Regulation of the Anaphase-promoting Complex/Cyclosome by *bimA*^{APC3} and Proteolysis of NIMA

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Submitted June 16, 1998; Accepted August 19, 1998
Monitoring Editor: Marc W. Kirschner

Surprisingly, although highly temperature-sensitive, the *bimA*^{APC3} anaphase-promoting complex/cyclosome (APC/C) mutation does not cause arrest of mitotic exit. Instead, rapid inactivation of *bimA*^{APC3} is shown to promote repeating oscillations of chromosome condensation and decondensation, activation and inactivation of NIMA and p34^{cdc2} kinases, and accumulation and degradation of NIMA, which all coordinately cycle multiple times without causing nuclear division. These *bimA*^{APC3}-induced cell cycle oscillations require active NIMA, because a *nimA5* + *bimA*^{APC3} double mutant arrests in a mitotic state with very high p34^{cdc2} H1 kinase activity. NIMA protein instability during S phase and G2 was also found to be controlled by the APC/C. The *bimA*^{APC3} mutation therefore first inactivates the APC/C but then allows its activation in a cyclic manner; these cycles depend on NIMA. We hypothesize that *bimA*^{APC3} could be part of a cell cycle clock mechanism that is reset after inactivation of *bimA*^{APC3}. The *bimA*^{APC3} mutation may also make the APC/C resistant to activation by mitotic substrates of the APC/C, such as cyclin B, Polo, and NIMA, causing mitotic delay. Once these regulators accumulate, they activate the APC/C, and cells exit from mitosis, which then allows this cycle to repeat. The data indicate that *bimA*^{APC3} regulates the APC/C in a NIMA-dependent manner.

INTRODUCTION

The *bimE* and *bimA* genes were originally defined by temperature-sensitive mutations in functions required for normal progression through mitosis in *Aspergillus nidulans* (Morris, 1976; Osmani *et al.*, 1988; Engle *et al.*, 1990; O'Donnell *et al.*, 1991) and were subsequently found to encode highly conserved proteins whose homologues have been isolated from many organisms ranging from fungi to humans (Hirano *et al.*, 1990, 1994; Mirabito and Morris, 1993; Lamb *et al.*, 1994;

Starborg *et al.*, 1994; King *et al.*, 1995; Tugendreich *et al.*, 1995). Most significantly, they were recently identified as components of the anaphase-promoting complex (APC) (King *et al.*, 1995; Peters *et al.*, 1996; Zachariae *et al.*, 1996). The APC is also known as the cyclosome (Sudakin *et al.*, 1995) and is therefore abbreviated here to APC/C (see Townsley and Ruderman, 1998, for review).

bimE encodes the largest subunit of the APC/C and has recently been termed APC1 (Peters *et al.*, 1996). We will use the designation *bimE*^{APC1} for the gene and BIME^{APC1} for the protein. *bimA* encodes a member of the tetratricopeptide repeat family of proteins, being most similar to *Schizosaccharomyces pombe* *nuc2* (Hirano *et al.*, 1990, 1994) and *Saccharomyces cerevisiae* CDC27 (Sikorski *et al.*, 1990; Lamb *et al.*, 1994), which was recently termed APC3 (Peters *et al.*, 1996). The

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APC/C functions as an E3 ubiquitin ligase, which specifically targets proteins (such as mitotic cyclins) for degradation in a destruction box (D box)-dependent manner (Glutzer *et al.*, 1991; King *et al.*, 1996b) through the ubiquitin-proteasome pathway, to promote initiation of anaphase and exit from mitosis into G1 (for reviews see Murray, 1995; King *et al.*, 1996a; Hershko, 1997; Townsley and Ruderman, 1998).

APC/C activity is cell cycle regulated (Hershko *et al.*, 1994; Zachariae and Nasmyth, 1996) potentially by reversible phosphorylation (Lahav-Baratz *et al.*, 1995) of certain subunits (Peters *et al.*, 1996). Recent data indicate that Polo-related kinases play a role in the activation of the APC/C during mitosis (Charles *et al.*, 1998; Descombes and Nigg, 1998; Shirayama *et al.*, 1998). In *S. cerevisiae*, the APC/C is activated during the metaphase to anaphase transition and remains active during G1 (Amon *et al.*, 1994), as also occurs in mammalian cells (Brandeis and Hunt, 1996). As cells progress into S phase from G1, mitosis-specific APC/C activity is turned off to allow accumulation of mitotic cyclins in preparation for the next mitosis.

It has been shown that the APC/C subunit BIME^{APC1} also has an interphase function to negatively regulate mitosis, as loss of BIME^{APC1} overrides the *nimA5* G2 arrest and, in combination with non-tyrosine-phosphorylated p34^{cdc2}, promotes premature mitosis from S phase (Osmani *et al.*, 1988; James *et al.*, 1995; Ye *et al.*, 1996). It has been proposed that some checkpoint controls over mitosis are switched from the APC/C during G1 to include tyrosine phosphorylation of p34^{cdc2} upon initiation of DNA replication (Ye *et al.*, 1997a). BIME^{APC1} may therefore normally play a role to prevent activation of NIMA kinase, and other mitotic regulators, during interphase to prevent premature mitosis.

