**BIMA\(^{APC3}\), a component of the *Aspergillus* anaphase promoting complex/cyclosome, is required for a G\(_2\) checkpoint blocking entry into mitosis in the absence of NIMA function**

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**SUMMARY**

Temperature sensitive (ts) nimA mutants of *Aspergillus nidulans* arrest at a unique point in G\(_2\) which is post activation of CDC2. Here we show that this G\(_2\) arrest is due to loss of nimA function and that it is dependent on BIMA\(^{APC3}\), a component of the anaphase promoting complex/cyclosome (APC/C). Whereas nimA single mutants arrested in G\(_2\) with decondensed chromatin and interphase microtubule arrays, nimA, bima\(^{APC3}\) double mutants arrested growth with condensed chromatin and aster-like microtubule arrays. nimA, bima\(^{APC3}\) double mutants entered mitosis with kinetics similar to bima\(^{APC3}\) single mutants and wild-type cells, indicating a checkpoint-like role for BIMA\(^{APC3}\) in G\(_2\). Even cells which had been depleted for NIMA protein and which contained insignificant levels of NIMA kinase activity entered mitosis on inactivation of bima\(^{APC3}\). BIMA\(^{APC3}\) was present in a >25S complex containing BIME\(^{APC1}\), and bima\(^{APC3}\) mutants were sensitive to elevated CYCLIN B expression, consistent with BIMA\(^{APC3}\) being a component of the APC/C. Inactivation of bima\(^{APC3}\) had little affect on the steady state levels of the B-type cyclin, NIME\(^{Cyclin B}\). Our results indicate that BIMA\(^{APC3}\), and most likely the APC/C itself, is activated in G\(_2\) in nimA mutants. We propose that APC/C activation is part of a novel, late G\(_2\) checkpoint, which responds to a defective process or structure in nimA mutants, and which prevents inappropriate entry into mitosis.

Key words: NIMA, APC, Cyclosome, Checkpoint

**INTRODUCTION**

The anaphase promoting complex or cyclosome (APC/C) is a ubiquitin ligase with multiple roles in cell cycle regulation. The APC/C was initially shown to be required for the cell cycle regulated ubiquitinylation of B-type cyclins in *S. solidissima* (Hershko et al., 1994), *Xenopus laevis* (King et al., 1995) and *Saccharomyces cerevisiae* (Zachariae et al., 1996b). The *X. laevis* APC/C is a 20S complex containing 8 distinct polypeptides whereas the *S. cerevisiae* APC/C is a 36S complex containing at least 13 different polypeptides (Yu et al., 1998; Zachariae et al., 1998b). Both complexes contain at least eight highly related proteins, designated APC1 through APC8 by Yu et al. (1998). Three of these proteins have also been identified in *Schizosaccharomyces pombe* (Hirano et al., 1988; Samejima and Yanagida, 1994; Yamashita et al., 1996), *A. nidulans* (Engle et al., 1990; O’Donnell et al., 1991; Table 1), and in mammals (Starborg et al., 1994; Tugendreich et al., 1995), indicating that the APC/C is likely to be widely conserved among eukaryotes.

The *S. cerevisiae* APC/C regulates multiple events in the mitotic cell cycle. APC/C dependent proteolysis of B-type cyclins is required at telophase for completion of mitosis (Surana et al., 1995; Irniger et al., 1995; Zachariae et al., 1996b) and in G\(_1\) to prevent inappropriate initiation of DNA synthesis (Irniger and Nasmyth, 1997). The APC/C also regulates destruction of PDS1 at metaphase, which is important for sister chromatid separation (Yamamoto et al., 1996a,b; Cohen-Fix et al., 1996). The spindle midzone component, ASE1, which is required for anaphase B (Pellman et al., 1995), is yet another substrate for the *S. cerevisiae* APC/C. ASE1 proteolysis at the end of mitosis and in G\(_1\) prevents the premature accumulation of ASE1, which otherwise causes a checkpoint arrest at the next metaphase (Juang et al., 1997). It is not yet known whether a single APC/C regulates each of the above events, however, CDC23\(^{APC8}\) is required in each of the above cases and
CDC16\textsuperscript{APC6} is required for ubiquitinylation of several B-type cyclins and PDS1.

Multiple roles in cell cycle control have also been noted for the \textit{S. pombe} APC/C. The B-type cyclin, CDC13, is an APC/C substrate (Yamashita et al., 1996). APC/C-dependent proteolysis of CUT2 is required for the metaphase to anaphase transition (Funabiki et al., 1997), making CUT2 analogous to PDS1 in \textit{S. cerevisiae}. CUT2 function is complex, however, as deletion of the CUT2 gene results in a metaphase block, and not premature sister chromatid separation. Loss of function mutations in CUT4\textsuperscript{APC1} or CUT9\textsuperscript{APC6} uncouple mitosis from cytokinesis (Samejima and Yanagida, 1994; Yamashita et al., 1996), suggesting that APC/C function also regulates cytokinesis. This is supported by the finding that overexpression of NUC2\textsuperscript{APC3} inhibits septation (Kumada et al., 1995). Although the APC/C substrate relevant to septation control is not known, nondegradable CUT2 blocks at metaphase and uncouples mitosis from cytokinesis (Funabiki et al., 1997), consistent with CUT2 being involved in septation control. NUC2\textsuperscript{APC3} is also required for the G1 arrest due to nitrogen starvation (Kumada et al., 1995), implicating the \textit{S. pombe} APC/C in pathways linking nutritional signals to the cell cycle.

Based on sequence comparisons, BIME\textsuperscript{APC1} and BIMA\textsuperscript{APC3} are two potential components of the \textit{A. nidulans} APC/C (Engle et al., 1990; O’Donnell et al., 1991). Recessive, conditionally lethal mutations in either \textit{bime}\textsuperscript{APC1} or \textit{bima}\textsuperscript{APC3} cause a cell cycle arrest in mitosis, consistent with a proposed role in the APC/C (Osmani et al., 1988; O'Donnell et al., 1991). Genetic analyses suggest an additional role for BIME\textsuperscript{APC1} in checkpoints regulating entry into mitosis. The temperature sensitive (ts) \textit{bime}\textsuperscript{APC1} mutation partially inactivates an S phase checkpoint, allowing chromosome condensation and spindle assembly in the presence of up to 100 mM hydroxyurea (Osmani et al., 1988; Ye et al., 1996). \textit{bime}\textsuperscript{APC1} also overcomes the interphase arrest due to ts mutations in \textit{nima}\textsuperscript{Q\textsuperscript{MCM2}}, \textit{nirM}, and \textit{nima}. Whereas \textit{ts nimQ\textsuperscript{MCM2}} or \textit{nimR21} mutants arrest before S phase, and \textit{ts nimA5} mutants arrest in late G2, double mutants carrying \textit{bime}\textsuperscript{APC1} in combination with \textit{nimQ\textsuperscript{MCM2}}, \textit{nimR21}, or \textit{nimA5} enter mitosis instead of arresting in interphase (Osmani et al., 1988, 1991b; James et al., 1995; Ye et al., 1997b). These results suggest that inactivation of NIMQ\textsuperscript{MCM2}, NIMR or NIMA activates a checkpoint which acts through BIME\textsuperscript{APC1} to prevent entry into mitosis.

