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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., 1998). 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

Table 1. Names of anaphase promoting complex/cyclosome components relevant to this work

General name	*A. nidulans*	*S. cerevisiae*	*S. pombe*	*X. laevis*
APC1	BIME	APC1	CUT4	BIME
APC3	BIMA	CDC27	NUC2	CDC27
APC6	BIMH*	CDC16	CUT9	CDC16
APC8	Not known	CDC23	Not known	APC8

*J. Cheng and P. Mirabito, unpublished results.

CDC16*APC6* is required for ubiquitinylation of several B-type cyclins and PDS1.

Multiple roles in cell cycle control have also been suggested for the *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 *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 CUT4APC1 or CUT9APC6 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 NUC2APC3 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. NUC2APC3 is also required for the G_1 arrest due to nitrogen starvation (Kumada et al., 1995), implicating the *S. pombe* APC/C in pathways linking nutritional signals to the cell cycle.

Based on sequence comparisons, BIMEAPC1 and BIMAAPC3 are two potential components of the *A. nidulans* APC/C (Engle et al., 1990; O'Donnell et al., 1991). Recessive, conditionally lethal mutations in either *bimEAPC1* or *bimAAPC3* 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 BIMEAPC1 in checkpoints regulating entry into mitosis. The temperature sensitive (ts) *bimE7APC1* 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). *bimE7APC1* also overcomes the interphase arrest due to ts mutations in *nimQMCM2*, *nimR*, and *nimA*. Whereas ts *nimQ20MCM2* or *nimR21* mutants arrest before S phase, and ts *nimA5* mutants arrest in late G_2, double mutants carrying *bimE7APC1* in combination with *nimQ20MCM2*, *nimR21*, or *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 NIMQMCM2, NIMR or NIMA activates a checkpoint which acts through BIMEAPC1 to prevent entry into mitosis.

Whereas little is yet known about the function of NIMQMCM2 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 *nimA* arrest cells in G_2 at a stage that is downstream of tyrosine dephosphorylation of the *A. nidulans* CDC2 homolog, NIMXCDC2 (Osmani et al., 1991a; Lu and Means, 1994; Ye et al., 1995). Dominant gain of function mutations in *nimA* induce chromosome condensation independent of NIMXCDC2 activation (O'Connell et al., 1994; Pu and Osmani, 1995). These data are consistent with a model in which NIMA functions downstream of NIMXCDC2/NIMECyclin B to promote chromosome condensation. Overexpression of NIMA results in loss of cytoplasmic microtubules (MTs) in *A. nidulans*, and *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 *bimE7APC1* with *nimA*$^-$ mutations together with the identification of BIMEAPC1 homologs in the APC/C suggests that the APC/C functions in a G_2 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 BIMEAPC1 and determined that BIMEAPC1 and BIMAAPC3 were present in a complex with a sedimentation rate consistent with that of the APC/C. We have also determined that BIMAAPC3 function was essential for arrest in G_2 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 G_2 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 *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 5×10^8 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-alc, which contained *alcA::nimA* as the only functional *nimA* gene, were constructed as follows. SWJ071 was transformed with pMO137 (O'Connell et al., 1994) to yield TSF2-1. TSF2-1 was crossed to SO8 to yield SFC161-9. SFC161-9 was transformed with pNIMAΔB linearized with *Kpn*I 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 *alcA::nimA* as their only copy of *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 *nimECyclin B* as its only *nimECyclin B* gene was constructed in two steps. SO25 was transformed with pMO126 to yield TMO126, which contained a single pMO126 integrated at the *nimECyclin B* locus. FOA resistant mitotic segregants were isolated and screened for the presence of HA-tagged *nimECyclin B* (*HA-nimECyclin B*) by Southern blot analysis. One such strain, MO78, contained *HA-nimECyclin B* as it's only *nimECyclin B* gene.

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

Strain	Genotype	Reference or derivation
FGSCA28	*biA1, pabaA1*	FGSC*
JCC51-6	*bimA9, argB2, nicA2, [argB+, alcA::nimE at argB]***	MLC489-8 × PMC892-19***
MLC1-19	*bimA9, pabaA1, nicA2, yA2*	GR5 × PMC449-1
MMB001	*bimE7, methB2, yA2*	SO4 × FGSCA272
MO68	*argB2, pabaA1, [argB+, alcA::nimE at argB]*	SWJ008 + pMO126‡
MO78	*biA1, pyrG89, pabaA1, methG11, sC12, choA1, chaA1, HA-nimE*	segregant of TMO126§
PM152	*bimA1, nimA5, pabaA1*	PM131 × SWJ014
PM156	*bimA1, pabaA1, wA2*	PM131 × SWJ014
PM161	*nimA5, pabaA1, wA2*	PM131 × SWJ014
PM211	*bimA1, nimE6, riboA1, wA2*	PM131 × SWJ195
PM221	*bimA1, nimT23, pabaA1, wA2*	PM131 × SWJ106
PM274	*nimA5, benA33, pabaA1, wA2, yA2*	RM24 × SWJ071
PMC462-5	*nimA5, pyrG89, [pyr4+, bimA-alc]*¶	SO6 × TPM200
PMC654-4	*nicA2, cnxE16, wA3, HA-nimE*	SWJ071 × MO78
PMC654-19	*nimA5, nicA2, cnxE16, HA-nimE, chaA1*	SWJ071 × MO78
PMC661-30	*bimA1, HA-nimE, choA1, yA2, chaA1*	PM131 × MO78
PMC693-10	*bimA1, nimA5, cnxE16, HA-nimE*	PMC661-27 × PMC654-19
PMC892-3	*bimA1, argB2, nicA2, pabaA1, [argB+, alcA::nimE at argB]*	PM144 × MO68
PMC892-19	*nicA2, argB2, [argB+, alcA::nimE at argB]*	PM144 × MO68
R153	*pyroA4, wA3*	FGSC
SFC11-3	*bimA9, HA-nimE, wA2, nicA2*	PMC654-19 × MLC1-54
SFC11-7	*bimA9, nimA5, wA2, HA-nimE*	PMC654-19 × MLC1-54
SFC161-9	*bimA1, pyrG89, nicA2, choA1, wA2, argB2, [argB+, alcA::nimA at argB]*	TSF2-1 × SO8
SFC466-48	*bimA1, argB2, pyrG89, nicA2, choA1, chaA1, [argB+, alcA::nimA at argB], [pyr4+, nimAΔ]*‖	TPM300-40 × FGSCA89
SFC466-201	*argB2, pyrG89, nicA2, wA2, [argB+, alcA::nimA at argB], [pyr4+, nimAΔ]*	TPM300-40 × FGSCA89
SO8	*bimA1, pyrG89, choA1, wA2*	O'Donnell et al., 1991
SWJ008	*argB2; pabaA1*	SWJ012 × FSCCA122
SWJ009	*bimE7;argB2; pabaA1; fwA1*	SWJ001 × FGSCA122
SWJ060	*argB2; pabaA1*	SWJ008 + pSJ61
SWJ063	*argB2; pabaA1*	SWJ008 + pSJ60
SWJ071	*nimA5, argB2, nicA2, wA2*	SJW015 × SWJ024
SWJ216	*nimT23, methB3, yA2*	SWJ166 × SWJ008
SWJ317	*bimE7;argB2; pabaA1; fwA1, [argB+, alcA::3HA-bimE at argB]*	SWJ009 + pbimE^{3xHA}
TMO126	*biA1, pyrG89, pabaA1, mehtG11, sC12, choA1, chaA1, nimE6, [pyr4+, HA-nimE at nimE]*	SO25 + pMO126
TPM200	*pyroA4, wA2, pryG89, [pyr4+, bimA-alc]*	Mirabito and Morris, 1993
TPM300-40	*bimA1, pyrG89, nicA2, choA1, wA2, argB2, [argB+, alcA::nimA at argB], [pyr4+, nimAΔ]*	SFC161-9 + pNIMΔB
TSF2-1	*nimA5, argB2, nicA2, wA2, [argB+, alcA::nimA at argB]*	SWJ071 + pMO137
TSJ36	*bimE7;argB2; pabaA1; fwA1*	SWJ009 + pSJ61
YYC67-25	*bimA1, nimX3, choA1, pabaA1, riboA1, yA2*	SFC4-14 × SO65
YYC36-38	*bimA1, nimA5, nimT23, pabaA1, yA2*	SFC4-1 × SFC4-5