The levels of NIME^{cyclinB} and NIMA proteins oscillate once each cell cycle in a strikingly similar manner (Evans *et al.*, 1983; Alfa *et al.*, 1990; Draetta *et al.*, 1990; Ghiara *et al.*, 1991; Hunt *et al.*, 1992; Richardson *et al.*, 1992; Grandin and Reed, 1993; Pu and Osmani, 1995; Ye *et al.*, 1995; Yamano *et al.*, 1996). Both proteins accumulate during late S to G2, peak at early M, and are rapidly degraded as cells progress out of mitosis with slightly different kinetics (Pu and Osmani, 1995; Ye *et al.*, 1995). If cells are arrested at mitosis, for instance by the *bimE7*^{APC1} mutation or by addition of nocodazole, then both NIME^{cyclinB} and NIMA proteins remain stable (Ye *et al.*, 1995). Additionally, degradation-deficient forms of either cyclin B (Murray *et al.*, 1989; Luca *et al.*, 1991; Gallant and Nigg, 1992; Surana *et al.*, 1993) or NIMA (O'Connell *et al.*, 1994; Pu and Osmani, 1995) prevent normal exit from mitosis. This raises the possibility that the degradation of NIME^{cyclinB} and NIMA proteins may be mediated, at least partly, by the same proteolytic pathway. Although the mitosis-specific proteolysis of cyclin B

through D box-directed ubiquitination has been well characterized in several systems (see Murray, 1995, for review), how NIMA kinase is specifically degraded during M phase progression is not understood. The determinants for mitotic instability of NIMA reside in its C-terminal domain, which contains PEST sequences and p34^{cdc2} phosphorylation sites (O'Connell *et al.*, 1994; Pu and Osmani, 1995).

In the present work, experiments using APC/C mutations and biochemical analysis of NIMA stability uncover a regulatory role for BIMA^{APC3} involving APC/C-dependent instability of NIMA.

MATERIALS AND METHODS

Aspergillus Strains and General Techniques

Strains of *A. nidulans* used in this study were R153 (*pyroA4; wA3*); GR5 (*pyrG89; pyroA4; wA3*); SO1 (*bimE7; pyrG89; pyroA4; wA2*); SO4 (*bimE7; pabaA1; wA2*); SO15 (*nimA5; bimE7; pabaA1; riboB2; wA2*); SO54 (*nimA5; wA2*); 5C (*alcA::nimA, pyr4⁺; pyrG89; benA22; pabaA1; fwA1*); RP5 (*alcA::nimA; pyrG89, pyr4⁺; nicB8; riboA1; yA2*); MAT68 (*alcA::nimE at argB; pabaA1*); AT62 (*bimA1; alcA::nimA, pyr4⁺; pyrG89; choA1; pabaA1*); AT92 (*alcA::nimE at argB; choA1; yA2*); AT114 (*bimE7; alcA::nimE at argB; yA2*); AT120 (*bimA1; alcA::nimE at argB; choA1; yA2*); AT128 (*bimE7; alcA::nimA, pyr4⁺ pyrG89; wA2*); and PM156 (*bimA1; pabaA1; wA2*). Media and general techniques for culture, transformation, light microscopy, protein extraction, immunoprecipitation, protein kinase assays, and Western blotting were as previously described (Ye *et al.*, 1995). NIMA and p34^{cdc2} H1 kinase activities were quantified, based on incorporation of ³²P into their *in vitro* substrates β -casein and histone H1, using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The relative amount of NIMA and NIME^{cyclinB} was measured after Western blotting with the use of a densitometer and ImageQuant software (Molecular Dynamics).

nimA Deletion

A 5.0-kb *KpnI* fragment containing the *nimA* open reading frame was cloned into pGEM giving rise to plasmid pWC1. A *PstI* fragment, 129 bp upstream of the start codon and 83 bp before the stop codon of *nimA*, was replaced with the *PstI* fragment of the *pyr4* gene from *Neurospora crassa*, which complements the *pyrG89* mutation of *A. nidulans*. Two plasmids containing opposite orientations of the *pyr4⁺* gene were obtained called *pnimA Δ A* and *pnimA Δ B*. A linear *KpnI* fragment from *pnimA Δ B*, as shown in Figure 7A, was used to delete *nimA* by transformation of GR5 and SO1.

Transformants were screened for heterokaryons (Osmani *et al.*, 1988) by streaking conidiospores (conidia) onto selective YAG without uridine and uracil. If a transformant is maintained as a heterokaryon conidia derived from such a transformant, it will not be able to grow in the absence of uridine and uracil. This is because conidia containing the parental nuclei (they are uninucleate) will not be able to germinate in the absence of uridine and uracil because of the *pyrG89* mutation. On the other hand, conidia containing deleted *nimA*, which can germinate in the absence of uridine and uracil because of the *pyr4⁺* gene, will not be able to undergo nuclear division and will cease growth as a single nucleate germling (Osmani *et al.*, 1988). Suspected heterokaryons were maintained as mycelial colonies on selective medium.

Southern blot analysis of selected heterokaryons was carried out to confirm specific *nimA* deletion. Isolated DNA was digested to completion with *XhoI*, separated by electrophoresis, and blotted onto nylon membranes. A 0.8-kb *KpnI*-*PstI* fragment was isolated from pWC1 and was used as probe for random primer labeling.

Figure 1. Temperature sensitivity of *bimA1*^{APC3} and *bimE7*^{APC1} strains. Strains containing no temperature-sensitive mutation (R153) or *bimA1*^{APC3} (PM156) or *bimE7*^{APC1} (SO4) were replica plated and incubated at permissive temperature (30°C) or semirestrictive temperature (35°C) for 3 d. Greater temperature sensitivity caused by *bimA1*^{APC3} when compared with *bimE7*^{APC1} is apparent.