Whereas little is yet known about the function of NIMQ\textsuperscript{MCM2} and NIMR, NIMA’s role in the regulation of mitosis has been extensively investigated (Osmani and Ye, 1996; Fry and Nigg, 1995). NIMA is a serine threonine protein kinase with an amino-terminal catalytic domain, several putative nuclear localization signals, and a PEST rich carboxyl terminal domain. Recessive and dominant negative mutations in \textit{nima} arrest cells in G2 at a stage that is downstream of tyrosine dephosphorylation of the \textit{A. nidulans} CDC2 homolog, NIMX\textsuperscript{CDC2} (Osmani et al., 1991a; Lu and Means, 1994; Ye et al., 1995). Dominant gain of function mutations in \textit{nima} induce chromosome condensation independent of NIMX\textsuperscript{CDC2} activation (O’Connell et al., 1994; Pu and Osmani, 1995). These data are consistent with a model in which NIMA functions downstream of NIMX\textsuperscript{CDC2}/NIME\textsuperscript{Cyclin B} to promote chromosome condensation. Overexpression of NIMA results in loss of cytoplasmic microtubules (MTs) in \textit{A. nidulans}, and \textit{S. pombe}, and nuclear envelope breakdown in mammalian cells, suggesting that microtubule dynamics and nuclear envelope structure may also be under NIMA control (O’Connell et al., 1994; Pu and Osmani, 1995; Lu and Hunter, 1995).

The genetic interaction of \textit{bime}\textsuperscript{APC1} with \textit{nima}\textsuperscript{ts} mutations together with the identification of BIME\textsuperscript{APC1} homologs in the APC/C suggests that the APC/C functions in a G2 checkpoint. This would reveal yet another role for the APC/C in cell cycle regulation. To directly investigate this possibility, we have generated antisera to BIME\textsuperscript{APC1} and determined that BIME\textsuperscript{APC1} and BIMA\textsuperscript{APC3} were present in a complex with a sedimentation rate consistent with that of the APC/C. We have also determined that BIMA\textsuperscript{APC3} function was essential for arrest in G2 due to loss of NIMA function. These results demonstrate that the APC/C is required to prevent mitosis in the absence of NIMA function. We propose that APC/C activation is part of a novel, late G2 checkpoint.

### MATERIALS AND METHODS

#### Strains, microbiological techniques and genetic analyses

Aspergillus strains used in this study are listed in Table 2. Standard conditions were used for \textit{Aspergillus} propagation (Morris, 1976; Kafer, 1977; Mirabito and Morris, 1993), genetics (Pontecorvo et al., 1953), and transformation (Osmani et al., 1987). Agar-solidified medium containing 0.08% sodium deoxycholate was used for analysis of colony growth rate (Morris, 1976). The kinetics of nuclear division were determined using flask cultures containing 5x10^8 spores in 40 ml liquid YG.

All transformants were shown to contain a single plasmid integrated at the chromosomal locus indicated in Table 2. Strains designated nima-allelic, which contained \textit{alcA::nimA} as the only functional \textit{nima} gene, were constructed as follows. SWJ071 was transformed with pMO137 (O’Connell et al., 1994) to yield TSLF2-1. TSLF2-1 was crossed to SO8 to yield SFC161-9. SFC161-9 was transformed with pNIMA linearized with KpnI and transformants were selected on medium containing glycerol as carbon source. Three of approximately 200 transformants were able to grow on medium containing glycerol but not glucose as carbon source. These strains were shown by Southern blot analysis to have undergone the predicted gene replacement and to harbor \textit{alcA::nimA} as their only copy of \textit{nima}. One of these transformants, TPM300-40, was out-crossed to FGSCA89 to yield SFC466-48 and SFC466-201.

A strain containing a hemagglutinin (HA) epitope-tagged version of \textit{nime}\textsuperscript{Cyclin B} as its only \textit{nime}\textsuperscript{Cyclin B} gene was constructed in two steps. SO25 was transformed with pMO126 to yield TMO126, which contained a single pMO126 integrated at the \textit{nime}\textsuperscript{Cyclin B} locus. FOA resistant mitotic segregants were isolated and screened for the presence of HA-tagged \textit{nime}\textsuperscript{Cyclin B} by Southern blot analysis. One such strain, M078, contained \textit{HA-nime}\textsuperscript{Cyclin B} as it’s only \textit{nime}\textsuperscript{Cyclin B} gene.
Plasmid constructions

Plasmids were constructed using standard techniques (Sambrook et al., 1989). The *bime<sup>APC1</sup>* nucleotide position numbers refer to Fig. 6 of Engle et al. (1990). pS19 is a 1,041 bp EcoRI-HindIII *bime<sup>APC1</sup>* cDNA fragment (nt 1,309-2,350) inserted into pATH3 (Hoerner et al., 1991). pS340 is a 999 bp EcoRI-SvII *bime<sup>APC1</sup>* cDNA fragment (nt 1,309-2,308) blunted and ligated into pUR292 (Ruther and Muller-Hill, 1983). pS61 contains an *alcA::bime<sup>APC1</sup>* fusion in a modified pKK12 (Kirk and Morris, 1983) in which the *alcA* promoter of pKK12 was replaced with the *alcA* promoter from pAL4 (Waring et al., 1989). pS61 contains the *alcA* ATG fused to nt 709 through nt 7,021 of the *bime<sup>APC1</sup>* cDNA which is fused to *bime<sup>APC1</sup>* genomic DNA sequences up to the *Scrl* site (Engle et al., 1990). pS60 is the same as pS61 except that an EcoRV fragment containing *bime<sup>APC1</sup>* cDNA from nt 4,293 to 5,655 was removed. pS160 is predicted to encode a truncated 170 kDa form of BIME<sup>APC1</sup>, pS195-3HA-bime<sup>APC1</sup> is pS61 with a NotI fragment encoding 3 tandem copies of the HA epitope (from pGTEPI, a gift from G. Tokiwa) inserted after the *pGTEPI* promoter of pKK12.

A full description of the construction of parent strains used in the derivation of the strains listed in this table will be described in the Fungal Genetics Newsletter (P. M. Mirabito, in preparation).

