A Role for NIMA in the Nuclear Localization of Cyclin B in Aspergillus nidulans

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Abstract. NIMA promotes entry into mitosis in late G2 by some mechanism that is after activation of the Aspergillus nidulans G2 cyclin-dependent kinase, NIMX^{CDC2}/NIME^{Cyclin B}. Here we present two independent lines of evidence which indicate that this mechanism involves control of NIMX^{CDC2}/NIME^{Cyclin B} localization. First, we found that NIME^{Cyclin B} localized to the nucleus and the nucleus-associated organelle, the spindle pole body, in a NIMA-dependent manner. Analysis of cells from asynchronous cultures, synchronous cultures, and cultures arrested in S or G2 showed that NIME^{Cyclin B} was predominantly nuclear during interphase, with maximal nuclear accumulation in late G2. NIMX^{CDC2} colocalized with NIME^{Cyclin B} in G2 cells. Although inactivation of NIMA using either the nimA1 or nimA5 temperature-sensitive mutations blocked cells in G2, NIMX^{CDC2}/NIME^{Cyclin B} localization was predominantly cytoplasmic rather than nuclear. Second, we found that nimA interacts genetically with sonA, which is a homologue of the yeast nucleocytoplasmic transporter GLE2/RAE1. Mutations in sonA were identified as allele-specific suppressors of nimA1. The sonA suppressor alleviated the nuclear division and NIME^{Cyclin B} localization defects of nimA1 cells without markedly increasing NIMX^{CDC2} or NIMA kinase activity. These results indicate that NIMA promotes the nuclear localization of the NIMX^{CDC2}/NIME^{Cyclin B} complex, by a process involving SONA. This mechanism may be involved in coordinating the functions of NIMX^{CDC2} and NIMA in the regulation of mitosis.

Entry into mitosis in Aspergillus nidulans is regulated by the coordinate function of two serine/threonine protein kinases, NIMX^{CDC2} and NIMA. NIMX^{CDC2} is an essential histone H1 kinase that is structurally and functionally homologous to fission yeast p34^{cdcl2} (Osmani et al., 1994). NIMA is a β-casein kinase and is structurally distinct from p34^{cdcl2}, containing an amino-terminal catalytic domain and a carboxyl-terminal regulatory domain (Osmani et al., 1988b; Lu et al., 1993; Pu and Osmani, 1995; Pu et al., 1995). Failure to properly activate either of these kinases in G2 prevents the initiation of mitosis, and the combined action of both kinases is critical for coordinating changes in chromosome, microtubule, and nuclear membrane structure during mitosis. For example, mutations preventing the activation of NIMX^{CDC2} in G2 normally arrests cells in late G2 (Osmani et al., 1991a; 1994). Although overexpression of NIMA can overcome this interphase arrest, the ensuing mitosis is disorganized such that chromosome condensation occurs but normal spindle assembly does not (O’Connell et al., 1994; Pu and Osmani, 1995). Likewise, nimA mutations normally arrest cells in late G2 (Osmani et al., 1987; 1991a). Although a bimE^{APC1} checkpoint mutation can overcome this G2 arrest, the ensuing mitosis is disorganized and includes aberrant spindle, chromatin, and nuclear membrane structure (Osmani et al., 1988a; 1991b).