Hybridization conditions were as previously described (Ye *et al.*, 1996).

Temperature Shifts

We usually do temperature upshift to 42°C by vigorously shaking the culture in a 60°C water bath. In this study, we also harvested *Aspergillus* cells by vacuum filtration through a layer of Miracloth (Calbiochem-Novabiochem, La Jolla, CA) and then transferred the cells directly to prewarmed medium at 42°C to cause instant temperature change of the culture.

Induction and Repression of NIME^{cyclinB} and NIMA Expression from the *alcA* Promoter

Induction and repression of NIMA and NIME^{cyclinB} expression from the *alcA* promoter were as previously described (Pu and Osmani, 1995). In this study, we also incorporated hydroxyurea (100 mM) into the medium to cause S phase arrest before ethanol induction.

RESULTS

Unsuccessful Cell Cycle Oscillations Are Induced by Rapid Inactivation of *bimA*^{APC3}

Temperature-sensitive mutations in two genes, *bimA*^{APC3} and *bimE*^{APC1}, which both encode components of the APC/C, have been previously identified in *A. nidulans* (Morris, 1976; Engle *et al.*, 1990; O'Donnell *et al.*, 1991; King *et al.*, 1995; Tugendreich *et al.*, 1995; Peters *et al.*, 1996; Zachariae *et al.*, 1996). When shifted to restrictive temperature, the *bimE7*^{APC1} mutation causes a rapid cell cycle arrest in mitosis (Morris, 1976; Osmani *et al.*, 1988; James *et al.*, 1995), consistent with this mutation causing inactivation of APC/C function and preventing mitotic exit. However, the *bimA1*^{APC3} mutation takes much longer to cause a mitotic arrest (O'Donnell *et al.*, 1991), even though this allele is more temperature sensitive than *bimE7*^{APC1} (Figure 1). The extreme temperature sensitivity caused by *bimA1*^{APC3}, and the lack of first cell cycle arrest in mitosis caused by this mutation, indicate that irreversible loss of APC/C function is not caused by the *bimA1*^{APC3} allele at restrictive temperature.

In an attempt to generate a more effective mitotic block with *bimA1*^{APC3}, we transferred cells, after filtration from 32°C medium, directly into medium prewarmed to 42°C with the expectation that this may more immediately inactivate APC/C function. This shortened the period to attain restrictive temperature from several minutes, using our standard technique, to a matter of a second or so. To our surprise, the instant temperature shift induced multiple synchronized mitotic oscillations, of increasing magnitude, with four peaks of chromosome mitotic index (CMI) occurring over 12 h (Figure 2A). This was specific for the *bimA1*^{APC3} mutation, as such synchronized mitotic oscillations were absent in wild-type and *bimE7*^{APC1} mutant cells when treated identically (our unpublished results). When *bimA1*^{APC3} mutant cultures were shifted to 42°C by shaking in a 60°C water bath (our standard temperature shift regimen), no cell cycle synchrony was apparent, but instead the CMI increased slowly to ~70% by 8 h after temperature shift.

As rapid temperature-shifted *bimA1*^{APC3} cultures underwent repeated rounds of chromosome condensation, we followed biochemically the abundance and activities of several mitotic regulators during these apparent cell cycle oscillations (Figure 2B). Both the activities of p34^{cdc2} and NIMA mitotic-promoting kinases oscillated in the same manner as the CMI values. When the CMI was low, the mitotic kinase activities were low, and when the CMI was high, so were the kinase activities, most noticeably for NIMA kinase activity (Figure 2B). The chromosome mitotic oscillations caused by rapid inactivation of *bimA1*^{APC3} are therefore clearly cell cycle oscillations driven by activation and inactivation of mitosis-promoting protein kinases. This conclusion was further ratified by the dramatic periodic accumulation and hyperphosphorylation, followed by degradation, of the NIMA protein revealed by Western blot analysis (Figures 2B and 4B, NIMA). No such oscillation in p34^{cdc2} abundance was detected, and only minor oscillations of NIME^{cyclinB} protein could be detected (Figure 2B).

The oscillations in cell cycle parameters caused by rapid inactivation of *bimA1*^{APC3} did not lead to successful nuclear division. If relatively young cultures, which contained germlings having two to four nuclei per cell, were shifted rapidly to restrictive temperature, virtually no increase in nuclear number was observed (Figure 2C). The germlings did continue to grow, but they were unable to complete successful mitotic division over a period in which at least two divisions would be expected to have taken place. In addition, it should be noted that no obvious increase in DNA ploidy occurred, based on DAPI staining, during the *bimA1*-induced cell cycle oscillations. During incubation at 42°C for 13 h (e.g., Figure 2) the normal 1n ploidy of *A. nidulans* should have increased to 16n if DNA synthesis was occurring without divi-

Figure 2. Mitotic oscillations induced by *bimA1^{APC3}* upon rapid temperature shift. (A) CMI of a *bimA1^{APC3}* mutant strain after rapid temperature shift. (B) Autoradiograms of p34^{cdc2} H1 and NIMA kinase activities and Western blot analysis of NIMA, NIME^{cyclinB}, and p34^{cdc2}. (C) Nuclear division and germling growth of the *nimA5*

sion. However, no such large nuclei were observed during these experiments (our unpublished results).