### Table 2. *A. nidulans* strain genotype and construction

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference or derivation</th>
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<tbody>
<tr>
<td>FGSCA28</td>
<td>bai1, pabaA</td>
<td>FGSC*</td>
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<tr>
<td>JCC51-6</td>
<td>bimA9, argB2, nicA2, [argB&lt;sup&gt;B&lt;/sup&gt;, alcA::nimE at argB]&lt;sup&gt;**&lt;/sup&gt;</td>
<td>MLC489-8 × PMC892-19&lt;sup&gt;***&lt;/sup&gt;</td>
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<td>MLC1-19</td>
<td>bimA9, pabaA, nicA2, ya2</td>
<td>GR5 × PMC449-1</td>
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<td>MMB001</td>
<td>bimE7, methB2, ya2</td>
<td>SO4 × FGSCA272</td>
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<td>MO68</td>
<td>argB2, pabaA, [argB&lt;sup&gt;B&lt;/sup&gt;, alcA::nimE at argB]</td>
<td>SWJ008 + pMO126‡</td>
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<td>MO78</td>
<td>bai1, pyrG99, bai1, methG11, sc12, choA1, chaA1, HA-nimE</td>
<td>segregant of TMO126§</td>
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<td>PM152</td>
<td>bimA1, nimA5, pabaA</td>
<td>PM131 × SWJ014</td>
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<td>PM156</td>
<td>bimA1, pabaA, ya2</td>
<td>PM131 × SWJ014</td>
</tr>
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<td>PM211</td>
<td>bimA1, nimE6, riboA1, wa2</td>
<td>PM131 × SWJ105</td>
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<td>PM221</td>
<td>bimA1, nimT23, pabaA1, wa2</td>
<td>PM131 × SWJ106</td>
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<td>PM274</td>
<td>nimA5, bimE APC1, pabaA1, wa2, ya2</td>
<td>RM24 × SWJ011</td>
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<td>PM66-25</td>
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<td>nicA2, cxxE16, wa3, HA-nimE</td>
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<td>PM654-19</td>
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<td>PM661-30</td>
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<td>PM693-10</td>
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<td>PMC661-27 × PMC654-19</td>
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<td>PM689-3</td>
<td>bimA1, argB2, [argB&lt;sup&gt;B&lt;/sup&gt;, alcA::nimE at argB]</td>
<td>PM144 × MO68</td>
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<tr>
<td>PM689-19</td>
<td>nicA2, argB2, [argB&lt;sup&gt;B&lt;/sup&gt;, alcA::nimE at argB]</td>
<td>PM144 × MO68</td>
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<tr>
<td>R153</td>
<td>pyroA4, wa3</td>
<td>FGSC</td>
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<td>PMC654-19 × MLIC1-54</td>
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<td>SFC11-7</td>
<td>bimA9, nimA5, wa2, HA-nimE</td>
<td>PMC654-19 × MLIC1-54</td>
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<td>SFC161-9</td>
<td>bimA1, pyrG99, nicA2, choA1, wa2, argB2, [argB&lt;sup&gt;B&lt;/sup&gt;, alcA::nimA at argB]</td>
<td>TSF2-1 × SO8</td>
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<td>SFC466-48</td>
<td>bimA1, argB2, pyrG99, nicA2, choA1, chaA1, [argB&lt;sup&gt;B&lt;/sup&gt;, alcA::nimA at argB], [pyr&lt;sup&gt;4&lt;/sup&gt;, nimAΔ]&lt;sup&gt;⁄&lt;/sup&gt;</td>
<td>TPM300-40 × FGSCA89</td>
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<tr>
<td>SFC466-201</td>
<td>bimA1, pyrG99, nicA2, wa2, [argB&lt;sup&gt;B&lt;/sup&gt;, alcA::nimA at argB], [pyr&lt;sup&gt;4&lt;/sup&gt;, nimAΔ]&lt;sup&gt;⁄&lt;/sup&gt;</td>
<td>TPM300-40 × FGSCA89</td>
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<td>S08</td>
<td>bimA1, pyrG99, choA1, wa2</td>
<td>O’Donnell et al., 1991</td>
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<td>SWJ008</td>
<td>argB2, pabaA</td>
<td>SWJ012 × FSCCA122</td>
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<td>bimE7, argB2; pabaA1; waF1</td>
<td>SWJ001 × FSCCA122</td>
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<td>SWJ060</td>
<td>argB2; pabaA</td>
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<td>SWJ063</td>
<td>argB2; pabaA</td>
<td>SWJ008 × pS61</td>
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<td>SWJ071</td>
<td>nicA5, bimE APC1, nicA2, wa2</td>
<td>SWJ015 × SWJ024</td>
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<td>SWJ1216</td>
<td>nimT23, methB3, ya2</td>
<td>SWJ116 × SWJ008</td>
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<td>SWJ317</td>
<td>bimE7, argB2; pabaA1; waF1, [argB&lt;sup&gt;B&lt;/sup&gt;, alcA::HA-bime&lt;sup&gt;APC1&lt;/sup&gt; at argB]</td>
<td>SO25 × pMO126</td>
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<td>TMO126</td>
<td>bimA1, pyrG99, bai1, methG11, sc12, choA1, chaA1, nimE6, [pyr&lt;sup&gt;4&lt;/sup&gt;, HA-nimE at nimE]</td>
<td>SO25 × pMO126</td>
</tr>
<tr>
<td>TPM200</td>
<td>pyro4A, wa2, pyrG99, [pyr&lt;sup&gt;4&lt;/sup&gt;, bima-alc]&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>Mirabito and Morris, 1993</td>
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<td>TPM300-40</td>
<td>bimA1, pyrG99, nicA2, choA1, wa2, argB2, [argB&lt;sup&gt;B&lt;/sup&gt;, alcA::nimA at argB], [pyr&lt;sup&gt;4&lt;/sup&gt;, nimAΔ]&lt;sup&gt;⁄&lt;/sup&gt;</td>
<td>SFC161-9 × pNMAB</td>
</tr>
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<td>TSF2-1</td>
<td>nicA5, bimE APC1, nicA2, [argB&lt;sup&gt;B&lt;/sup&gt;, alcA::nimA at argB]</td>
<td>SWJ071 + pMO137</td>
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<td>TSJ36</td>
<td>bimE7, argB2; pabaA1; waF1</td>
<td>SWJ099 + pS61</td>
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<td>YYC67-25</td>
<td>bimA1, nimX3, choA1, pabaA1, riboA1, ya2</td>
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<td>YYC36-38</td>
<td>bimA1, nich5, nimT23, pabaA1, ya2</td>
<td>SFC4-1 × SFC4-5</td>
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**Genotype entered in brackets indicates that the strain carries a plasmid which contains the *A. nidulans* markers indicated and which is integrated at the chromosomal locus. For example [argB<sup>B</sup>, alcA::nimE at argB] indicates that JCC51-6 carries a plasmid with the argB<sup>B</sup> gene and an alcA::nimE gene fusion integrated at the argB locus. The original transformant is either listed in this table or the reference describing its construction is indicated.

***MLC489-8 × PMC892-19 indicates that the strain JCC51-6 is a meiotic segregant of a cross between these two strains.

‡SO6 × TPM200 indicates that the strain MO68 is derived from transformation of strain SWJ008 with plasmid pMO126.

‡‡SWJ008 × pMO126 indicates that the strain MO68 is derived from transformation of strain SWJ008 with plasmid pMO126.

Represents a FOA resistant, pyrG<sup>99</sup> mitotic segregant from TMO126 as described in Materials and Methods. This strain carries an HA epitope-tagged nimE gene (HA-nimE) as it’s only nimE gene

¶SO6 × TPM200 indicates that the strain carries an alcA::bime<sup>APC1</sup> fusion as it’s only functional bime<sup>APC1</sup> gene (Mirabito and Morris, 1993).