To ensure the coordinated function of NIMX^{CDC2} and NIMA, each kinase must somehow be sensitive to the function of the other. Phosphorylation of NIMA by NIMX^{CDC2} is likely to be involved in making NIMA function sensitive to NIMX^{CDC2} at the G2 to M transition (Ye et al., 1995). Before activation of NIMX^{CDC2} in late G2, NIMA is hypophosphorylated and active as a β-casein kinase. Upon activation of NIMX^{CDC2}, NIMA is converted to a hyperphosphorylated, slightly more active form that reacts well with the antiphosphoprotein antibody, MPM2. The finding that NIMX^{CDC2} is necessary for NIMA hyperphosphorylation and MPM2 reactivity in vivo, and is sufficient for NIMA hyperphosphorylation and MPM2 reactivity in vitro, is consistent with a direct role for NIMX^{CDC2} in NIMA hyperphosphorylation (Ye et al., 1995).
The role of NIMA hyperphosphorylation in NIMA’s function as a mitotic regulator remains to be determined. Hyperphosphorylated NIMA is detectable coincident with the initiation of mitosis in synchronous cultures, consistent with hyperphosphorylation playing a positive role in NIMA’s function as a mitotic inducer (Ye et al., 1995). This stimulatory effect could be at the level of NIMA activity, since hyperphosphorylation causes a twofold increase in NIMA’s β-casein kinase activity. Hyperphosphorylation could also regulate NIMA function at other levels, for example, by regulating NIMA localization or NIMA proteolysis. The identification of several consensus CDC2 phosphorylation sites in NIMA’s carboxyl terminus (Osmani et al., 1988b; Fry and Nigg, 1995) and the requirement of the carboxyl terminus for NIMA proteolysis (O’Connell et al., 1994; Pu and Osmani, 1995) is suggestive of hyperphosphorylation playing a role in regulating NIMA turnover.

If there is a mechanism making NIMXCDC2 function sensitive to NIMA activity, it does not involve regulation of NIMXCDC2 activation (Osmani et al., 1991a). Like most eukaryotic cells (Nurse, 1990), activation of CDC2 during G2 in Aspergillus is mediated by its binding to the Cyclin B homologue, NIME\textsubscript{Cyclin B}, which is the principle B-type cyclin associated with activated NIMXCDC2 during G2 (Bergen et al., 1984; Osmani et al., 1994; James et al., 1995). NIMXCDC2/NIME\textsubscript{Cyclin B} is activated by dephosphorylation of tyrosine residue 15 on NIMXCDC2 (O’Connell et al., 1992; Osmani et al., 1994). Tyrosine phosphorylation of NIMXCDC2 requires the function of the p105\textsuperscript{Wee1} homologue, ANKA\textsubscript{Wee1}, (Ye et al., 1996), and tyrosine dephosphorylation requires the function of the p80\textsuperscript{Ddc2} homologue, NIMT\textsuperscript{Ddc2} (O’Connell et al., 1992). nima mutations cause a specific cell cycle arrest in G2 very close to the nimT\textsuperscript{Ddc2} mutant arrest point, yet they do not prevent formation of a NIMXCDC2/NIME\textsubscript{Cyclin B} complex, dephosphorylation of NIMXCDC2 on tyrosine 15, or activation of the NIMXCDC2/NIME\textsubscript{Cyclin B} complex as a histone H1 kinase (Osmani et al., 1991a; Pu et al., 1995). Furthermore, the nima5 mutation prevents mitosis even in strains expressing a mutant form of NIMXCDC2 which cannot be phosphorylated on threonine 14 or tyrosine 15 (Ye et al., 1996). Thus, loss of NIMA function prevents mitosis by some mechanism other than regulation of the activity of NIMXCDC2/NIME\textsubscript{Cyclin B}.

One way in which NIMXCDC2 function could be affected by NIMA would be if NIMA function was required for proper localization of activated NIMXCDC2. It is known that CDC2/cyclin localization is regulated for certain cyclin-dependent kinase complexes (for example see Pines and Hunter, 1991; Gallant and Nigg, 1992; Ookata et al., 1992; Maridor et al., 1993; Ookata et al., 1995). Here we present evidence from two independent lines of investigation supporting a role for NIMA in the subcellular localization of NIMXCDC2/NIME\textsubscript{Cyclin B}. First, using indirect immunofluorescence analysis of fixed cells, we found that NIMXCDC2 and NIME\textsubscript{Cyclin B} localized to the nucleus and the nuclear-associated organelle, the spindle pole body (SPB)\textsuperscript{1}, in a NIMA-dependent manner. Second, using suppressor analysis, we found that mutations in a homologue of the nucleocytoplasmic transporter GLE2/RAE1 (Brown et al., 1995; Murphy et al., 1996) act as allele-specific suppressors of the nimA1 mutation. Together, these results suggest a role for NIMA in the nuclear localization of the NIMXCDC2/NIME\textsubscript{Cyclin B} and they provide evidence for a mechanism by which NIMXCDC2/NIME\textsubscript{Cyclin B} function is made sensitive to NIMA to coordinate the action of these two mitotic promoting kinases.