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).

*FGSC = Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas 66160-7420, USA.

**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 indicated. For example *[argB+, alcA::nimE at argB]* indicates that JCC51-6 carries a plasmid with the *argB+* 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.

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

§MO78 is a FOA resistant, *pyrG89* 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.

¶*[pyr4, bimA-alc]* indicates that the strain carries an *alcA::bimA* fusion as it's only functional *bimA* gene (Mirabito and Morris, 1993).

‖*[pyr4+, nimAΔ]* indicates that the *nimA* gene has been deleted and replaced with the *pyr4* gene as described in Materials and Methods.

Plasmid constructions

Plasmids were constructed using standard techniques (Sambrook et al., 1989). The *bimE^{APC1}* nucleotide position numbers refer to Fig. 6 of Engle et al. (1990). pSJ19 is a 1,041 bp *Eco*RI-*Hin*dIII *bimE^{APC1}* cDNA fragment (nt 1,309-2,350) inserted into pATH3 (Koerner et al., 1991). pSJ40 is a 999 bp *Eco*RI-*Sst*II *bimE^{APC1}* cDNA fragment (nt 1,309-2,308) blunted and ligated into pUR292 (Ruther and Muller-Hill, 1983). pSJ61 contains an *alcA::bimE^{APC1}* fusion in a modified pKK12 (Kirk and Morris, 1993) in which the *alcA* promoter of pKK12 was replaced with the *alcA* promoter from pAL4 (Waring et al., 1989). pSJ61 contains the *alcA* ATG fused to nt 709 through 7,021 of the *bimE^{APC1}* cDNA which is fused to *bimE^{APC1}* genomic DNA sequences up to the *Sca*I site (Engle et al., 1990). pSJ60 is the same as pSJ61 except that an *Eco*RV fragment containing *bimE^{APC1}* cDNA from nt 4,293 to 5,655 was removed. pSJ60 is predicted to encode a truncated 170 kDa form of BIME^{APC1}. p3HA-bimE^{APC1} is pSJ61 with a *Not*I fragment encoding 3 tandem copies of the HA epitope (from pGTEPI, a gift from G. Tokiwa) inserted after the *bimE^{APC1}* ATG. A *Not*I site was added at *bimE^{APC1}* nt 709 using the pSELECT system (Promega Corp., Madison, WI) and the oligonucleotide 5′ TGG-GAGCTCGTCGACTCAAAATGGGCGGCCGCAACGCCGCCTC-TAAA 3′. The *alcA::bimE^{APC1}* constructs in plasmids pSJ61 and p3HA-bimE^{APC1} were shown to be functional *bimE^{APC1}* genes by complementation of a *bimE7^{APC1}* mutant. pSJ61 was transformed into ASJ009 to make TSJ36 and p3HA-bimE^{APC1} was transformed into ASJ009 to make SWJ317. TSJ36 and SWJ317 were unable to grow at 43˚C on glucose containing medium but were able to grow at 43˚C on medium containing ethanol or glycerol as carbon source. pMO120

is pKK12 containing the *nimE*$^{Cyclin\ B}$ coding sequence (O'Connell et al., 1992) fused to the *alcA* promoter. pMO126 was made by ligating the oligonucleotide, 5′ CGCGTTACCCCTACGACGTCCCCGACTACGCCG 3′, which encodes a single HA epitope, into the *Mlu*I site between codons 11 and 12 of *nimE*$^{Cyclin\ B}$ in pNIM11 (O'Connell et al., 1992). pNIMAΔB (a gift from S. A. Osmani) is a pUC19 clone of a 6 kb *Kpn*I genomic *nimA* fragment (Osmani et al., 1987) in which the 2.1 kb *nimA Pst*I fragment was replaced with the *Neurospora crassa pyr4* gene. This removed all but the last 24 codons of the *nimA* coding sequence.

Antibody production and purification

trpE::bimE^{APC1} and *lacZ::bimE*^{APC1} encoded fusion proteins were isolated as inclusion bodies from *E. coli* cultures carrying pSJ19 or pSJ40 using standard procedures (Koerner et al., 1991; Rio et al., 1986). The *trpE::bimE*^{APC1} 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 *lacZ::bimE*^{APC1} 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, choline-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 *alcA::bimE*^{APC1} 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^{APC3} and BIME^{APC1} 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 at 32°C. This treatment arrested *nimA-alc* strains in interphase. The cells were then transferred to 43°C and incubated for 3 hours. For analysis of NIME$^{Cyclin\ B}$ levels (see Fig. 8), cultures were incubated overnight in YSC glucose at 25°C, shifted to 43°C gradually over a 30 minute period, and then incubated for up to 6 hours at 43°C.