∥pyr<sup>4</sup>, bima-alc<sup>‡</sup> indicates that the strain carries an alcA::bime<sup>APC1</sup> fusion as it’s only functional bime<sup>APC1</sup> gene (Mirabito and Morris, 1993).
is pKK12 containing the nimE<sub>Cyclin B</sub> coding sequence (O’Connell et al., 1992) fused to the <i>alcA</i> promoter. pMO126 was made by ligating the oligonucleotide, 5’ GCGTTAACCCTTACGAGCTCCGGGAGTCCGGCGATGACCC -TAGCCG 3’, which encodes a single HA epitope, into the MluI site between codons 11 and 12 of nimE<sub>Cyclin B</sub> in pNIM11 (O’Connell et al., 1992). pNIMA<sub>Δ</sub>B (a gift from S. A. Osmani) is a plC19 clone of a 6 kb KpnI genomic nimA fragment (Osmani et al., 1987) in which the 2.1 kb nimA <i>PstI</i> fragment was replaced by the <i>Neurospora crassa</i> pyr4<sup>+</sup> gene. This removed all but the last 24 codons of the <i>nimA</i> coding sequence.

**Antibody production and purification**

<i>trpE::bimE</i><sub>APC1</sub> and <i>lacZ::bimE</i><sub>APC1</sub> encoded fusion proteins were isolated as inclusion bodies from <i>E. coli</i> cultures carrying pSJ19 or pSJ40 using standard procedures (Koerner et al., 1991; Rio et al., 1986). The <i>trpE::bimE</i><sub>APC1</sub> fusion protein was purified by SDS-PAGE, electroeluted, and used to immunize guinea pigs. Immunizations, serum collections, and serum processing were performed by Hazleton Research Products, Inc. (Denver, PA). The resulting antisera were purified on affinity columns containing <i>lacZ::bimE</i><sub>APC1</sub> fusion protein using standard procedures.

**Fluorescence microscopy**

Cells were fixed and stained with 4’,6-diamidino-2-phenylindole (DAPI) as described (Osmani et al., 1987). Cells were fixed and prepared for indirect immunofluorescence microscopy as described (Mirabito and Morris, 1993) with the following exceptions. Cell walls were removed using 40 mg/ml NOVO 234, 80 mg/ml Driselase (Interspex Products, Inc. Foster City, CA), 1 mM DIFP, 2 µg/ml leupeptin, 40 µg/ml aprotinin (Sigma). Lipids were extracted using –20°C methanol for 8 minutes followed by –20°C acetone for 30 seconds. Lipid extraction using room temperature methanol or 0.2% NP-40 yielded similar results. Cover slips were mounted on mounting medium (90% glycerol in TBS containing 1 mg/ml p-phenylenediamine). DM1A (Sigma) was used at 1:100 and CY3-labeled, goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) was used at 1:500.

**Culture media, preparation of cell extracts, western blot analysis and NIMA kinase assays**

SC is minimal medium supplemented with riboflavin, pyridoxine-HCl, p-aminobenzoic acid, biotin, methionine, nicotinic acid, chlorine-HCl, arginine (Kafer, 1977). YSC is SC supplemented with 0.5% yeast extract. YSC+UU is YSC supplemented with uracil and uridine (Kafer, 1977). Carbon sources were either 50 mM glucose, 50 mM glycerol, or 200 mM ethanol.

For induction of <i>alcA::bimE</i><sub>APC1</sub> transcription (see Fig. 1), cells were cultured overnight at 25°C in YSC+UU glucose, washed free of glucose with sterile saline, and then incubated in YSC+UU ethanol for 3 hours. For characterization of BIMA<sub>APC3</sub> and BIME<sub>APC1</sub> in extracts from wild-type cells (see Fig. 2), cultures were grown in YSC+UU glucose overnight at 25°C. For analysis of NIMA kinase levels (see Fig. 7C), strains were incubated at 25°C for 18 hours in SC glycerol. The cells were harvested by centrifugation, washed twice with YSC glucose, and then incubated in YSC glucose for 3 hours. For characterization of BIMA<sub>APC3</sub> and BIME<sub>APC1</sub> in extracts from the control strain and BIMA<sub>APC3</sub> and BIME<sub>APC1</sub> are components of an APC/C-like complex

** RESULTS**

BIMA<sub>APC3</sub> and BIME<sub>APC1</sub> are components of an APC/C-like complex

BIMA<sub>APC3</sub> and BIME<sub>APC1</sub> homologs are components of the APC/C in several organisms (King et al., 1996). If BIMA<sub>APC3</sub> and BIME<sub>APC1</sub> are part of the A. nidulans APC/C, we would expect them to be present in a large, 20S to 40S complex. We prepared antiserum specific to BIME<sub>APC1</sub> (Materials and Methods) to investigate possible BIMA<sub>APC3</sub>/BIME<sub>APC1</sub> interactions. The BIME<sub>APC1</sub> antiserum detected a doublet at 225 kDa (Fig. 1, lanes 1 and 6), consistent with the size predicted for BIME<sub>APC1</sub> (Engle et al., 1990). The antiserum also bound to a 120 kDa protein and some lower molecular mass proteins.

To investigate the antiserum specificity, we probed extracts from strains engineered to overexpress various forms of BIME<sub>APC1</sub>. Under the culture conditions used to induce BIME<sub>APC1</sub> overexpression, extracts from the control strain contained approximately normal levels of the anti-BIME<sub>APC1</sub>, reactive bands (Fig. 1, lanes 2, 7). The strain engineered to overexpress full length BIME<sub>APC1</sub> accumulated high levels of the 225 kDa doublet and multiple lower molecular mass bands (Fig. 1, lane 3). A doublet at 170 kDa and multiple lower molecular mass proteins also accumulated in a strain.
Fig. 1. Characterization of BIMEAPC1. Western blots containing protein from cells grown in glucose medium (lanes 1 and 11) or ethanol medium (lanes 2-10 and 12). Blots were probed with BIMEAPC1 antisera (lanes 1-5, 11 and 12) or with 12CA5 (lanes 6-10) to visualize 3HA-BIMEAPC1. Lanes 1, 2, 6 and 9, wild type (R153); lanes 3 and 10, BIMEAPC1 overexpressor (SWJ060); lanes 4 and 11, truncated BIMEAPC1 overexpressor (SWJ063); lanes 5 and 12, 3HA-BIMEAPC1 overexpressor (SWJ317). Lanes 6 and 7 are longer exposures of lanes 1 and 2. The asterisk indicates a protein(s) that cross reacts with the BIMEAPC1 antisera (see lanes 6 and 7). The dot indicates a protein that reacts with the 2’ Ab alone. The arrowheads indicate the BIMEAPC1 doublet.

expressing a truncated form of BIMEAPC1 (Fig. 1, lane 4). The high molecular mass doublet and the lower molecular mass isoform also accumulated in a strain expressing BIMEAPC1 fused to 3 copies of the hemagglutinin epitope (3HA-BIMEAPC1). Most of these proteins were also recognized by the anti-HA mAb, 12CA5 (Fig. 1, lane 12). These results indicated that BIMEAPC1 is present in two approximately 225 kDa isoforms. The nature of the difference in these BIMEAPC1 isoforms has not been determined.