Materials and Methods

Strains, Microbiological Techniques, and Genetic Analyses

Aspergillus strains used in this study are listed in Table I. Standard conditions were used for Aspergillus propagation (Morrise, 1976; Kafir, 1977), genetics (Pontecorvo et al., 1953), and transformation (Osmani et al., 1987; Gems et al., 1991, 1994). The conditions and procedures used to grow A. nidulans cultures and isolate protein extracts were as described previously (Ye et al., 1995) except where noted in the text. For cytological analyses, A. nidulans cells were grown in liquid YG (Morrise, 1976) on coverslips as previously described (Mirabito and Morris, 1992).

Fluorescence Microscopy

Cells were fixed and stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei as previously described (Osmani et al., 1997). Cells were fixed and prepared for indirect immunofluorescence microscopy as previously described (Mirabito and Morris, 1993) with the following exceptions. Cell walls were removed using 40 mg/ml novozyme 234 (NOVO 234) (Interspex Products, San Mateo, CA), 80 mg/ml Driselase (Interspex Products, Foster City, CA), 1 mM diisopropyl fluorophosphate (DFP) (Sigma Chemical Co., St. Louis, MO), 2 μg/ml leupeptin, and 40 mg/ml Aprotinin (Sigma Chemical Co.). Lipids were extracted using 20°C methanol for 8 min followed by 20°C acetone for 30 s. Lipid extraction using room temperature methanol or 1% NP-40 yielded similar results. Coverslips were mounted on mounting medium (90% glycerol in TBS containing 1 mg/ml p-phenylenediamine).

Primary Abs used were 12CAS (Berkeley Antibody Co., Richmond, CA) at 10 mg/ml; rabbit anti-NIME\textsubscript{Cyclin B} serum E8 (Osmani et al., 1994) at 1:1,000; preimmune rabbit serum E8 at 1:1,000; rabbit anti-NIMXCDC2 serum E77 (Osmani et al., 1994) at 1:1,000; preimmune rabbit serum E77 at 1:1,000; MPM2 (gift of J. Kuang, M.D. Anderson Cancer Center, Houston, TX) at 1:1,000; affinity-purified rabbit anti-γ-tubulin (gift of B. Oakland, Ohio State University, Columbus, OH) at 1:100; anti-histone H1 mouse mAb (gift of A. Epstein, University of Southern California, School of Medicine, Los Angeles, CA) at 1:1,000; and DMI1A (Sigma Chemical Co.) at 1:100. Secondary Abs (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were CY3-labeled, goat anti-mouse IgG at 1:500, dihydroorotiazinylaminofluorescein (DTAF)-labeled, goat anti-mouse IgG at 1:250, CY3-labeled, goat anti-rabbit IgG at 1:500, and DTAF-labeled, goat anti-rabbit IgG at 1:250. Photomicrographs of cells stained with 12CAS within each figure were produced using identical conditions of fixation, staining, exposure, and enlargement.

For colocalization of NIME\textsubscript{Cyclin B} and SPB or nuclear antigens, some experiments involved double staining using two mouse mAbs (12CAS and anti-histone H1 or 12CAS and MPM2). For these experiments, fixed cells were incubated first in 12CAS and then in a conjugated, anti-mouse secondary antibody, and then in 20 mg/ml of unconjugated, anti-mouse Fab fragments (Jackson ImmunoResearch Laboratories, Inc.) before incubation in the second mouse mAb. This treatment effectively blocked all the anti-mouse IgG sites on the 12CAS mAb; no further binding to 12CAS was detectable in control experiments. Colocalization of NIME\textsubscript{Cyclin B} and SPBs was confirmed using 12CAS and the affinity-purified, rabbit anti-γ-tubulin Ab.

Photomicrographs were captured using either a Photometrics charge-coupled device (CCD) camera (model Sensys; Tucson, AZ) and manipulated using Phase 3 Imaging Systems software (Sterling Heights, MI) (see Fig. 8) or a Diagnostics Instruments Inc. (Media Cybernetics, Silver Spring, MD) CCD camera and then manipulated using Adobe Photoshop 4.0 (Adobe Systems, Inc., San Jose, CA).