Cells were harvested, disrupted, and assayed for NIMA kinase activity using the NIMA antiserum, E14 (a gift from S. A. Osmani) and standard procedures (Lu et al., 1993; Ye et al., 1997a). For western blot analyses, total mycelial protein was isolated using the following procedure. Cells were harvested by filtration, washed with TBS + 1 mM DIFP, rapidly dried by blotting between paper towels, and placed in ice-cold protein isolation buffer (PIB) which contained 50 mM Tris-HCl, pH 7.5, 15 mM EDTA, 1 mM DTT, 1% NP-40, 1 mM DIFP, 40 μg/ml aprotinin, 2 μg/ml leupeptin, 60 mM β-glycerophosphate, 0.1 mM sodium vanadate, and 0.6 μM microcystin LR. Cell were disrupted on ice using a ground glass dounce homogenizer. For most experiments, extracts were cleared of cell debris by centrifugations at 16,000 ***g*** at 4°C for 5 minutes, the supernatant fraction was collected, the centrifugation was repeated. Immunoprecipitations were carried out at 4°C using standard procedures (Harlow and Lane, 1988). For each immunoprecipitation, 2.5 mg protein from cleared extracts was mixed with either 2 μg purified polyclonal anti-BIMA^{APC3} antibody (Mirabito and Morris, 1993), 5 μg BIME^{APC1} antiserum (see above), or 5 μg 12CA5 (BAbCo, Berkeley, CA). The immune complexes were adsorbed to PIB-equilibrated Protein A beads (Pierce, Rockford, IL). Protein A bound immune complexes were washed with 150 mM NaCl, 0.2% Tween-20. Increasing the salt concentration of the wash solution to 1 M KCl gave identical results.

SDS-PAGE and western blotting were carried out as described by Mirabito and Morris (1993) except that protein bands were visualized using chemiluminescence using procedures as recommended by the manufacturer (Pierce, Rockford, IL). Secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were HRP-labeled goat anti-mouse IgG used at 1:30,000 and HRP-labeled goat anti-rabbit used at 1:20,000.

For S value determination, extracts were fractionated on linear 15-40% sucrose gradients. Sucrose was dissolved in protein isolation buffer minus microcystin and aprotinin. Extracts (0.5 ml) were layered onto 12 ml gradients which were then centrifuged at 37,000 rpm for 14 hours at 4°C in a Beckman SW41 rotor. Samples (0.4 ml) were collected manually from the top of the gradient. Size standards were catalase (11S), apoferritin (17S), and purified 18S and 25S *A. nidulans* rRNAs. The results shown in Fig. 2 were obtained using extracts cleared with 16,000 ***g*** centrifugation for 5 minutes at 4°C. Similar results were obtained using extracts cleared by centrifugation at 109,000 ***g*** at 4°C for 1 hour (using a Beckman TLA-45 rotor in a Beckman TL-100 ultracentrifuge, k factor = 99). Under these conditions, the majority of the BIMA^{APC3} and BIME^{APC1} did not pellet.

RESULTS

BIMA^{APC3} and BIME^{APC1} are components of an APC/C-like complex

BIMA^{APC3} and BIME^{APC1} homologs are components of the APC/C in several organisms (King et al., 1996). If BIMA^{APC3} and BIME^{APC1} 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^{APC1} (Materials and Methods) to investigate possible BIMA^{APC3}/BIME^{APC1} interactions. The BIME^{APC1} antiserum detected a doublet at 225 kDa (Fig. 1, lanes 1 and 6), consistent with the size predicted for BIME^{APC1} (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^{APC1}. Under the culture conditions used to induce BIME^{APC1} overexpression, extracts from the control strain contained approximately normal levels of the anti-BIME^{APC1}-reactive bands (Fig. 1, lanes 2, 7). The strain engineered to overexpress full length BIME^{APC1} 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

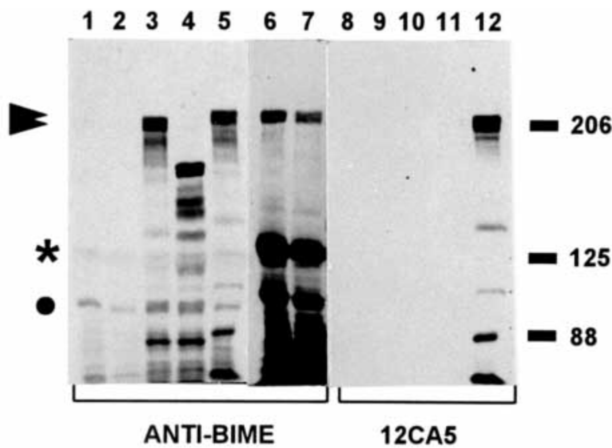


Fig. 1. Characterization of BIME^{APC1}. Western blots containing protein from cells grown in glucose medium (lanes 1 and 11) or ethanol medium (lanes 2-10 and 12). Blots were probed with BIME^{APC1} antiserum (lanes 1-5, 11 and 12) or with 12CA5 (lanes 6-10) to visualize 3HA-BIME^{APC1}. Lanes 1, 2, 6 and 9, wild type (R153); lanes 3 and 10, BIME^{APC1} overexpressor (SWJ060); lanes 4 and 11, truncated BIME^{APC1} overexpressor (SWJ063); lanes 5 and 12, 3HA-BIME^{APC1} 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 BIME^{APC1} antiserum (see lanes 6 and 7). The dot indicates a protein that reacts with the 2° Ab alone. The arrowheads indicate the BIME^{APC1} doublet.

expressing a truncated form of BIME^{APC1} (Fig. 1, lane 4). The high molecular mass doublet and the lower molecular mass proteins also accumulated in a strain expressing BIME^{APC1} fused to 3 copies of the hemagglutinin epitope (3HA-BIME^{APC1}). Most of these proteins were also recognized by the anti-HA mAB, 12CA5 (Fig. 1, lane 12). These results indicated that BIME^{APC1} is present in two approximately 225 kDa isoforms. The nature of the difference in these BIME^{APC1} isoforms has not been determined.

To determine if BIMA^{APC3} and BIME^{APC1} are physically associated, we isolated immune complexes using antisera specific to either BIMA^{APC3} or BIME^{APC1} and probed each complex for the presence of both proteins. BIMA^{APC3} antiserum immunoprecipitated BIMA^{APC3} and BIME^{APC1} (Fig. 2A, lanes 1-6) and BIME^{APC1} antiserum immunoprecipitated BIME^{APC1} and BIMA^{APC3} (Fig. 2A, lanes 7-12). The BIMA^{APC3} and BIME^{APC1} immune complexes were stable in solutions containing 1% NP-40 and 1 M KCl (data not shown). Given that BIMA^{APC3} antiserum did not cross react with BIME^{APC1} and that BIME^{APC1} antiserum did not cross react with BIMA^{APC3} (Fig. 2A), the co-immunoprecipitation of BIMA^{APC3} and BIME^{APC1} indicated that they are present in one or more complexes. To confirm the specificity of this result, we isolated immune complexes from 3HA-BIME^{APC1} containing extracts using 12CA5 or BIMA^{APC3} antiserum. BIMA^{APC3} and 3HA-BIME^{APC1} were present in both types of immune complexes (data not shown).