To determine if BIMAAPC3 and BIMEAPC1 are physically associated, we isolated immune complexes using antisera specific to either BIMAAPC3 or BIMEAPC1 and probed each complex for the presence of both proteins. BIMAAPC3 antisera immunoprecipitated BIMAAPC3 and BIMEAPC1 (Fig. 2A, lanes 1-6) and BIMEAPC1 antisera immunoprecipitated BIMAAPC3 and BIMAAPC1 (Fig. 2A, lanes 7-12). The BIMAAPC3 and BIMEAPC1 immune complexes were stable in solutions containing 1% NP-40 and 1 M KCl (data not shown). Given that BIMAAPC3 antisera did not cross react with BIMEAPC1 and that BIMEAPC1 antisera did not cross react with BIMAAPC3 (Fig. 2A), the coinmunoprecipitation of BIMAAPC3 and BIMEAPC1 indicated that they are present in one or more complexes. To confirm the specificity of this result, we isolated immune complexes from 3HA-BIMEAPC1 containing extracts using 12CA5 or BIMAAPC3 antisera. BIMAAPC3 and 3HA-BIMEAPC1 were present in both types of immune complexes (data not shown).

To determine the size of the BIMAAPC3/BIMEAPC1 complex, we fractionated extracts from wild-type strains on sucrose gradients and assayed gradient fractions for BIMAAPC3 and BIMEAPC1 by western blot analysis. BIMAAPC3 and BIMEAPC1 cosedimented in a broad peak representing S values larger than 25S (Fig. 2B). Both proteins were also present in fractions nearer the top of the gradient, including another peak of cosedimentation at 15S. Multiple independent experiments confirmed co-sedimentation of the majority of BIMAAPC3 and BIMEAPC1 at >25S with a consistent, minor peak at 15 S. BIMAAPC3 and BIMEAPC1 immunoprecipitated from fractions containing the >25S complex, whereas we were unable to demonstrate immunoprecipitation of BIMAAPC3 and BIMEAPC1 from the 15S complex (data not shown). Identical profiles of BIMAAPC3 and BIMEAPC1 sedimentation were obtained using extracts that were first cleared by centrifugation at 109,000 g for 1 hour at 4°C (data not shown). Mutations affecting APC/C function in S. cerevisiae have been shown to confer sensitivity to overexpression of B-type cyclins (Irniger et al., 1995). To determine if bimaAAPC3 and bimaEAPC1 mutants were sensitive to B-type cyclin overexpression, we constructed strains that were ethanol-inducible for overexpression of the major G2 B-type cyclin, NIME Cyclin B (alcA::nimE Cyclin B strains). The mutant and wild-type strains grew well on glucose medium and the wild-type strains grew well on ethanol medium (Fig. 2C). In contrast, the growth of alcA::nimE Cyclin B strains containing mutations in either bimaAAPC3 or bimaEAPC1 were severely inhibited on ethanol medium.

**bimaAAPC3 mutations relieve the G2 arrest of the nimA5 mutation**

The bimaEAPC1 mutation causes defects in multiple cell cycle checkpoints, including the G2 cell cycle arrest caused by ts mutations in nimA (Osmani et al., 1988; James et al., 1995; Ye et al., 1996). This suggests a role for BIMEAPC1, and therefore, for the A. nidulans APC/C, in a G2 checkpoint activated by ts mutations in nimA. To determine if mutations in bimaAAPC3 also alter this checkpoint, we examined nuclear division in nimA5 and bimaAAPC3 single mutants, and nimA5, bimaAAPC3 double mutants during germination of G1 arrested spores at restrictive temperature. The nimA5 spores germinated and increased in size severalfold but did not enter mitosis (Fig. 3). Instead, they arrested with decondensed chromatin in an elongated, interphase like nucleus (Fig. 4B) or an abnormal nucleus (Fig. 4C) and interphase MTs (Fig. 4N). Half the bimaAAPC3 cells entered and arrested in mitosis by 6 hours (Fig. 3). The kinetics of mitotic cell accumulation was similar to the timing for nuclear division in wild-type control strains. The majority of the mitotic cells contained a single mass of condensed chromatin (Fig. 4D), however, cells with two separated chromatin masses were detectable (Fig. 4E). The other cells contained nuclei that were abnormal but not obviously mitotic based on DAPI staining (Fig. 4F). Half the cells contained a mitotic spindle and no interphase MTs (Fig. 4M) whereas the other half contained interphase microtubule arrays (data not shown).

Cells of the nimA5, bimaAAPC3 double mutant entered and arrested in mitosis with kinetics indistinguishable from the bimaAAPC3 single mutant (Fig. 3). At 7 hours, the double mutant culture accumulated a higher percentage of mitotic cells than the single bimaAAPC3 mutant (75% vs 50%). Most of the mitotic cells contained a single mass of condensed chromatin (Fig. 4G), however, cells with two or more chromatin masses also accumulated (Fig. 4H). The nonmitotic cells contained...
Fig. 2. Characterization of the <i>A. nidulans</i> APC/C. (A) Co-immunoprecipitation of BIMA<sup>APC3</sup> and BIME<sup>APC1</sup>. (A) Western blot of an immunoprecipitation of BIMA<sup>APC3</sup> using BIMA<sup>APC3</sup> antiserum (lane 2) shows coprecipitation of BIME<sup>APC1</sup> (lane 5). The reciprocal immunoprecipitation experiment, using BIME<sup>APC1</sup> antiserum, shows coprecipitation of BIME<sup>APC1</sup> (lane 11) and BIMA<sup>APC3</sup> (lane 8). Lanes 1, 4, 7, and 10 are samples of whole cell extracts. Lanes 3, 6, 9, and 12 are samples of the supernatant remaining after immunoprecipitation. Lanes 1-3 and 7-9 were probed with BIMA<sup>APC3</sup> antiserum and lanes 4-6 and 9-12 were probed with BIME<sup>APC1</sup> antiserum. (B) Characterization of the BIMA<sup>APC3</sup>/BIME<sup>APC1</sup> complex by sucrose gradient centrifugation. Western blot containing samples from a cell extract fractionated on a linear 15-40% sucrose gradient. Arrowheads indicate the peak fraction of the sedimentation markers catalase (11s), 18S <i>A. nidulans</i> rRNA, and 25S <i>A. nidulans</i> rRNA. Lanes 1-3 were probed with BIME antiserum and lanes 4-6 were probed with BIMA<sup>APC3</sup> antiserum. (C) Elevated expression of NIME Cyclin B inhibits the growth of bimA<sup>APC3</sup> and bimE<sup>APC1</sup> mutants. 10<sup>6</sup> spores were spotted onto glucose or ethanol medium and incubated at 32˚C for 3 days. Strain key: 1, FGSCA28 (wild type); 2, MO68 (alcA::nimE<sup>Cyclin B</sup>); 3, PM156 (bimA<sup>APC3</sup>); 4, PMC892-3 (bimA<sup>APC3</sup>, alcA::nimE<sup>Cyclin B</sup>); 5, MLC1-19 (bimA<sup>APC3</sup>); 6, JCC51-6 (bimA<sup>APC3</sup>, alcA::nimE<sup>Cyclin B</sup>); 7, MMB001 (bimE<sup>APC1</sup>); 8, SFC138-6 (bimE<sup>APC1</sup>, alcA::nimE<sup>Cyclin B</sup>).