To determine the role of NIMA in the cell cycle oscillations caused by inactivation of *bimA^{APC3}*, a double *bimA1^{APC3} + nimA5* mutant strain was constructed and subjected to the rapid temperature shift protocol. In this double mutant no significant oscillation of p34^{cdc2} kinase activity was apparent, but instead p34^{cdc2} kinase activity dramatically increased 10-fold, and the chromosome mitotic index also increased to a high sustained level with only minor variations (Figure 3A). Additionally, no oscillations were observed in the level or phosphorylation state of NIMA or NIME^{cyclinB}; rather, these two proteins accumulated after the temperature shift (Figure 3B). These data indicate that active NIMA is required to generate the cell cycle oscillations observed after rapid inactivation of *bimA^{APC3}*. The data also indicate that rapid inactivation of *bimA^{APC3}* can apparently override the G2 arrest caused by *nimA5* in a manner reminiscent of how *bimE7^{APC1}* also overrides the *nimA5* G2 arrest (Osmani *et al.*, 1988, 1991b; James *et al.*, 1995) (see below).

The mitotic oscillations promoted by rapid temperature shift of *bimA1^{APC3}*-containing cells were also tested for sensitivity to checkpoint control mechanisms. Mitotic oscillations were instigated by rapid temperature shift to 42°C. After 4 h incubation, cells were tested for the DNA damage checkpoint by using methyl methanesulfonate (MMS) and were also treated with the microtubule poison nocodazole, which is known to generate a pseudomitotic arrest. No effect was observed on the oscillations of either the CMI or NIMA or p34^{cdc2} kinase activities after treatment with MMS (Figure 4, A and B), whereas, as noted previously, the wild-type delays the cell cycle after sustaining DNA damage by inactivating p34^{cdc2} (Ye *et al.*, 1997b). Again the oscillations observed after MMS treatment were particularly apparent for the abundance and phosphorylation state of NIMA. In contrast, addition of nocodazole largely suppressed the cell cycle oscillations, and these cells arrested with high CMI values (Figure 4A) and also high levels of NIMA, NIME^{cyclinB}, and NIMA and p34^{cdc2} kinase activities (our unpublished results). These results suggest that, after rapid temperature shift, the *bimA1^{APC3}* mutant cells have lost the DNA damage checkpoint but still have an intact spindle assembly checkpoint. The ability of nocodazole to trap *bimA1^{APC3}* cells in mitosis

Figure 2 (cont). + *bimA1^{APC3}* mutant cells after rapid temperature upshift. Spores were first germinated at 32°C for 7.5 h, and, after harvesting by centrifugation, the germlings were rapidly transferred to new medium prewarmed to 42°C and incubated at 42°C for 8 h. The number of nuclei and germling length were measured before and 8 h after temperature shift.

Figure 3. *nimA* dependence of the mitotic oscillations induced by *bimA1*^{APC3}. (A) CMI of a *nimA5* + *bimA1* double mutant strain after rapid temperature shift. (B) Autoradiogram of p34^{cdc2} H1 kinase activity and Western blots of NIMA, NIME^{cyclinB}, and p34^{cdc2} after rapid temperature shift.

also confirms that this mutation causes a reversible delay to mitotic progression and not an arrest in mitosis.

NIMA and NIME^{cyclinB} Are Stabilized in Cells with Inactive bimE^{APC1} and bimA^{APC3}

Previous work has demonstrated that NIMA protein abundance is regulated in a cell cycle-dependent manner, being low during S phase and maximal at mitosis, similar to cyclin B (Ye *et al.*, 1995). Additionally, degradation of NIMA, also like cyclin B, is required for exit from mitosis (O'Connell *et al.*, 1994; Pu and Osmani, 1995), and the APC/C is known to regulate the stability of other mitotically unstable proteins (for review see Cohen-Fix and Koshland, 1997; Townsley

Figure 4. Effects of addition of nocodazole and MMS on the *bimA1*^{APC3}-induced mitotic oscillations. Nocodazole or MMS was added to *bimA1*^{APC3} mutant cells 4 h after temperature shift and remained in the culture during the duration of the experiment. (A) CMI. (B) Autoradiograms of p34^{cdc2} H1 and NIMA kinase activities and NIMA Western blot in the MMS-treated *bimA1*^{APC3} cells.

and Ruderman, 1998). These observations, and the dramatic oscillations in NIMA abundance caused by rapid inactivation of the APC/C subunit *bimA*^{APC3} (Figures 2B and 4B), prompted us to investigate the potential role of the APC/C in the regulation of NIMA stability.