To determine the size of the BIMA^{APC3}/BIME^{APC1} complex, we fractionated extracts from wild-type strains on sucrose gradients and assayed gradient fractions for BIMA^{APC3} and BIME^{APC1} by western blot analysis. BIMA^{APC3} and BIME^{APC1} 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 BIMA^{APC3} and BIME^{APC1} at >25S with a consistent, minor peak at 15 S. BIMA^{APC3} and BIME^{APC1} coimmunoprecipitated from fractions containing the >25S complex, whereas we were unable to demonstrate coimmunoprecipitation of BIMA^{APC3} and BIME^{APC1} from the 15S complex (data not shown). Identical profiles of BIMA^{APC3} and BIME^{APC1} sedimentation were obtained using extracts that were first cleared by centrifugation at 109,000 ***g*** for 1 hour at 4°C (data not shown).

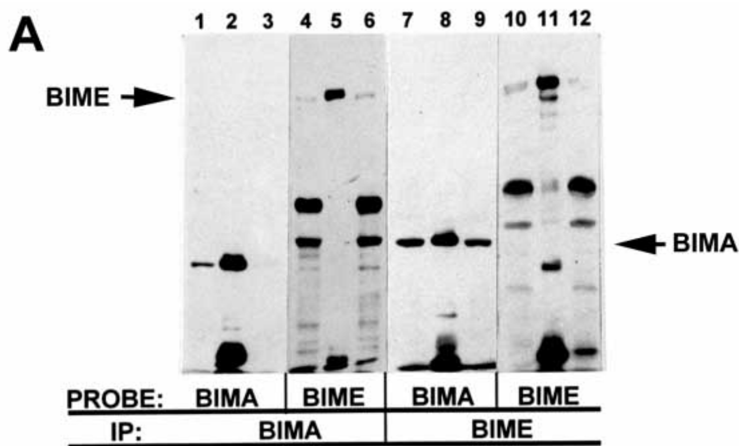
Mutations affecting APC/C function in *S. cerevisiae* have been shown to confer sensitivity to overexpression of B-type cyclins (Irniger et al., 1995). To determine if *bimA^{APC3}* and *bimE^{APC1}* mutants were sensitive to B-type cyclin overexpression, we constructed strains that were ethanol-inducible for overexpression of the major G_2 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 *bimA^{APC3}* or *bimE^{APC1}* were severely inhibited on ethanol medium.

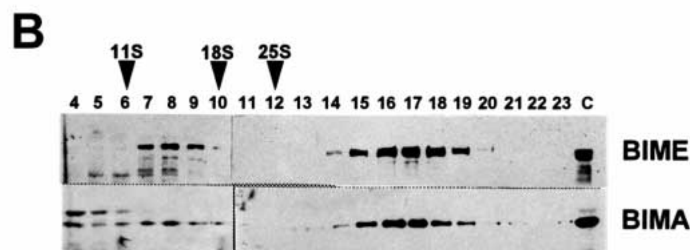
bimA^{APC3} mutations relieve the G_2 arrest of the *nimA5* mutation

The *bimE7^{APC1}* mutation causes defects in multiple cell cycle checkpoints, including the G_2 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 BIME^{APC1}, and therefore, for the *A. nidulans* APC/C, in a G_2 checkpoint activated by ts mutations in *nimA*. To determine if mutations in *bimA^{APC3}* also alter this checkpoint, we examined nuclear division in *nimA5* and *bimA1^{APC3}* single mutants, and *nimA5, bimA1^{APC3}* double mutants during germination of G_1 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 *bimA1^{APC3}* 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*, *bimA1^{APC3}* double mutant entered and arrested in mitosis with kinetics indistinguishable from the *bimA1^{APC3}* single mutant (Fig. 3). At 7 hours, the double mutant culture accumulated a higher percentage of mitotic cells than the single *bimA1^{APC3}* 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





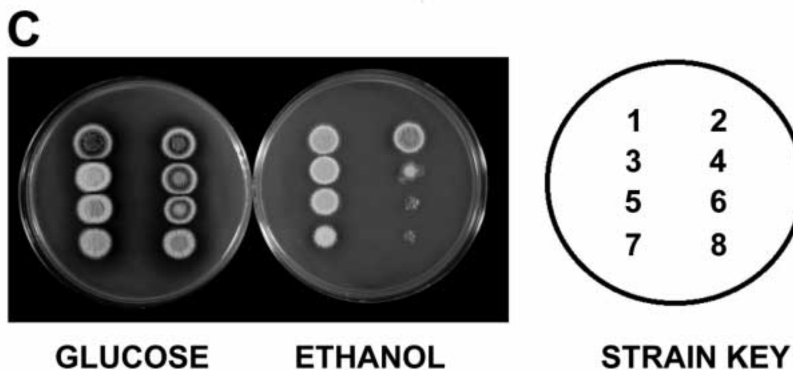


Fig. 2. Characterization of the *A. nidulans* APC/C. (A) Co-immunoprecipitation of BIMA^{APC3} and BIME^{APC1}. (A) Western blot of an immunoprecipitation of BIMA^{APC3} using BIMA^{APC3} antisera (lane 2) shows coprecipitation of BIME^{APC1} (lane 5). The reciprocal immunoprecipitation experiment, using BIME^{APC1} antiserum, shows coprecipitation of BIME^{APC1} (lane 11) and BIMA^{APC3} (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^{APC3} antiserum and lanes 4-6 and 9-12 were probed with BIME^{APC1} antiserum. (B) Characterization of the BIMA^{APC3}/BIME^{APC1} complex by sucrose gradient centrifugation. Western blot containing samples from a cell extract fractionated on a linear 15-40% sucrose gradient. Fraction numbers are indicated at the top. Arrowheads indicate the peak fraction of the sedimentation markers catalase (11s), 18S *A. nidulans* rRNA, and 25S *A. nidulans* rRNA. The top panels were probed with BIME antiserum and the bottom panels were probed with BIMA^{APC3} antiserum. (C) Elevated expression of NIME$^{Cyclin\ B}$ inhibits the growth of bimA^{APC3} and bimE^{APC1} mutants. 10^6 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*$^{Cyclin\ B}$); 3, PM156 (*bimA1*^{APC3}); 4, PMC892-3 (*bimA1*^{APC3},*alcA::nimE*$^{Cyclin\ B}$); 5, MLC1-19 (*bimA9*^{APC3}); 6, JCC51-6 (*bimA9*^{APC3}, *alcA::nimE*$^{Cyclin\ B}$); 7, MMB001 (*bimE7*^{APC1}); 8, SFC138-6 (*bimE7*^{APC1},*alcA::nimE*$^{Cyclin\ B}$).