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\textsuperscript{1} Abbreviations used in this paper: CCD, charge-coupled device; cs, cold sensitive; DAPI, 4',6-diamidino-2-phenylindole; HA, hemagglutinin; ORF, open reading frame; SPB, spindle pole body; ts, temperature sensitive.
Culture Conditions for NIME Localization Studies

For analysis of NIME\textsuperscript{Cyclin B} and NIME\textsuperscript{DC2} localization in exponentially growing asynchronous cultures, cells were incubated in YG on coverslips at 32°C for 8–12 h. For analysis of NIME localization in cells arrested in S phase, cells were incubated in YG at 32°C for 6 h and then hydroxyurea was added at 50 mM and incubated was continued for an additional 3 h at 32°C. For analysis of NIME\textsuperscript{Cyclin B} and NIME\textsuperscript{DC2} localization in cells shifted to restrictive temperature, cells were incubated in YG liquid at permissive temperature until they contained an average of 2–4 nuclei, and then the cultures were placed in a 44°C incubator for up to 6 h. To generate synchronous cultures, nim\textsuperscript{T672} or nim\textsuperscript{A} mutants were germinated for 2 h at 32°C, shifted to 44°C for 5 h to arrest cells in Go of their first cell cycle. The cells were shifted back to either 32°C or room temperature by replacing the medium. Samples were taken before the shift and at the times after the shift as indicated in the legends to Figs. 3 and 5.

Isolation and Characterization of Strains Carrying the sonA1 Suppressor Mutation

Spores of the temperature-sensitive (ts) nimA strain, LPW2, were mutagenized with 4-nitroquinoline-1-oxide as described (Harris et al., 1994) to achieve a kill rate of 80–95%. The survivors were plated on MAG medium (2% malt extract, 0.2% peptone, 1% dextrose, 2% agar) and incubated at 42°C for 3 d. revertants were isolated at a frequency of 1,487 revertants out of 4 × 10\textsuperscript{6} survivors plated. revertants were patched to MAG plates and incubated at 20°C for 8 d to screen for cold sensitivity. Out of 1,487 revertants, 35 were cold sensitive. To screen for extragenic suppressors, each cold-sensitive (cs) revertant was crossed to A612 and random meiotic progeny were selected and scored for the ts and cs phenotypes. Three out of the 35 cs revertants yielded both ts and cs meiotic progeny and demonstrated linkage of the cs and nimA suppression phenotypes. This indicated that these revertants contained the nimA mutation and a cs, extragenic suppressor of nimA1. Complementation analysis in diploids and genetic crosses indicated that three alleles of a single suppressor gene had been isolated. We named this gene sonA as suppressor of nimA1. Dominance/recessiveness was determined by generating diploids between each revertant and strain AI54. The suppressor mutation was assigned to chromosome VIII using standard parasexual analysis (Pontercorvo et al., 1953).

Cloning and Characterization of sonA

Standard procedures were used for DNA preparation and manipulation (Sambrook et al., 1989). Wild-type sonA was cloned by complementation of the cs phenotype of LPW16 using a chromosome VIII–specific cosmid library (Brody et al., 1991) and the autonomously replicating plasmid pAR1-library (Brody et al., 1991) and the autonomously replicating plasmid of the cs phenotype of LPW16 using a chromosome VIII–specific cosmid (Verdoes et al., 1994) using standard procedures (Gems et al., 1991, 1994). Individual cosmids capable of complementing sonA1 were identified by sib selection. A common 2.4-kb HindIII fragment was the smallest restriction fragment from these cosmids capable of complementing sonA1 in trans. This fragment was sequenced using an Applied Biosystems DNA sequencer (model 373A; Foster City, CA) using procedures recommended by the manufacturer. Open reading frame (ORF) identification and sequence alignments were preformed using the Applied Biosystems Sequence Analysis Program. Database searches were preformed using BLAST (Altschul et al., 1990).