To directly assess the role of the APC/C in NIMA degradation, we measured the stability of NIMA expressed from the heterologous *alcA* promoter in S phase-arrested cells, as we have shown previously that NIMA is very unstable during S phase arrest (Ye *et al.*, 1995; Ye *et al.*, 1996). The stability of NIME^{cyclinB} when expressed from the *alcA* promoter was similarly determined. Three conditions were used: stability with normal APC/C function, stability with *bimA*^{APC3} inactivated, and stability with *bimE*^{APC1} inactivated. As shown in Figure 5, A and B, in the presence of APC/C function both NIME^{cyclinB} and NIMA are unstable in the hydroxyurea (HU)-arrested S phase

Figure 5. APC/C-dependent stabilization of NIMEcyclinB and NIMA. (A) Cells of the wild-type (wt = R153) and *bimA1*^{APC3} cells containing a copy of *alcA*-driven *nimE*^{cyclinB} were grown at 32°C to early log phase in acetate media (repressing for *alcA*). HU (100 mM) was added to the cells for 2 h to cause S phase arrest before rapid transfer to inducing medium (ethanol) also containing 100 mM HU but at 42°C. One hour after transfer to inducing medium at 42°C, glucose was added to repress expression from the *alcA* promoter. The abundance of NIMEcyclinB was determined by Western blotting. The same blots were also subsequently detected for p34^{cdc2} as loading control. (B) The experimental procedures for NIMA induction and repression in HU-arrested cells were as described in A by using a strain with *alcA* inducible *nimA*. NIMA was detected by Western blot after immunoprecipitation. No general proteolysis was observed in the samples from which NIMA was isolated, and the level of p34^{cdc2} was found by Western blotting to be constant. However, in addition to full-length NIMA (top band) numerous smaller NIMA degradation products were observed.

cells with a half-life of <20 min. In contrast, both NIMEcyclinB and NIMA are greatly stabilized when the APC/C subunit BIMA^{APC3} was inactive, and their half-lives were increased to >60 min. Similar results were obtained when the BIME^{APC1} APC/C component was inactivated and both NIMEcyclinB and NIMA proteins were both highly stabilized (our unpublished results). These results provide strong biochemical evidence for a role of the APC/C in NIMA degradation at a point in the cell cycle where NIMA is known to be unstable.

Mutations of the APC/C Override the *nimA5* G2 Arrest by Elevating p34^{cdc2} H1 Kinase Activity and Making *nimA5* Leaky

The *nimA5* mutation arrests cells in G2 at restrictive temperature even though p34^{cdc2} kinase activity in-

creases to mitotic levels (Osmani *et al.*, 1991a; Ye *et al.*, 1995). Our current data (Figure 3A) and previous studies (Osmani *et al.*, 1991b; James *et al.*, 1995) indicate that mutation of the APC/C allows cells to bypass the *nimA5* G2 arrest and enter mitosis, albeit a defective mitosis (Osmani *et al.*, 1991b). To further understand this phenomenon we have carried out two types of experiment. First, biochemical analysis was completed to see whether mitosis was occurring without NIMA kinase activity in APC⁻ + *nimA5* double mutant strains. Second, *nimA* was deleted from *bimE7*^{APC1} cells to see whether an APC/C mutation could override complete lack of NIMA function.

Strains containing single *nimA5*, *bimA1*^{APC3}, or *bimE7*^{APC1} mutations, and those containing *nimA5* + *bimA1*^{APC3} and *nimA5* + *bimE7*^{APC1} were subjected to temperature shift and analyzed for CMI, NIMA, and p34^{cdc2} H1 kinase activities (Figure 6). Two features of the analysis are particularly interesting. First, it is clear that both the *bimA1*^{APC3} and *bimE7*^{APC1} mutations allow some activation of the mutant NIMA kinase to a level higher than that observed in the single *nimA5* mutant strain (Figure 6B). This partial activation could contribute to the defective mitosis seen in *bimE7*^{APC1} + *nimA5* mutant strains. Second, dramatic activation of the p34^{cdc2} H1 kinase was promoted in both *bimE7*^{APC1} + *nimA5* and *bimA1*^{APC3} + *nimA5* strains to a level much higher than that observed in normal mitosis and greater than that recorded in cells blocked in mitosis by the *bimE7*^{APC1} mutation or at the restrictive temperature for *bimA1*^{APC3} or *nimA5* (Figure 6C). Such high activation of p34^{cdc2} H1 kinase is likely to play a role in the ability of *bimE7*^{APC1} and *bimA1*^{APC3} mutations to override the *nimA5* G2 arrest (Figure 6A).

Deletion of *nimA* Prevents an APC/C Mutant from Entering Mitosis

To see whether some NIMA activity is required to allow mitotic events when *bimE*^{APC1} is inactivated, we removed *nimA* by targeted deletion in both a wild-type strain and a *bimE7*^{APC1} strain. A linear DNA deletion strategy was used (Figure 7A) in which *nimA* was replaced with the *pyr4* nutritional marker from *Neurospora crassa*, which encodes orotidine-5'-phosphate decarboxylase and complements the *pyrG89* mutation in *A. nidulans*. The deleted allele in the homokaryotic state is lethal (see below) and was therefore maintained in heterokaryons as previously described (Osmani *et al.*, 1988; Oakley and Osmani, 1993).

After transformation with the linear deletion construct and preliminary screening of transformants for heterokaryons (see MATERIALS AND METHODS), Southern blot analysis identified several heterokaryons containing both a wild-type allele and a clean deleted allele (Figure 7, A and B) termed *nimAΔ*. It

Figure 6. APC/C mutations override the *nimA5* G2 arrest with very high p34^{cdc2} H1 kinase activity. (A) Mutant cells, as indicated, were grown to early log phase at 32°C and shifted to 42°C at time 0. At the times indicated the CMI was determined after DAPI staining and fluorescent microscopy. p34^{cdc2} H1 (B) and NIMA kinase (C) activities were assayed after immunoprecipitation with specific antibodies.

was noticeable that all deleted heterokaryons contained much lower levels of the deleted allele. This is most likely due to selection for the wild-type allele, as NIMA is expressed at a low level, is cell cycle regulated, and is very unstable. It is therefore likely that a 50:50 ratio of *nimA* to *nimA*Δ nuclei in a heterokaryon would not supply enough NIMA to enable normal cell cycle progression within that heterokaryon. This therefore leads to the selection for high ratios of the wild-type allele.