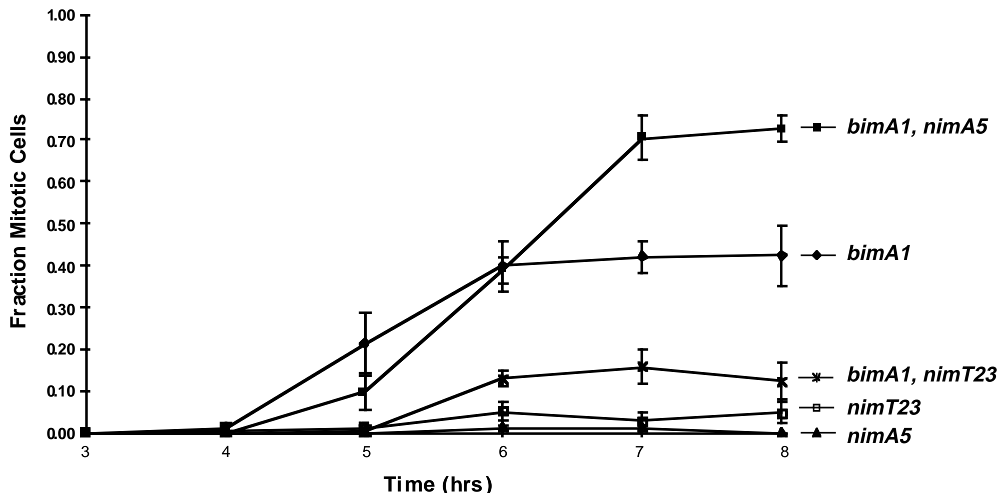


Fig. 3. Mitosis in cell cycle mutants during germination at restrictive temperature. Cultures were prepared and incubated at 43°C and the fraction mitotic cells were determined. Strains are: PM156 (*bimA1*^{APC3}), PM152 (*bimA1*^{APC3}, *nimA5*), SWJ216 (*nimT23*^{cdc25}), PM161 (*nimA5*), PM221 (*bimA1*^{APC3}, *nimT23*^{cdc25}). Each data point represents the average of 3 independent cell counts. Error bars represent 1 s.d.

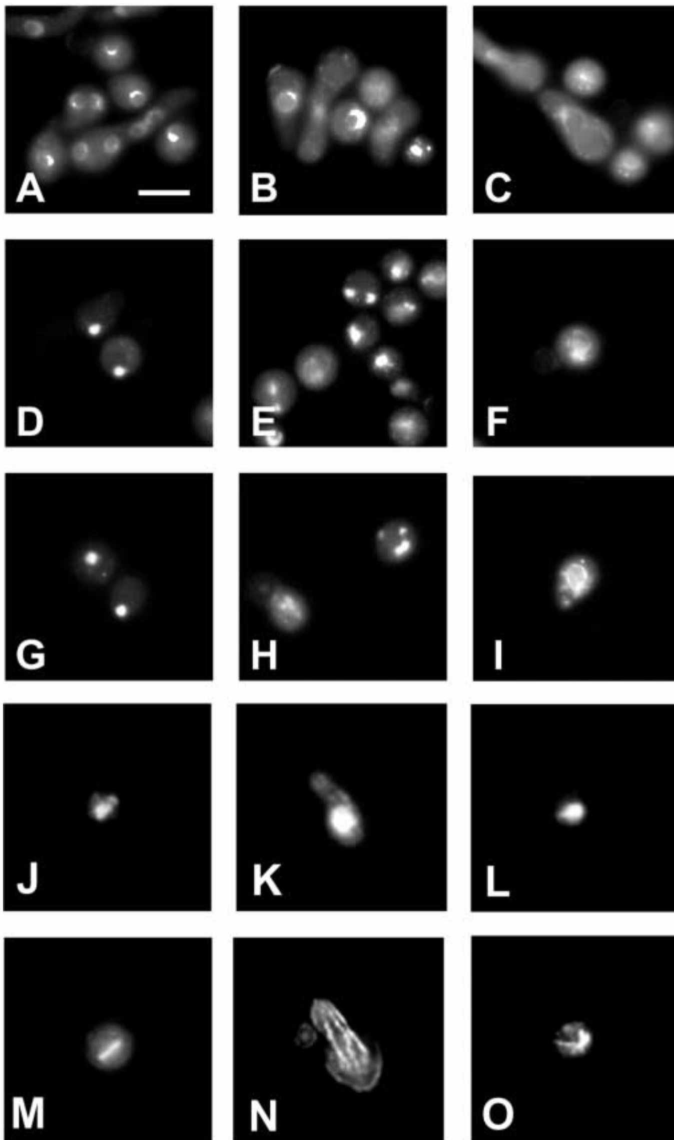


Fig. 4. Cytology of wild-type and cell cycle mutants germinated at restrictive temperature. (A-I) Examples of DAPI stained cells from the experiment described in Fig. 3, at 7 hours. (J-l) DAPI staining and (M-O) microtubule staining of cells germinated on coverslips for 7 hours at 43°C. (A) Wild type (R153); (B,C,K,N) *nimA5* (PM161); (D-F,J,M) *bimA1^{APC3}* (PM156); (G-I,L,O) *bimA1^{APC3}, nimA5* (PM152). Bar, 10 μm.

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

Table 3. Predominant nuclear phenotype at terminal arrest in cell cycle mutants

Condensed/mitotic chromatin	Decondensed/interphase chromatin
bimA1^{APC3}	*nimT23^{cdc25}*
bimA1^{APC3}, nimA5	*nimE6$^{Cyclin\ B}$*
bimA9^{APC3}, nimA5	*nimX3^{cdc2}*
bimA^{APC3}-alc	*nimA5*
bimA^{APC3}-alc, nimA5	*nimA-alc*
bimA1^{APC3}, nimA-alc	*bimA1^{APC3}, nimT23^{cdc25}*
benA33$^{\beta\text{-tubulin}}$	*bimA1^{APC3}, nimA5, nimT23^{cdc25}*
	bimA9^{APC3}
	bimA1^{APC3}, nimE6$^{Cyclin\ B}$
	bimA1^{APC3}, nimX3^{cdc2}
	benA33$^{\beta\text{-tubulin}}$, nimA5

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 *bimA^{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 G_2 checkpoint defect in *bimA^{APC3}* mutants is not allele specific

To determine if the interaction between *bimA^{APC3}* and *nimA* is specific to the *bimA1^{APC3}* allele, we examined nuclear division in two additional *nimA5*, *bimA^{APC3}* double mutants: nimA5, bimA9^{APC3}, and nimA5, bimA^{APC3}-alc. The ts *bimA9^{APC3}*