The sonA cDNA was isolated by PCR of A. nidulans total cDNA using Vent polymerase (New England BioLabs, Inc., Beverly, MA). The isolated cDNA covers the entire ORF of SONA, starting at 90 bp upstream from the start codon and ending 32 bp downstream from the stop codon. The sequence for the forward primer was 5'-GCTCTTGATAC-GGGGCTATCCCTTAGCTGCGCCTGAGGAGGAG-3' and for the reverse primer was 5'-CGAATGTAGATCTTAGCCTGGAAG-3'.

To determine whether the cloned gene was sonA or a suppressor of sonA1, we integrated the cloned gene at its homologous locus in the sonA1 strain, LPW16, using a two-step gene replacement procedure (O’Connell et al., 1992). In the first step, plasmid pRSW2, containing the sonA1 complementing gene and the A. nidulans pyrG gene, was used to transform LPW16, converting LPW16 from cs to wild-type growth at 20°C. Plasmid pRSW2 was constructed by inserting an EcoRI/BamHI genomic fragment (containing the wild-type sonA gene from 284 bp upstream of the ATG to 140 bp downstream from the stop codon) into pRG3 (Waring et al., 1989). In the second step, a transformant was plated on medium containing uracil and 5-fluoroorotic acid to select for plasmid loss. Both cs and wild-type isolates were obtained as 5-fluoroorotic acid-resistant sectors and plasmid loss was confirmed by Southern blot analysis. Wild-type isolates were confirmed to have lost the sonA1 mutation by standard genetic analysis.

A strain expressing endogenous SONA1 and a recombinant SONA fused to two tandem copies of the hemagglutinin (HA) epitope at its carboxyl terminus (SONA-HA) was created by transforming pMTW2 into LPW16. Plasmid pMTW2, containing sonA fused to two tandem HA epitope, was constructed as follows: a sonA PCR fragment containing 284 bps upstream from the start codon and 140 bps downstream from the stop codon was inserted into pRG3 as a BamHI/EcoRI fragment. A sequence encoding two tandem HA epitopes (5'-TACCATCATTGAGCGCATGATCCACACTGAGGACGACTGCGACGGGAA-3') was inserted into the sonA gene immediately before the stop codon by site-directed mutagenesis using the Quick Change\textsuperscript{TM} mutagenesis kit (Stratagene, La Jolla, CA). Expression of SONA-HA complemented the cs phenotype of sonA1 and was verified by Western blot analysis using 12CA5.

Analysis of Protein Kinase Levels

To prepare cultures for protein kinase studies, 10\textsuperscript{6} spores/ml were inoculated into flasks containing YG liquid medium and then were shaken at 250 rpm at 32°C or 10 h. For analysis of asynchronous cultures, samples were collected and processed after 10 h at 32°C. For analysis of cultures shifted to restrictive temperature, cultures were brought to 42°C and sampled at the times indicated in Fig. 10. For analysis of cultures shifted back to permissive temperature, 3h, 42°C cultures were brought back to 32°C and sampled at the times indicated in Fig. 10. Procedures for sampling, protein isolation, and protein kinase assays were as previously described (Osmani et al., 1991a; Ye et al., 1995).

Results

NIME\textsuperscript{Cyclin B} Localized Predominantly to the Nucleus

To determine the subcellular localization of NIME\textsuperscript{Cyclin B}, we have created a number of A. nidulans strains (Table I) in which the wild-type NIME\textsuperscript{Cyclin B} gene (nimE\textsuperscript{Cyclin B}) has been replaced with an HA-tagged version of NIME\textsuperscript{Cyclin B} (HA-nimE\textsuperscript{Cyclin B}) (Osmani et al., 1994). These strains are phenotypically identical to coisogenic strains containing the untagged version (data not shown), and we can detect HA-NIME\textsuperscript{Cyclin B} using the mAb, 12CA5. HA-NIME\textsuperscript{Cyclin B} localized predominantly to the nucleus in rapidly growing wild-type cells (Fig. 1, top row). Approximately half of the cells in exponentially growing asynchronous cultures showed detectable nuclear HA-NIME\textsuperscript{Cyclin B} staining, whereas essentially every cell showed nuclear staining with anti-histone H1 mAb. The percentage of cells in asynchronous cultures which exhibited nuclear HA-NIME\textsuperscript{Cyclin B} staining was consistent in multiple experiments. The nuclear NIME\textsuperscript{Cyclin B} staining was punctate and included chromatin, the nucleolus, and the SPB, as judged by staining with 12CA5, DAPI, and either anti-γ-tubulin Ab or MPM2 (Figs. 1 and 2; data not shown). Faint staining of the cytoplasm in cells with bright nuclear staining was also detected. Double staining with 12CA5 and the anti-tubulin Ab, DM1A, showed that all of the cells with nuclear HA-NIME\textsuperscript{Cyclin B} contained cytoplasmic microtubules and that cells with mitotic spindles lacked nuclear-specific HA-NIME\textsuperscript{Cyclin B} staining (Fig. 1, bottom two rows).