Fig. 3. Mitosis in cell cycle mutants during germination at restrictive temperature. Cultures were prepared and incubated at 43˚C and the fraction mitotic cells were determined. Strains are: PM156 (bimA<sup>APC3</sup>), PM152 (bimA<sup>APC3</sup>, nimA5), SWJ216 (nimT23<sup>16kD</sup>,), PM161 (nimA5), PM221 (bimA<sup>APC3</sup>, nimT23<sup>16kD</sup>), Each data point represents the average of 3 independent cell counts. Error bars represent 1 s.d.
Regulation of mitosis by the APC/C

abnormal nuclei (Fig. 4I) and interphase-like microtubule arrays (data not shown). Although some cells contained bipolar spindles, the vast majority of the mitotic cells contained an abnormal microtubule array resembling a monopolar spindle or mitotic aster (Fig. 4O). MPM2 stained only one discrete focus at the center of these asters, consistent with the presence of duplicated but unseparated SPBs (data not shown). Similar results were obtained when asynchronous, log phase cultures of nimA5 and bimA1 APC3 single and double mutants were shifted to restrictive temperature (data not shown).

To determine whether the interaction of nimA5 with mitotic mutations is specific to mutations affecting the APC/C or if it is a consequence of leak through the nimA5 arrest point, we examined a strain containing nimA5 and the benA33 mutation. benA33 is a ts mutation in a β-tubulin gene which causes a metaphase-like mitotic arrest (Oakley and Morris, 1981). Germination of the nimA5, benA33 double mutant at restrictive temperature gave results which were essentially identical to those for the nimA5 single mutant (data not shown). This indicated that the mitotic arrest in the nimA5, bimA1 APC3 double mutant is not due to leak through the nimA5 arrest point.

Initiation of mitosis in bimA1 APC3 mutants is dependent on NIMX CDC2/NIME Cyclin B function

Initiation of mitosis in bimE APC1 mutants was shown to be dependent on NIMX CDC2/NIME Cyclin B function (James et al., 1995). To determine if mitotic initiation in bimA1 APC3 mutants was also dependent on NIMX CDC2/NIME Cyclin B, we examined nuclear division in strains containing bimA1 APC3 and either nimT23 cdc25, nimE6 Cyclin B, or nimX3 CDC2. In contrast to bimA1 APC3, nimA5 double mutants, the majority of bimA1 APC3, nimT23 cdc25 double mutants arrested in interphase of the first nuclear division (Fig. 3). The accumulation of 20% mitotic bimA1 APC3, nimT23 cdc25 cells corresponded to cells which had leaked through the nimT23 cdc25 interphase arrest (data not shown; also see James et al., 1995). Similar results were obtained for the bimA1 APC3, nimE6 Cyclin B double mutant and bimA1 APC3, nimX3 CDC2 double mutant (see Table 3).

The results above demonstrated that entry into mitosis occurred without delay in bimA1 APC3, nimA5 double mutants but not in strains containing mutations in bimA1 APC3 and either nimT23 cdc25, nimE6 Cyclin B, or nimX3 CDC2. This implies that activation of NIMX CDC2/NIME Cyclin B is required for mitosis in the bimA1 APC3, nimA5 double mutant. To investigate this, we examined nuclear division in a bimA1 APC3, nimA5, nimT23 cdc25 triple mutant. The results for the triple mutant were essentially identical to those for the bimA1 APC3, nimT23 cdc25 double mutant: at restrictive temperature, the majority of cells arrested with a single interphase nucleus and 20% of the cells leaked through the interphase arrest into mitosis (data not shown).

The G2 checkpoint defect in bimA1 APC3 mutants is not allele specific

To determine if the interaction between bimA1 APC3 and nimA is specific to the bimA1 APC3 allele, we examined nuclear division in two additional nimA5, bimA1 APC3 double mutants: nimA5, bimA9 APC3, and nimA5, bimA1 APC3-alc. The ts bimA9 APC3
mutation was very leaky for nuclear division, as all cells divided at least once (Fig. 5A,B). Nuclear division eventually stopped by 10 hours, with cells containing up to 8 abnormally shaped nuclei (data not shown). Barely 20% of the \textit{bimA9 APC3} cells were mitotic at any time during the experiment. In contrast, very few of the \textit{bimA9 APC3}, \textit{nimA5} double mutant cells divided and 60% entered and arrested in mitosis with wild-type kinetics. Similar results were obtained when asynchronous cultures of \textit{nimA5} and \textit{bimA9 APC3} single and double mutants were shifted to restrictive temperature (data not shown).

In \textit{bimA APC3-alc} strains, the only functional \textit{bimA APC3} is under control of the \textit{alcA} promoter (Mirabito and Morris, 1993). These strains cannot grow on glucose medium but grow well on ethanol medium. In glucose medium at 43°C, the majority of \textit{bimA APC3-alc} cells arrested growth with abnormal nuclei containing decondensed chromatin (data not shown), with 30% of the cells arrested in mitosis (Fig. 5C,D). One nuclear division occurred in half of the \textit{bimA APC3-alc} cells. We were unable to detect BIMA APC3 on western blots of protein isolated from \textit{bimA APC3-alc} mutants germinated under these culture conditions (data not shown). By 7 hours, up to 80% of the \textit{nimA5}, \textit{bimA APC3-alc} double mutant cells had entered and arrested in mitosis. These results indicated that the interaction between \textit{nimA5} and \textit{bimA APC3} is not specific to \textit{bimA1 APC3}.

Furthermore, they demonstrated that the affect of \textit{bimA9 APC3} and \textit{bimA APC3-alc} mutations on exit from mitosis are enhanced when combined with \textit{nimA5}.

**The interphase arrest in \textit{nimA} mutants is due to loss of NIMA function**

The finding that the \textit{bimA1 APC3}, \textit{nimA5}, \textit{nimT23 cdc25} triple mutants arrest in interphase clearly indicated that NIMX\textsuperscript{CDC2/NIME}\textsubscript{Cyclin B} is required for mitosis in \textit{bimA APC3}, \textit{nimA5} double mutants. Given that NIMA accumulates in \textit{nimA5} strains incubated at restrictive temperature (Osmani et al., 1991a), it was possible that NIMA activity might also be required for mitosis in \textit{nimA5}, \textit{bimA APC3} mutants. To address this issue, we constructed strains in which the only \textit{nimA} gene was the \textit{nimA} cDNA under control of the \textit{alcA} promoter. We used these strains, which are analogous to the \textit{bimA APC3-alc} strains described above, to determine the phenotype of \textit{nimA} single and \textit{nimA}, \textit{bimA APC3} double mutants in the absence of NIMA expression.

