been shown to affect cell cycle progression in a human cell line [40]. Transient expression of kinase-negative NIMA, or the C-terminal portion of NIMA, led to an increase in the percentage of cells in G_2 . By deletion analysis a small region of NIMA just C-terminal to the catalytic domain (residues 345–395) has been implicated in the ability both of dominant-negative alleles of NIMA to cause G_2 delay and of wild-type NIMA to promote mitotic events in human cells (Figure 5). This region also contains two potential bipartite nuclear localization signals which may be functional, as NIMA expressed in human cells is localized to the nucleus. Removal of the nuclear localization motifs both decreases the nuclear localization of active NIMA and eliminates the chromatin condensation phenotype. Their removal also impairs the dominant-negative effects of inactive NIMA. Nuclear localization of NIMA is therefore potentially important to its function. However, artificially targeting truncated NIMA to the nucleus, by tagging it with a Simian Virus 40 nuclear localization sequence, did not restore the capacity of truncated NIMA to promote mitosis. Some other structural feature of NIMA, in

addition to the nuclear localization signal, is therefore important for its ability to promote mitotic events [40].

Truncation analysis of NIMA in *A. nidulans* also identified a region in the C-terminus of the kinase domain (residues 295–381) as being important to NIMA function [64]. As noted above, deletion of the C-terminal domain of NIMA generates a stable protein kinase which promotes mitotic events and, due to its increased stability, is very toxic. However, if this construct undergoes further deletion to leave just the kinase domain then the protein is no longer toxic and it cannot functionally replace *nimA5*. Clearly the non-catalytic domain of NIMA in the region of residues 295–400 contains important functional features. One such motif, found not only in NIMA but also in several NIMA-related kinases (NIM-1, NEK1, NEK2 and KIN3), is coiled-coil domains (Figure 6) [125]. Among other functions, such motifs have been shown to be involved in both hetero- and homo-dimerization [126,127]. When one considers the similarities of the phenotypes caused by overexpression of active NIMA, or of dominant-negative NIMA, in fungi and human cells, the potential binding partner(s) for these coiled-coil domains are of great interest as these may be conserved components of cell cycle regulation. It is likely that such binding partners will soon be isolated, either in genetic screens or by utilizing the two-hybrid system in yeast.

We thank Henk-Jan Bussink, Liping Wu, Russell Fincher, Aysha Osmani and Matthew Puncher for their comments on this Review, and Gang Xu for the coiled-coil analysis. This work was supported by NIH grant GM 42564 and by funds from the Geisinger Clinic Foundation.

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