The ability to maintain nuclei containing a deletion of an essential gene in the heterokaryotic state in *A. nidulans* allows the analysis of the null phenotype because the heterokaryotic state is broken when conidia are formed, as they are uninucleate (Osmani *et al.*, 1988). Phenotypic analysis of germinated conidia derived from heterokaryons having a clean deletion of *nimA* identified a low percentage of conidia (~3.0%) that germinated to produce polarized germlings with a single interphase nucleus (Figure 7C), which is the

Figure 7. Targeted *nimA* deletion. (A) Restriction maps of the *nimA* locus and *nimAΔ* locus after targeted deletion. The *PstI* fragment of *nimA* was replaced with a *PstI* fragment containing the *pyr4* gene. The linear genomic *KpnI* fragment containing the *pyr4* insert was used for transformation in a wild-type (wt = GR5) and a *bimE7^{APC1}* strain. Diagnostic *XhoI* fragments of 4.0 kb (wt) and 4.5 kb (*nimAΔ*) when probed with the *PstI*–*KpnI* fragment are indicated. (B) Autoradiogram of a Southern blot of *XhoI*-digested genomic

classic *nim* (never in mitosis) phenotype of *A. nidulans*. These cells clearly contain the deleted allele, as they are *pyr4⁺*. They also showed the expected phenotype for deletion of an essential cell cycle gene. All other conidia from the heterokaryon are parental *pyrG89*-containing cells because they did not form germlings on media lacking uridine and uracil. The low recovery of the *nimAΔ* allele in conidia from the heterokaryons confirms the Southern blot analysis, indicating that there is selection for the wild-type allele in these heterokaryons. We conclude that the *nimA5* phenotype is very similar to the *nimAΔ* null allele and that *nimA* is an essential gene.

Phenotypic analysis of conidia derived from a heterokaryon containing a clean deletion of *nimA* in a *bimE7^{APC1}* background was used to determine whether inactivation of *bimE^{APC1}* could cause mitosis in the absence of *nimA*. Conidia were plated on media lacking uridine and uracil and were germinated at 32°C for a period to allow identification of those containing the *nimAΔ* allele. After temperature shift to 42°C to inactivate *BIME^{APC1}*, DAPI staining revealed that all the *nimAΔ* + *bimE7^{APC1}* cells (>150) remained arrested in interphase (Figure 7D). The experiment was repeated by germinating directly at 42°C, but the *nimAΔ* allele still prevented *bimE7^{APC1}* from promoting cells into mitosis. It is therefore necessary to have some NIMA activity or cells remain arrested before mitosis when the APC/C is inactivated by *bimE7^{APC1}*.

DISCUSSION

The abundance of the NIMA kinase is regulated through the cell cycle, and its degradation is required for exit from mitosis (Osmani and Ye, 1996). Mitotic degradation of other regulatory proteins (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996; Juang *et al.*, 1997; Michaelis *et al.*, 1997; Charles *et al.*, 1998; Shirayama *et al.*, 1998) is also required for normal exit from mitosis, and these proteins are

Figure 7 (cont). DNA isolated from putative heterokaryons after deletion of *nimA* probed with the radiolabeled *PstI*–*KpnI* fragment indicated in A. 10, 13, 32, 76, 109, and 131 are deletions in GR5, and 19R and 30R indicate deletions in a *bimE7^{APC1}* strain. Heterokaryons 10, 13, 109, 19R, and 30R all contain both a specific deletion and the wild-type allele. (C) Micrographs of DAPI-stained germlings. The wild-type germling is from an 8-h sample and contains eight interphase nuclei. The *nimAΔ* germlings are derived from a heterokaryon germinated for 8 and 15 h, respectively, at 32°C. (D) Representative micrographs of DAPI-stained germlings from strains with the indicated pertinent genotypes. Spores were first allowed to germinate at 32°C for 7 h before shifting to 42°C for 4 h. The *nimA5* mutant germling was grown at 42°C directly for 7 h. It is to be noted that the single nucleus of the large *nimAΔ* + *bimE7^{APC1}* germling remained at interphase upon shift to 42°C for 4 h, whereas nuclei in the *bimE7^{APC1}* or *nimA5* + *bimE7^{APC1}* mutant germlings all became condensed. The arrow indicates an ungerminated spore carrying the parental genotype (GR5 = *pyrG89*) derived from the *nimAΔ* + *bimE7^{APC1}* heterokaryon.

targeted by the APC/C for degradation. In the current work we provide evidence that BIMA^{APC3} plays a regulatory role in APC/C function and show that NIMA kinase is likely a target of the APC/C.

Although the *bimA1*^{APC3} allele causes tight temperature sensitivity, it does not cause an irreversible arrest in mitosis, which would be expected for a mutation causing inactivation of the APC/C at restrictive temperature. Remarkably, we demonstrate that inactivation of *bimA1*^{APC3} instead induces repeating rounds of mitotic events characterized by chromosome condensation and decondensation, activation and inactivation of the p34^{cdc2} and NIMA protein kinases, accumulation and degradation of NIMA, and cyclic rounds of phosphorylation of NIMA. The tight correlation observed between these events strongly indicates that rapid inactivation of *bimA1*^{APC3} promotes cyclic activation and inactivation of the mitosis-promoting kinases, which then drive nuclei in and out of a mitotic state.