In order to determine if the \textit{alcA:nima} gene was functional and if glucose medium could repress \textit{alcA:nima} function, we compared the growth of a \textit{nima5} mutant (SWJ071), a \textit{nima5} mutant containing an ectopic copy of \textit{alcA:nima} (TSF2-1), and a \textit{nima} deletion strain containing an ectopic copy of \textit{alcA:nima} (SFC466-201) on glucose and ethanol media at

**Fig. 5.** Nuclear division and mitosis in cell cycle mutants during germination under restrictive culture conditions. Cultures were prepared and incubated at 43°C and the average nuclear number and fraction of mitotic cells were determined. Each data point was determined by counting more than 300 cells. Strains were: (A and B) MLC1-19 (\textit{bimA9 APC3}) and SFC11-7 (\textit{bimA9 APC3}, \textit{nimA5}). (C and D) TPM200 (\textit{bimA-alc APC3}) and PMC462-5 (\textit{bimA-alc APC3}, \textit{nimA5}).
32˚C and 43˚C (Fig. 6A). The wild-type control grew well on both media at both temperatures. Like SWJ071, TSF2-1 grew on both media at 32˚C. In addition, TSF2-1 grew well at 43˚C on ethanol medium whereas SWJ071 did not, indicating that alcA::nimA can complement nimA5. Furthermore, TSF2-1 could not grow on glucose medium at 43˚C, indicating that repression of alcA::nimA expression was sufficient to prevent complementation of nimA5. SFC466-201 grew well on ethanol medium at either 32˚C or 43˚C but did not grow at either temperature on glucose medium. This indicated that repression of alcA::nimA is lethal and, therefore, that nimA is an essential gene. We have used nimA-alc to indicate the genotype of strains containing alcA::nimA as their only nimA gene. As with nimA5 mutants germinated at restrictive temperature, nimA-alc mutants germinated in glucose medium arrested with a single, interphase nucleus and cytoplasmic microtubule arrays (Fig. 6B,C).

To determine if BIMA APc3 is required for the interphase arrest in nimA-alc mutants, we analyzed nuclear division in a nimA-alc single mutant and a nimA-alc, bimA1APc3 double mutant. In glucose medium, the nimA-alc single mutant arrested with a single interphase nucleus, whereas the nimA-alc, bimA1APc3 double mutant entered mitosis with wild-type kinetics (Fig. 7A,B). Essentially all cells of the double mutant arrested in mitosis with aster-like microtubule arrays (data not shown).

Although the alcA promoter is known to be tightly repressed in strains grown on glucose medium (Gwynne et al., 1987), it was possible that accumulation of significant NIMA kinase activity may have occurred in the nimA-alc, bimA1APc3 double mutant, leading to entry into mitosis. To investigate this possibility, we measured the NIMA kinase activity of the nimA-alc single mutant and the nimA-alc, bimA1APc3 double mutant under conditions which repress nimA-alc expression and are restrictive for the bimA1APc3 mutation (see Materials and Methods). We compared these levels to NIMA kinase levels in asynchronous wild-type cells, bimA1APc3 cells at restrictive temperature, and in cells from a synchronous culture arrested in mitosis by benomyl addition. Under these conditions, the cell cycle arrest of the nimA-alc single mutant and the nimA-alc, bimA1APc3 double mutant were similar to that described above (Fig. 8). Little NIMA kinase activity was detected in either the nimA-alc single mutant or the nimA-alc, bimA1APc3 double mutant, consistent with the depletion of NIMA under these conditions. The difference between the NIMA kinase levels in the benomyl arrested mitotic culture and the nimA-alc, bimA1APc3 mitotic culture was striking. Even cells from the asynchronous wild-type control culture accumulated
Fig. 8. nimA-alc, bimA1APC3 double mutants enter mitosis but do not accumulate NIMA kinase activity. Cultures were first shifted from glycerol to glucose medium to repress NIMA expression in nimA-alc strains and then shifted to 43°C for 3 hours to inactivate bimA1APC3 (see Materials and Methods). NIMA-specific kinase activity was measured in NIMA immunoprecipitates using standard procedures (Lu et al., 1993; Ye et al., 1997). NIMA kinase activity is expressed relative to that in the wild-type control sample. The results in the inset are the average of 3 independent experiments (error bars = one standard deviation). Strains were PMC654-4 (wild type), SFC4-2 (bimA1APC3), SFC466-201 (nimA-alc), and SFC466-48 (nimA-alc, bimA1APC3). The control mitotic culture was obtained by first arresting a nimT2Scdc25 mutant (SFC4-21) in G2 at 43°C and then releasing it into mitosis in the presence of 5 μg/ml benomyl for 15 minutes (Ye et al., 1995).

considerably more activity than either of the nimA-alc mutant strains. Furthermore, there was little to no significant difference between the NIMA activity detected in the nimA-alc single mutant and the nimA-alc, bimA1APC3 double mutant (Fig. 8, inset). Thus, the mitotic arrest of the nimA-alc, bimA1APC3 double mutant is not explained by accumulation of significant NIMA kinase activity.

**bimA1APC3** mutations have little effect on steady state NIMEcyclin B levels

Given that BIMA1APC3 appeared to be a component of the APC/C, we considered the possibility that *bimA1APC3* mutations may lead to abnormal accumulation of the B-type cyclin, NIMEcyclin B, and that this may underlie the abnormal mitosis observed in nimA-alc, bimA1APC3 double mutants. To investigate this, we examined NIMEcyclin B levels in wild-type and mutant strains cultured at permissive or restrictive temperature. In order to specifically detect NIMEcyclin B, we constructed strains which contained a HA-tagged nimEcyclin B (HA-nimEcyclin B) in place of the endogenous nimEcyclin B gene. HA-NIMEcyclin B reacts with polyclonal NIMEcyclin B antiserum and with the mAB, 12CA5 (Fig. 9A). None of the strains examined demonstrated a dramatic increase in the steady state levels of HA-NIMEcyclin B after shift to restrictive temperature (Fig. 9B), including the bimA1APC3, nimA5 double mutant. Thus, the phenotype of *bimA1APC3* mutants is not due to large increases in the steady state levels of NIMEcyclin B.

**DISCUSSION**

*bimE2APC3* was one of the first mutations shown to cause cell cycle checkpoint defects (Osmani et al., 1988). This mutation inactivates checkpoints thought to coordinate mitosis with DNA replication (Osmani et al., 1988; Ye et al., 1996) and NIMA function (Osmani et al., 1988, 1991a). The identification of BIMEAPC1 homologs in the APC/C led to the hypothesis that the *A. nidulans* APC/C is involved in these checkpoints (Peters et al., 1996; Ye et al., 1997). Our results strongly support this hypothesis, and they indicate a central role for the APC/C component, BIMA1APC3, in a G2 checkpoint regulating entry into mitosis.

**Identification of the A. nidulans APC/C**

BIMA1APC3 and BIMEAPC1 cosedimented in a large, >25S complex which almost certainly corresponds to the APC/C (Fig. 2). Like the *S. cerevisiae* APC/C (Zachariae et al., 1996b),

![Image](image-url)

**Fig. 9.** Western blot analysis of NIME. Cultures were prepared as described in Materials and Methods. (A) Characterization of a strain expressing HA-NIMEcyclin B. Western blots containing cell extracts isolated from a HA-nimEcyclin B strain (+) and a no HA control (−). The blots were probed with either NIMEcyclin B antiserum (left) or 12CA5 (right). The HA-tagged NIMEcyclin B migrates at a position slightly above wild-type NIMEcyclin B. (B) Western blot analysis of HA-NIMEcyclin B levels in wild-type and cell cycle mutants. Western blots containing protein from cultures before (0) and 2, 4, and 6 hours after a shift to restrictive temperature. The blots were first probed with 12CA5 to visualize HA-NIMEcyclin B (top panel) and then were probed with DM1A to visualize alpha tubulin (bottom panel). Strains analyzed are indicated above the blots: wild type, PMC654-4; bimA9APC3, SFC11-3; bimA1APC3, PMC661-30; nimA5, PMC654-19; nimA5, bimA1APC3, PMC693-10.
the A. nidulans APC/C is considerably larger than that identified in X. laevis (King et al., 1995) and S. pombe (Yamashita et al., 1996). A minor peak of BIMA APC3 and BIME APC1 cosedimentation was consistently identified at 15S. We have not yet determined the nature of the difference between the 15S and >25S complexes. Preliminary experiments indicate that cells blocked at different stages of the cell cycle contain BIMA APC3 and BIME APC1 complexes similar to that shown in Fig. 2, suggesting that the difference in size is not cell cycle specific (C. M. Lies and P. M. Mirabito, unpublished results). It is possible that the smaller complex represents an intermediate in APC/C assembly. Alternatively, given the pleiotropic phenotype of bimA APC3 and bimE APC1 mutants, it is tempting to speculate that the two complexes represent functionally distinct forms of the APC/C.