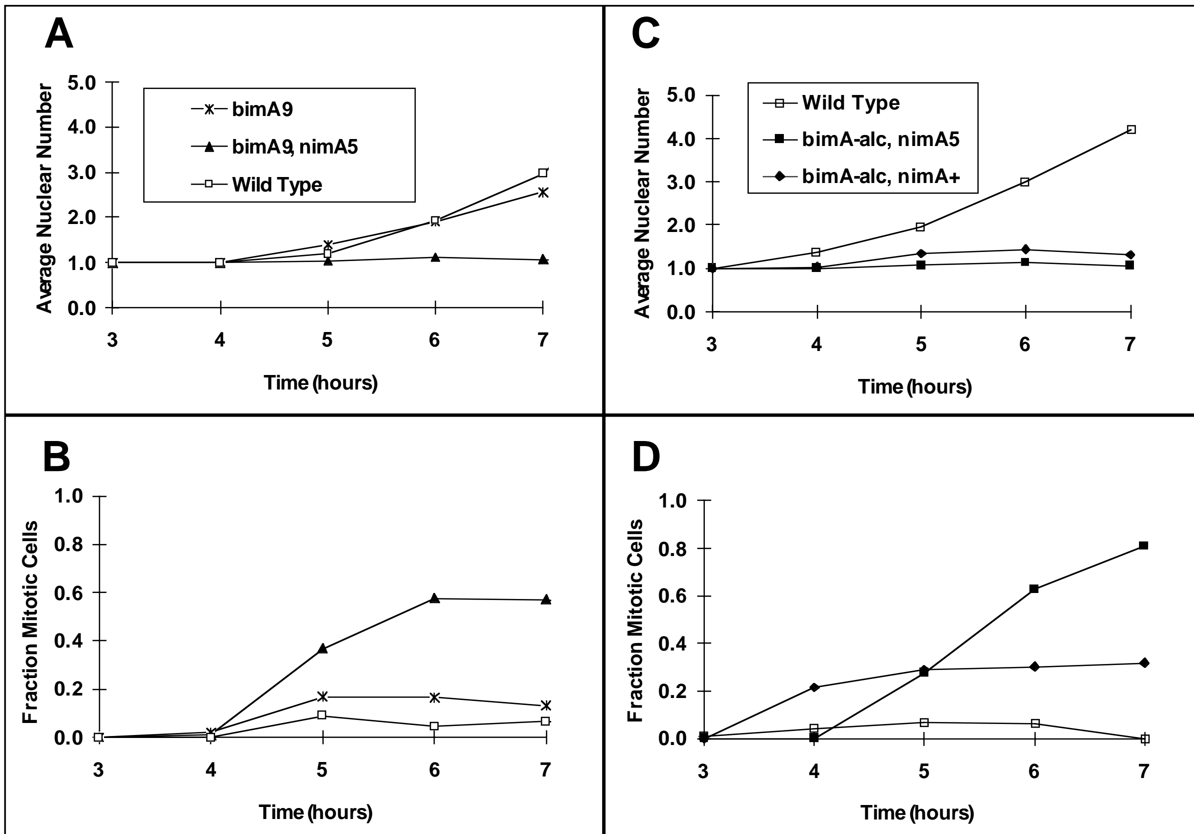


Fig. 5. Nuclear division and mitosis in cell cycle mutants during germination under restrictive culture conditions. Cultures were prepared and incubated at 43˚C and the average nuclear number and fraction of mitotic cells were determined. Each data point was determined by counting more than 300 cells. Strains were: (A and B) MLC1-19 (*bimA9*^{APC3}) and SFC11-7 (*bimA9*^{APC3}, *nimA5*). (C and D) TPM200 (*bimA-alc*^{APC3}) and PMC462-5 (*bimA-alc*^{APC3}, *nimA5*).

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 *bimA9*^{APC3} cells were mitotic at any time during the experiment. In contrast, very few of the *bimA9*^{APC3}, *nimA5* double mutant cells divided and 60% entered and arrested in mitosis with wild-type kinetics. Similar results were obtained when asynchronous cultures of *nimA5* and *bimA9*^{APC3} single and double mutants were shifted to restrictive temperature (data not shown).

In *bimA*^{APC3}*-alc* strains, the only functional *bimA*^{APC3} is under control of the *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 *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 *bimA*^{APC3}*-alc* cells. We were unable to detect BIMA^{APC3} on western blots of protein isolated from *bimA*^{APC3}*-alc* mutants germinated under these culture conditions (data not shown). By 7 hours, up to 80% of the *nimA5*, *bimA*^{APC3}*-alc* double mutant cells had entered and arrested in mitosis. These results indicated that the interaction between *nimA5* and *bimA*^{APC3} is not specific to *bimA1*^{APC3}. Furthermore, they demonstrated that the affect of *bimA9*^{APC3} and *bimA*^{APC3}*-alc* mutations on exit from mitosis are enhanced when combined with *nimA5*.

The interphase arrest in *nimA*$^{-}$ mutants is due to loss of NIMA function

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

In order to determine if the *alcA::nimA* gene was functional and if glucose medium could repress *alcA::nimA* function, we compared the growth of a *nimA5* mutant (SWJ071), a *nimA5* mutant containing an ectopic copy of *alcA::nimA* (TSF2-1), and a *nimA* deletion strain containing an ectopic copy of *alcA::nimA* (SFC466-201) on glucose and ethanol media at

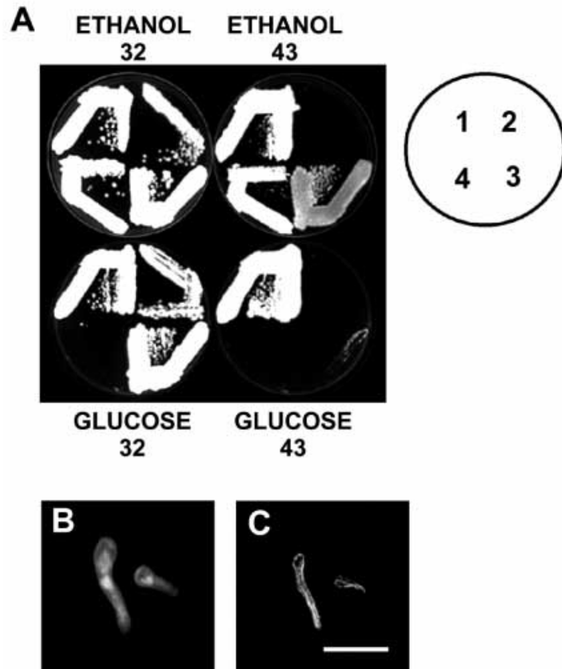


Fig. 6. Characterization of *nimA*-alc strains. (A) 10^6 spores of the strains indicated were streaked on glucose or ethanol containing medium and incubated at 32˚C or 43˚C for 3 days. Strain key: 1, R153 (wild type); 2, SWJ071 (*nimA5*); 3, TSF2-1 (*nimA5, alcA::nimA*); 4, SFC466-201 (*nimA-alc*). (B and C) SFC466-201 cells were grown in glucose medium and prepared for immunocytology as described in Materials and Methods. (B) DAPI staining, (C) microtubule staining. Bar, 10 µm.