The lack of nuclear NIME\textsuperscript{Cyclin B} staining in all cells of an asynchronous culture suggested that nuclear NIME\textsuperscript{Cyclin B} localization might be cell cycle stage-specific. To preliminarily investigate this possibility, we localized HA-NIME\textsuperscript{Cyclin B} in cells blocked in S phase with hydroxyurea or in late G2
with a ts mutation in nimTcdc25. Both treatments gave essentially the same result: the frequency of nuclear HA-NIME Cyclin B staining increased to essentially 100% when cells were blocked in S or G2. Fig. 2 shows examples of the results for cells blocked in G2 by the nimTcdc25 mutation. As cells accumulated at the nimTcdc25 G2 arrest point, nuclear staining became increasingly brighter. The 12CA5 staining result was confirmed by double staining with 12CA5 and polyclonal rabbit anti-NIME Cyclin B serum (Osmani et al., 1994). Both Abs stained nuclei, including chromatin, the nucleolus, and SPB-like dots (Fig. 2, top row). To determine if NIMX CDC2 colocalized with NIME Cyclin B, we performed double labeling experiments using 12CA5 and polyclonal rabbit anti-NIMX CDC2 specific antisera (Osmani et al., 1994). NIME Cyclin B and NIMX CDC2 colocalized on nuclei (Fig. 2, middle row). Preimmune serum controls for the anti-NIME and anti-NIMX antisera showed no nuclear specific staining (data not shown). Nuclear staining was also obtained using rabbit anti-NIME Cyclin B and anti-NIMX CDC2 sera on a nimTcdc25 mutant strain which expressed only untagged NIME Cyclin B (data not shown).

To determine if the nuclear-associated dots were indeed SPBs, we performed double labeling experiments using polyclonal rabbit anti-NIME Cyclin B serum and the mouse mAb, MPM2, which stains the SPB of G2 cells (Martin et al., 1997). Anti-NIME Cyclin B and MPM2 staining colocalized on SPBs and the nucleolus in nimTcdc25-arrested cells (Fig. 2, bottom row). Double labeling with 12CA5 and anti-γ-tubulin, or with anti-NIMX CDC2 sera and MPM2 gave similar results (data not shown).

To further characterize the cell cycle dependency of NIME Cyclin B localization, we determined the localization of HA-NIME Cyclin B in cycling cells from synchronous cultures. We generated synchronous cultures by blocking cells in late G2 using a nimTcdc25 mutation and then releasing the cell cycle arrest. Immediately before release from the G2 arrest, 100% of the cells showed nuclear HA-NIME Cyclin B staining (Fig. 3). Within 10 min after release from the G2 arrest, 90% of the cells entered mitosis and showed no nuclear HA-NIME Cyclin B staining, whereas the cells in the same sample that had not yet entered mitosis continued to show nuclear HA-NIME Cyclin B staining (Fig. 3 B). By 20 min after release, all the cells had finished mitosis (Fig. 3 A) and no cells (out of more than 300 examined) exhibited nuclear HA-NIME Cyclin B staining. Cells with nuclear HA-NIME Cyclin B began to accumulate 80 min after the release and almost all of the cells exhibited nuclear HA-NIME Cyclin B staining (Fig. 3, A and B) just before initiation of the second mitosis. As with the first mitosis, nuclear HA-NIME Cyclin B staining was lost coincident with the initiation of mitosis. Out of more than a thousand mitotic cells examined, only two showed very faint nuclear NIME HA staining. Essentially all cells showed SPB staining at the initial G2 arrest point and in G2 just before the second division (data not shown). SPB staining was not distinguishable from chromatin or nucleolar staining in cells at other time points.