How does inactivation of an APC/C subunit cause cell cycle oscillations of the mitosis-promoting kinases? Some insight into this phenomenon is provided by data indicating that NIMA could be a target of the APC/C.

It is known that inhibition of DNA synthesis by addition of hydroxyurea to dividing *A. nidulans* cells causes S phase arrest and disappearance of NIMA protein (Ye *et al.*, 1996). By expression of *nimA* from an inducible promoter after S phase arrest, we have shown that, like NIME^{cyclinB}, NIMA is rendered unstable in an APC/C-dependent manner. We have no direct evidence that this involves the E3 ubiquitin ligase activity of the APC/C, but this is an obvious possibility.

We have also found that the APC/C plays a role in NIMA stability at G2 because the previously reported ability of the *bimE7*^{APC1} mutation to override the *nimA5* G2 arrest (Osmani *et al.*, 1988) was found to be shared with the *bimA1*^{APC3} mutation. Thus mutations of subunits of the APC/C allow entry into mitosis when NIMA is defective. Biochemical analysis was used to reveal the molecular basis for this effect. First, the kinase activity of p34^{cdc2} is greatly increased in *nimA5* + *bimE7*^{APC1} and *nimA5* + *bimA1*^{APC3} double mutant strains to a level much higher than that found during normal mitosis. This increased level of p34^{cdc2} activity is at least in part because NIME^{cyclinB} accumulates to higher levels in these double mutants. Increases in NIME^{cyclinB} at the G2 arrest point of *nimA5* can cause immediate elevation of p34^{cdc2} H1 kinase activity, as p34^{cdc2} is not subjected to inhibitory tyrosine phosphorylation at this point in the cell cycle (Osmani *et al.*, 1991a). Second, inactivation of *bimA*^{APC3} or *bimE*^{APC1} renders the *nimA5* mutation slightly leaky, giving rise to marginally elevated levels of NIMA kinase activity. This is, at least in part, due to an accumulation of NIMA protein to higher levels but could also be caused by the marked elevation of p34^{cdc2} H1 kinase activity observed in these strains.

The data indicate that at G2 NIMA and NIME^{cyclinB} proteins are held in check via an APC/C-dependent mechanism. Therefore, APC/C mutations make the *nimA5* mutation leaky and also superinduce p34^{cdc2} H1 kinase activity to promote defective mitotic events. Mutation of the APC/C cannot, however, override complete loss of NIMA function, as deletion of *nimA* arrests cells in G2 even after inactivation of *bimE7*^{APC1}.

While this work was in preparation, it has been reported that promoter rundown of *nimA*, which causes a G2 arrest, can be overridden by inactivation of *bimA1*^{APC3} in cells containing "insignificant levels" of NIMA kinase. This experiment has been interpreted to mean that APC/C mutations can promote mitotic events in the complete absence of NIMA function (Lies *et al.*, 1998). This apparent discrepancy with our data may be due to the different experimental approaches used. However, deletion of *nimA* from the genome generates a true null phenotype. Additionally, the insignificant levels of NIMA kinase reported (Lies *et al.*, 1998) may be significant, because when NIMA and the APC/C are inhibited, p34^{cdc2} H1 kinase levels are induced to higher than mitotic levels (Figure 6). Additionally, as NIMA is likely a substrate of the APC/C (see below), promoter rundown of NIMA is probably less effective when the turnover rate of NIMA is reduced by inactivation of the APC/C.

The fact that mutation of APC/C subunits causes elevation of NIMA at the *nimA5* arrest point and also renders NIMA more stable during S-phase arrest suggests that NIMA requires the APC/C for instability during interphase and indicates that NIMA is a substrate of the APC/C. In further support of this contention, we note that NIMA is degraded during mitosis and that it is stabilized when the spindle assembly checkpoint is engaged, which is thought to be imposed by inhibition of the APC/C (Osmani and Ye, 1996). Additionally, we have previously shown that forced expression of NIMA enhances the *bimE*^{APC1} mutation, which is also consistent with NIMA being a substrate of the APC/C (Ye *et al.*, 1996). We now show that the *bimA1*^{APC3} mutation causes amazing oscillations in the abundance of NIMA protein, indicating that this APC/C mutation promotes repeated rounds of inactivation and then activation of the APC/C toward NIMA. With these observations in mind we propose the following to help explain the oscillatory effects caused by *bimA1*^{APC3}.

The *bimA1*^{APC3} mutation may uncouple an oscillatory clock mechanism that controls the APC/C and thus cell cycle progression. By rapid inactivation of *bimA1*^{APC3}, perhaps this regulatory clock is reset to a base level. By restarting the clock from the same regulatory point in the cell cycle, all cells would then proceed through the cell cycle in a synchronous manner, resulting in the observed cell cycle-specific

changes we documented regarding chromosome condensation, kinase activities, and protein abundance.