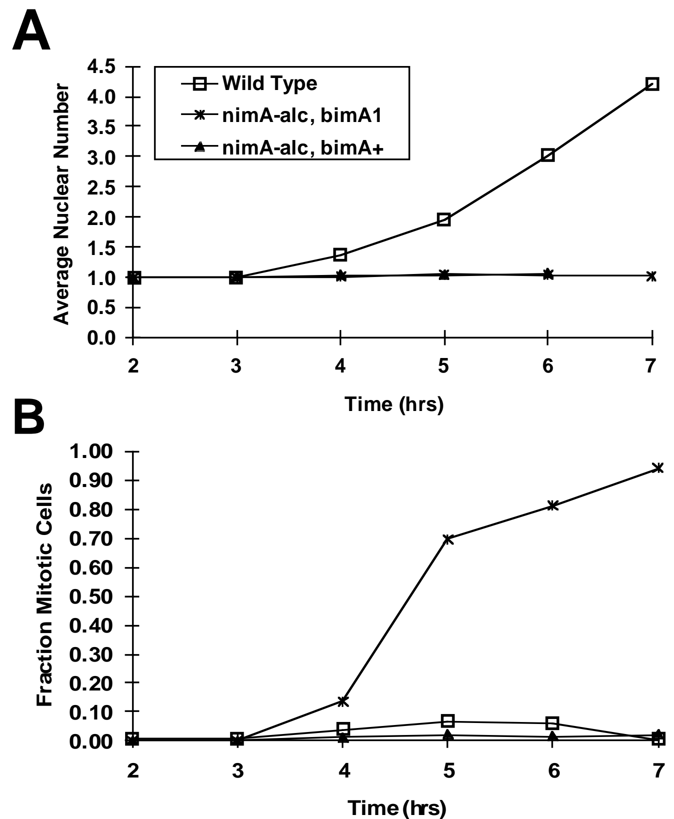


Fig. 7. BIMA^{APC3} is required for the G_2 checkpoint arrest of *nimA-alc* mutants even in the absence of NIMA expression. (A and B) Nuclear division and mitotic index in *nimA-alc* mutants and control strains. Cultures were prepared and incubated at 43˚C and the average nuclear number and fraction mitotic cells were determined as described in Materials and Methods. Each data point was determined by counting more than 300 cells. Strains were: R153 (wild type), SFC466-201 (*nimA-alc*), SFC466-48 (*bimA1, nimA-alc*).

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*, *bimA1^{APC3}* double mutant. In glucose medium, the *nimA-alc* single mutant arrested with a single interphase nucleus, whereas the *nimA-alc*, *bimA1^{APC3}* 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*, *bimA1^{APC3}* 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*, *bimA1^{APC3}* double mutant under conditions which repress *nimA-alc* expression and are restrictive for the *bimA1^{APC3}* mutation (see Materials and Methods). We compared these levels to NIMA kinase levels in asynchronous wild-type cells, *bimA1^{APC3}* 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*, *bimA1^{APC3}* 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*, *bimA1^{APC3}* 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*, *bimA1^{APC3}* mitotic culture was striking. Even cells from the asynchronous wild-type control culture accumulated

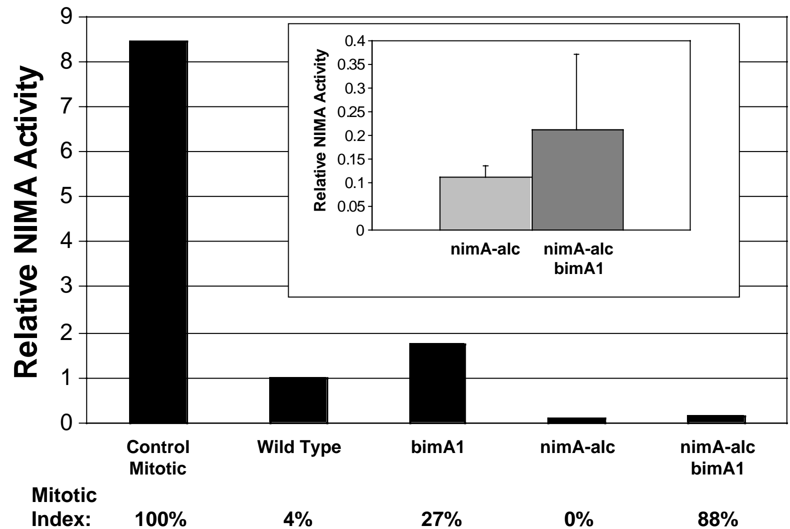


Fig. 8. *nimA-alc*, *bimA1*^{APC3} double mutants enter mitosis but do not accumulate NIMA kinase activity. Cultures were first shifted from glycerol to glucose medium to repress NIMA expression in *nimA-alc* strains and then shifted to 43°C for 3 hours to inactivate *bimA1*^{APC3} (see Materials and 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 (*bimA1*^{APC3}), SFC466-201 (*nimA-alc*), and SFC466-48 (*nimA-alc*, *bimA1*^{APC3}). The control mitotic culture was obtained by first arresting a *nimT23*^{CDC25} mutant (SFC4-21) in G_2 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*, *bimA1*^{APC3} double mutant (Fig. 8, inset). Thus, the mitotic arrest of the *nimA-alc*, *bimA1*^{APC3} double mutant is not explained by accumulation of significant NIMA kinase activity.

bimA^{APC3} mutations have little affect on steady state NIME$^{Cyclin\ B}$ levels

Given that BIMA^{APC3} appeared to be a component of the APC/C, we considered the possibility that *bimA*^{APC3} mutations may lead to abnormal accumulation of the B-type cyclin, NIME$^{Cyclin\ B}$, and that this may underlie the abnormal mitosis observed in *nimA*$^{-}$, *bimA*^{APC3} double mutants. To investigate this, we examined NIME$^{Cyclin\ B}$ levels in wild-type and mutant strains cultured at permissive or restrictive temperature. In order to specifically detect NIME$^{Cyclin\ B}$, we constructed strains which contained a HA-tagged *nimE*$^{Cyclin\ B}$ (*HA-nimE*$^{Cyclin\ B}$) in place of the endogenous *nimE*$^{Cyclin\ B}$ gene. HA-NIME$^{Cyclin\ B}$ reacts with polyclonal NIME$^{Cyclin\ B}$ antiserum and with the mAB, 12CA5 (Fig. 9A). None of the strains examined demonstrated a dramatic increase in the steady state levels of HA-NIME$^{Cyclin\ B}$ after shift to restrictive temperature (Fig. 9B), including the *bimA1*^{APC3}, *nimA5* double mutant. Thus, the phenotype of *bimA*^{APC3} mutants is not due to large increases in the steady state levels of NIME$^{Cyclin\ B}$.

DISCUSSION

bimE7^{APC1} 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 BIME^{APC1} 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, BIMA^{APC3}, in a G_2 checkpoint regulating entry into mitosis.