The G2 arrest of nimA mutants is dependent on the APC/C

nimA ts mutants arrest very late in G2, post activation of CDC2 (Osmani et al., 1991; Ye et al., 1995). This arrest is somewhat perplexing, given that activation of CDC2 is widely accepted as a universally conserved, ultimate trigger for initiation of mitosis. Our results demonstrate that the G2 arrest in nimA mutants is due to loss of nimA function and that it is dependent on BIMA APC3. Loss of function mutations in bimA APC3 relieved the dependence of mitotic initiation on nimA, resulting in entry into an aberrant mitosis in the absence of NIMA function. Together with the demonstration of BIMA APC3 and BIME APC1 as APC/C components, these results support a model in which APC/C is required in G2 to prevent inappropriate entry into mitosis in nimA mutants.

An APC/C-dependent G2 checkpoint regulating entry into mitosis

In developing a model to explain the role of the APC/C in the G2 arrest of nimA mutants, we have made two key assumptions. First, we assumed that the APC/C is not active in G2 of a normal, nimA+ cell cycle. Second, we assumed that activation of the APC/C leads to the ubiquitinylation and subsequent proteolysis of one or more target proteins. Both assumptions are supported by studies in frog and yeast (King et al., 1995; Zachariae and Nasmyth, 1996a) and are entirely consistent with the contemporary view of APC/C function. Based on these assumptions, and on our results showing that the G2 arrest of nimA mutants is dependent on APC/C function, we propose that loss of NIMA function leads to APC/C activation, and that the APC/C then targets the destruction of one or more proteins required for initiation of mitosis (Fig. 10).

One novel prediction of this model is that some defect in nimA mutants specifically leads to activation of the APC/C in G2. APC/C activation may be the result of a checkpoint signal elicited by a defective process or structure which accumulates in nimA mutants. Given that entry into mitosis in the absence of NIMA function results in aberrant microtubule organization (Fig. 4), it is possible that defects in microtubule or spindle pole body (SPB) structure accumulate in nimA mutants and are detected by a G2 checkpoint. The localization of BIMA APC3 to the SPB is consistent with its role in such a checkpoint involving SPB function.

Another potential mechanism by which the checkpoint could sense loss of NIMA function is that NIMA directly phosphorylates and inhibits the function of a component of the checkpoint pathway. In this case, NIMA function is essentially part of the checkpoint, which would program the checkpoint to respond rapidly to loss of NIMA function. This hypothesis predicts that one or more components of the checkpoint would be substrates of NIMA.

Another novel prediction of this model is that the APC/C ubiquitinylates protein(s) involved in initiation of mitosis. Since inactivation of NIMXCDC2/NIME Cyclin B prevents mitotic initiation in checkpoint defective strains, it is possible that the function of NIMXCDC2/NIME Cyclin B is the ultimate target for this checkpoint. If so, then NIMXCDC2/NIME Cyclin B function must be indirectly inhibited, since mitotic levels of active NIMXCDC2/NIME Cyclin B accumulate in nimA mutants (Osmani et al., 1991a; Ye et al., 1995). One way in which the APC/C could indirectly inhibit NIMXCDC2/NIME Cyclin B is by affecting its accumulation at important subcellular locations. For example, BIMA APC3 and the NIMXCDC2/NIME Cyclin B complex both localize to the spindle and nucleus and NIMXCDC2/NIME Cyclin B mislocalizes to the cytosol in nimA- mutants (Mirabito and Morris, 1993; L. Wu, S. Osmani, and P. Mirabito, unpublished). The APC/C G2 checkpoint could target protein(s) required for proper NIMXCDC2/NIME Cyclin B localization, thus preventing mitosis without resulting in dramatic decreases in bulk NIMXCDC2/NIME Cyclin B activity.

Differential roles for BIME APC1 and BIMA APC3 in APC/C function

Mutations in bimE APC1 and bimA APC3 have different effects on cell cycle progression. For example, bimE APC1 mutants undergo a first cycle, metaphase arrest (Osmani et al., 1988) whereas bimA APC3 mutants never arrest in the first cycle and...
only 20% of bimA9APC3 cells have a mitotic terminal arrest phenotype (Fig. 5). Thus, bimE7APC1 has a more severe impact than bimA9APC3 on exit from mitosis. Conversely, the bimE7APC1 mutation relieves the G2 arrest in nimA mutants only after a considerable delay (Osmani et al., 1988; James et al., 1995), whereas the bimA9APC3 mutation relieves the G2 arrest of nimA mutants without delay (Fig. 5). It is unlikely that quantitative differences in the effect of bimE7APC1 and bimA9APC3 on APC/C function can satisfactorily explain these contradictory results. Rather, the results imply that BIMA APC3 and BIME APC1 have overlapping but distinct roles in APC/C function. BIMA APC3 may be particularly important for the proposed G2 checkpoint whereas BIME APC1 may be more important for progression past metaphase. For example, BIMA APC3 and BIME APC1 may be directly involved in distinct pathways of APC/C activation or in the recognition of different APC/C substrates. This is consistent with the diverse effects of mutations in different APC/C genes of S. pombe (Yamada et al., 1997) and it would provide a rationale for the yet unexplained complexity of the APC/C.

It is interesting that the bimA9APC3, nimA5 double mutant not only enters mitosis but also arrests in mitosis, even though the bimA9APC3 single mutant divides at least once before terminal arrest (Fig. 5). The mitoses in bimA9APC3 mutants produce abnormal nuclei resembling those seen in aneuploids, suggesting a defect in mitotic chromosome transmission (Harris and Hamer, 1995). nimA mutants are likewise defective in chromosome transmission (Upshall and Mortimore, 1984). The synthetic mitotic arrest phenotype of nimA5 with bimA9APC3 implies that NIMA and BIMA APC3 interact to regulate chromosome segregation and exit from mitosis.

In summary, the data presented here show that the G2 arrest in nimA mutants is dependent on the APC/C. Although our results do not address the mechanism which underlies this dependence, they clearly indicate an intimate relationship between NIMA function and APC/C dependent proteolysis. Genetic and biochemical experimental approaches are currently underway to identify additional components of the proposed G2 checkpoint, and these studies should provide insight into the function of the APC/C in G2. Given the evolutionary conservation of the APC/C and the increasing evidence for NIMA-related function in other organisms (O’Connell et al., 1994; Lu and Hunter, 1995), it is tempting to speculate that the proposed G2 checkpoint may be a conserved feature of mitotic regulation.

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