Second, to help explain why multiple oscillations occur, we suggest that the *bimA1^{APC3}* mutation makes the APC/C partially resistant to activation by its substrates, such as cyclin B, Polo, and NIMA, during mitosis. This would cause cells to delay in mitosis and accumulate APC/C substrates until a critical threshold level could accumulate and eventually activate the partially defective APC/C. After APC/C activation, cyclin B, Polo, and NIMA would be degraded and allow exit from mitosis. In this manner an extended mitotic delay would be imposed followed by progression through interphase into another mitotic delay until the defective APC/C could be activated again after accumulation of substrates, and so on. In this manner multiple cell cycle oscillations could be generated between interphase and mitotic states.

We have shown that NIMA is likely a substrate of the APC/C and that its level and activity oscillate widely after rapid inactivation of *bimA1^{APC3}*. For this reason it is possible that NIMA accumulation plays a key role in the activation of the APC/C seen after rapid inactivation of *bimA1^{APC3}* (Figure 8). This possibility is further supported by the fact that in a *nimA5* + *bimA1^{APC3}* double mutant the APC/C is not activated, and cells remain in an extended mitotic state with elevated levels of both NIMA and NIME^{cyclinB}. Thus, *nimA* is required for the activation of the APC/C after inhibition of *bimA1^{APC3}*. This then explains why the *nimA5* + *bimA1^{APC3}* double mutant arrests in mitosis but the single *bimA1^{APC3}* mutant oscillates in and out of mitosis. In *bimA1^{APC3}* strains, NIMA protein could accumulate with normal activity and help activate the APC/C to allow degradation of mitotic substrates and promote exit from mitosis (Figure 8B). By contrast, in *nimA5* + *bimA1^{APC3}* strains, although NIMA5 protein accumulates and has enough activity to allow cells into mitosis, it does not properly activate the APC/C, thus causing the extended mitotic arrest observed (Figure 8C).

Of course it is possible that both p34^{cdc2}/cyclin B and NIMA are involved in the activation of the APC/C at mitosis, along with other regulators such as Polo-like kinases (Charles *et al.*, 1998; Descombes and Nigg, 1998; Shirayama *et al.*, 1998). The important point is that *bimA1^{APC3}* apparently plays a regulatory role in APC/C function. We propose that *bimA1^{APC3}* could be part of a cell cycle clock mechanism and, additionally, that it is required for normal activation of the APC/C at mitosis in response to accumulation and activation of APC/C substrates during mitosis.

Finally, it should be noted that the cyclic oscillations of cell cycle events caused by inactivation of *bimA1^{APC3}* are unusual in several respects. Most noticeably they are not successful in dividing nuclei, despite repeated rounds of activation and inactivation

Figure 8. Relationship between NIMA and BIM1^{APC3}. To help explain why the *nimA5* mutation in combination with *bimA1^{APC3}* causes an effective mitotic arrest phenotype, whereas the *bimA1^{APC3}* mutation generates repeating rounds of mitotic oscillation, we propose the following. (A) Substrates of the APC/C, such as cyclin B, Polo-like kinases, and NIMA, accumulate to a threshold during mitosis, which activates the APC/C to trigger their demise and also to exit from mitosis. (B) The *bimA1^{APC3}* mutation increases the threshold of activation of the APC/C. This results in a mitotic delay during which NIMA protein and activity accumulate. When NIMA activity reaches the higher threshold level of activation, the APC/C is activated, and cells can exit mitosis. (C) In the *bimA1* + *nimA5* double mutant, although p34^{cdc2} H1 kinase activity and NIMA5 activity are increased to allow entry into mitosis, the level of NIMA5 activity stays below that required to activate the APC/C. This then causes an extended mitotic arrest, because cells cannot exit mitosis.

of p34^{cdc2} and NIMA. Perhaps some APC/C substrates, such as Pds1 (Cohen-Fix *et al.*, 1996), Cut2 (Funabiki *et al.*, 1996), Scc1 (Michaelis *et al.*, 1997), and Ase1 (Juang *et al.*, 1997), that need to be removed to

allow normal progression out of mitosis are not destroyed when *bimA*^{APC3} is inhibited. The cell cycle oscillations are also not responsive to the DNA damage checkpoint. They are, however, responsive to the spindle assembly checkpoint activated by microtubule depolymerization. Because the *bimA*^{APC3}-induced cyclic oscillations are arrested at mitosis after microtubule depolymerization, it is indicated that the APC/C is still inhibited by Mad2p, which mediates this checkpoint arrest (He *et al.*, 1997; Li *et al.*, 1997).

Our data therefore indicate that *bimA*^{APC3} inactivation prevents degradation of anaphase substrates of the APC/C while allowing normal regulation of the APC/C during the spindle checkpoint. This suggests that the regulation of the APC/C is disturbed in a relatively specific manner by the *bimA*^{APC3} mutation. Perhaps the cyclic rounds of chromosome condensation and decondensation promoted by *bimA*^{APC3} inactivation are driven primarily by cycles of NIMA activity, with most other cell cycle-specific events remaining unchanged. This could occur if the *bimA*^{APC3} mutation interferes with the activation of the APC/C toward NIMA but not toward substrates required for anaphase progression and passage through other phases of the cell cycle. Future experiments will be aimed at validating our proposed regulatory role for *bimA*^{APC3}, which should further our understanding of the regulation of the APC/C, particularly with regard to its substrate specificity during cell cycle progression and checkpoint control.

ACKNOWLEDGMENTS

We thank the members of our laboratory for constructive suggestions on this work and Drs. Peter Mirabito and Matthew O'Connell for *A. nidulans* strains. This work was supported in part by a grant from the National Institutes of Health (GM-42564) to S.A.O.

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