Identification of the *A. nidulans* APC/C

BIMA^{APC3} and BIME^{APC1} 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),

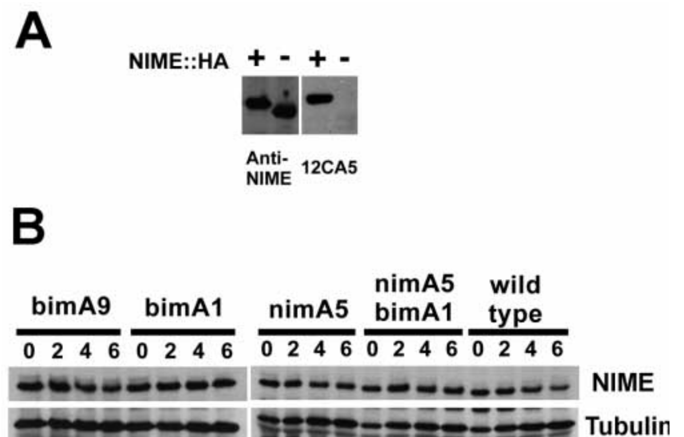


Fig. 9. Western blot analysis of NIME. Cultures were prepared as described in Materials and Methods. (A) Characterization of a strain expressing HA-NIME$^{Cyclin\ B}$. Western blots containing cell extracts isolated from a *HA-nimE*$^{cyclin\ B}$ strain (+) and a no HA control (–). The blots were probed with either NIME$^{Cyclin\ B}$ antiserum (left) or 12CA5 (right). The HA-tagged NIME$^{Cyclin\ B}$ migrates at a position slightly above wild-type NIME$^{Cyclin\ B}$. (B) Western blot analysis of HA-NIME$^{Cyclin\ B}$ 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-NIME$^{Cyclin\ B}$ (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; *bimA9*^{APC3}, SFC11-3; *bimA1*^{APC3}, PMC661-30; *nimA5*, PMC654-19; *nimA5*, *bimA1*^{APC3}, 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 G_2 arrest of *nimA* mutants is dependent on the APC/C

nimA ts mutants arrest very late in G_2, 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 G_2 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 G_2 to prevent inappropriate entry into mitosis in *nimA* mutants.

An APC/C-dependent G_2 checkpoint regulating entry into mitosis

In developing a model to explain the role of the APC/C in the G_2 arrest of *nimA* mutants, we have made two key assumptions. First, we assumed that the APC/C is not active in G_2 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 G_2 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 G_2. 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 G_2 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.

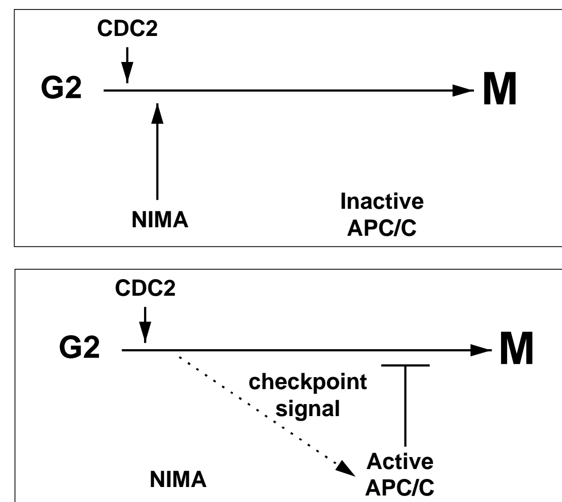


Fig. 10. A model for a late G_2 checkpoint involving the anaphase promoting complex/cyclosome. Top panel represents events in a wild-type cell and the bottom panel represents events in a *nimA* mutant. Arrows indicate positive regulation and T's indicate negative regulation. The dotted line in the bottom panel represents the checkpoint signalling pathway which senses loss of NIMA function and activates the APC/C.

Another novel prediction of this model is that the APC/C ubiquitinylates protein(s) involved in initiation of mitosis. Since inactivation of $NIMX^{CDC2}/NIME^{Cyclin\ B}$ prevents mitotic initiation in checkpoint defective strains, it is possible that the function of $NIMX^{CDC2}/NIME^{Cyclin\ B}$ is the ultimate target for this checkpoint. If so, then $NIMX^{CDC2}/NIME^{Cyclin\ B}$ function must be indirectly inhibited, since mitotic levels of active $NIMX^{CDC2}/NIME^{Cyclin\ B}$ accumulate in *nimA* mutants (Osmani et al., 1991a; Ye et al., 1995). One way in which the APC/C could indirectly inhibit $NIMX^{CDC2}/NIME^{Cyclin\ B}$ is by affecting its accumulation at important subcellular locations. For example, $BIMA^{APC3}$ and the $NIMX^{CDC2}/NIME^{Cyclin\ B}$ complex both localize to the SPB and the nucleus and $NIMX^{CDC2}/NIME^{Cyclin\ B}$ mislocalizes to the cytosol in $nimA^-$ mutants (Mirabito and Morris, 1993; L. Wu, S. Osmani, and P. Mirabito, unpublished). The APC/C G_2 checkpoint could target protein(s) required for proper $NIMX^{CDC2}/NIME^{Cyclin\ B}$ localization, thus preventing mitosis without resulting in dramatic decreases in bulk $NIMX^{CDC2}/NIME^{Cyclin\ B}$ 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, $bimE7^{APC1}$ mutants undergo a first cycle, metaphase arrest (Osmani et al., 1988) whereas $bimA9^{APC3}$ mutants never arrest in the first cycle and

only 20% of *bimA9*^{APC3} cells have a mitotic terminal arrest phenotype (Fig. 5). Thus, *bimE7*^{APC1} has a more severe impact than *bimA9*^{APC3} on exit from mitosis. Conversely, the *bimE7*^{APC1} mutation relieves the G_2 arrest in *nimA* mutants only after a considerable delay (Osmani et al., 1988; James et al., 1995), whereas the *bimA9*^{APC3} mutation relieves the G_2 arrest of *nimA* mutants without delay (Fig. 5). It is unlikely that quantitative differences in the effect of *bimE7*^{APC1} and *bimA9*^{APC3} 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 G_2 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 *bimA9*^{APC3}, *nimA5* double mutant not only enters mitosis but also arrests in mitosis, even though the *bimA9*^{APC3} single mutant divides at least once before terminal arrest (Fig. 5). The mitoses in *bimA9*^{APC3} 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 *bimA9*^{APC3} implies that NIMA and BIMA^{APC3} interact to regulate chromosome segregation and exit from mitosis.

In summary, the data presented here show that the G_2 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 G_2 checkpoint, and these studies should provide insight into the function of the APC/C in G_2. 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 G_2 checkpoint may be a conserved feature of mitotic regulation.

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