**NIME Cyclin B Localizes Predominantly to the Cytoplasm in nimA Mutants**

To investigate the possibility that NIMA is involved in NIMX CDC2/NIME Cyclin B localization, we constructed strains expressing HA-NIME Cyclin B and carrying the ts nimA5 or nimA1 mutations. We determined the localization of HA-NIME Cyclin B in these strains at restrictive and permissive temperature. At permissive temperature,
about half the cells from asynchronous cultures of nimA5 or nimA1 mutants showed nuclear HA-NIMECyclin B localization (data not shown). In contrast, after the shift to restrictive temperature, the nimA5 and nimA1 cells showed little nuclear-specific HA-NIMECyclin B staining (Fig. 4). Instead of being concentrated at nuclei, HA-NIMECyclin B staining was diffuse throughout the cell. Although nimA and nimTcdc25 mutants arrest at essentially the same point in late G2, their NIMECyclin B staining patterns are dramatically different (Fig. 4, compare top row with second and third rows). Antibody access to the nuclei of these cells was confirmed by staining with MPM2 (Fig. 4, middle column) and anti-histone H1 antibodies (data not shown).

It was possible that the above results were caused by masking of the single HA epitope of HA-NIMECyclin B in nimA mutants. To address this possibility, we stained nimA5 cells cultured at restrictive temperature with polyclonal rabbit anti-NIME Cyclin B and anti-NIMX CDC2 sera. As with 12CA5 staining, anti-NIME Cyclin B staining was diffuse throughout the cells of nimA mutants (Fig. 4, second row). Similar results were obtained with anti-NIMX CDC2 serum (Fig. 4, bottom row).

Like the nimTcdc25 mutation, the nimA5 mutation is readily reversible. A few minutes after return to permissive temperature, nimA5 mutants leave G2 and enter mitosis synchronously (Oakley and Morris, 1983; Osmani et al., 1991a). If nuclear NIME Cyclin B localization is important for mitotic initiation, then one would predict that NIME Cyclin B would reaccumulate on nuclei of nimA mutants as cells approach mitosis after return to permissive temperature. To test this, we compared the nuclear HA-NIME Cyclin B staining profiles of a nimA5 and a nimTcdc25 mutant at their arrest point in G2 and after release from the G2 arrest into room temperature medium. Release from the G2 arrest under these conditions is relatively slow, allowing for ex-
NIME Cyclin B Localization Defects of nimA1

As part of a search for nimA-interacting genes, we have attempted to identify extragenic suppressors of nimA mutations by isolating induced revertants of the nimA1 heat-sensitive (ts) mutation (refer to Materials and Methods). nimA1 is a tight ts mutation, with colony formation severely inhibited at 42°C, a temperature at which wild-type strains grow well (Fig. 6). We isolated a number of revertants which were simultaneously converted from heat sensitive (ts) to cs, being able to form colonies normally at 42° but not at 20°C (see LPW29 in Fig. 6). For three of these revertants, suppression of nimA1 was unlinked to nimA and tightly linked to the cs phenotype, demonstrating that the original revertants contained a cs, extragenic suppressor of nimA1 (data not shown). These suppressor mutations also conferred a cs phenotype in a nimA1 background (see LPW16 in Fig. 6). All three cs mutations were recessive and in the same complementation group (data not shown), which we designated sonA for suppressor of nimA1. One of these mutations, sonA1, was chosen for further study.

We cloned the wild-type sonA gene by complementation of the cs defect of a sonA1 mutant (refer to Materials and Methods). A cosmId clone capable of complementing sonA1 was recovered and the complementing sequence was localized to a 2.6-kb genomic fragment. A cDNA clone, generated by PCR, was also sufficient for complementation of sonA1. Sequence of the genomic and cDNA clones indicated that the complementing fragment encoded a 1.2-kb ORF interrupted by a single, 65-bp intron (Fig. 7 B). The cloned gene was sonA and not a suppressor of sonA1 because it was tightly linked to sonA (refer to Materials and Methods) and because the sequence of this gene in the sonA1 strain contained a single C to G mutation resulting in a P to R change at amino acid residue 205 in the ORF (Fig. 7 A).

Conceptual translation of the sonA ORF predicts that the sonA polypeptide (SONA) contains 362 amino acid residues corresponding to a mol wt of ~39.5 kD. Sequence database searches revealed that SONA is highly similar to Schizosaccharomyces pombe RAE1 (Brown et al., 1995) and Saccharomyces cerevisiae GLE2 (Murphy et al., 1996). SONA is 84.3% similar (58.8% identical) to RAE1 and is 84.3% similar (48.9% identical) to GLE2. Although the four putative β-transducin/WD repeats in RAE1 and GLE2 are well conserved in SONA, the similarity between SONA and RAE1 and GLE2 extends well outside of these repeats along the entire peptide sequences (Fig. 7 B).

To determine the localization of SONA, we constructed strains expressing an HA epitope-tagged version of SONA (SONA-HA). SONA-HA was functional as its expression complemented the cs phenotype of sonA1 (data not shown). Exponentially growing cells expressing SONA-HA showed punctate staining appearing as a ring at the periphery of each nucleus (Fig. 8). A no-HA control strain showed no nuclear ring staining (data not shown; also refer to Fig. 1). SONA-HA staining was not completely coincident with DAPI staining in that it was limited to the nuclear periphery and was evident at the nuclear periphery in regions next to nucleoli, where DAPI staining was excluded. The SONA-HA staining pattern was strikingly similar to the nuclear pore-like staining seen for GLE2 (Murphy et al., 1996).

sonA1 Suppresses the Nuclear Division and NIME Cyclin B Localization Defects of nimA1 without Markedly Increasing NIMA or NIMX Kinase Activity

Colonies of sonA1, nimA1 double mutants exhibited...
slower growth at 42°C than either wild-type or sonAI single mutant strains (refer to Fig. 6). To examine the suppression of nimA1 by sonAI in more detail, we followed nuclear division and nuclear morphology during spore germination in strains that were either wild-type, nimA1, sonA1, or nimA1 plus sonA1. Fig. 9 shows examples of cells stained with DAPI to visualize nuclei. At 32°C (permissive growth temperature for nimA1 and sonA1), essentially all of the spores from all the strains examined germinated and underwent nuclear division at approximately wild-type rates. At 42°C, the majority of nimA1 mutant spores failed to undergo a single nuclear division even after 10 h, whereas the majority of spores from wild type had undergone 2–4 divisions. Essentially all the spores of the nimA1, sonA1 double mutant germinated and underwent apparently normal nuclear divisions at 42°C. The rate of nuclear division in this strain was somewhat slower than that in wild type (data not shown). This is consistent with the reduced colony growth rate of sonA1, nimA1 strains, indicating that sonA1 does not suppress nimA1 function to wild-type, nimA1 levels.

It was previously shown that nimA1 mutants arrest in G2 even though they accumulate partially active NIMA and fully activated NIMX CDC2 (Pu et al., 1995). It was possible that sonA1 suppresses nimA1 by somehow increasing the level of NIMA or NIMX CDC2 activity. To investigate these possibilities, we measured the levels of NIMA and NIMX CDC2 kinase activities in a wild-type strain, a nimA1

Figure 4. Nuclear-specific NIME Cyclin B and NIMX CDC2 localization was prevented by nimA mutations. Cells of the nimT cdc25 mutant, SFC4-21, the nimA5 mutant, PMC654-19, and the nimA1 mutant, SFC403-19, were cultured, fixed, and then prepared for immunocytology as described in Materials and Methods. Left, strain identity. The images corresponding to Ab or DAPI staining are labeled at the top of the panels. Arrows, position of nuclei in the images showing NIME Cyclin B and NIMX CDC2 localization. Bar, 10 μm.
A mutant, a sonA1 mutant, and a nimA1, sonA1 double mutant. Samples were analyzed from asynchronous cultures, from cultures shifted to restrictive temperature, and from cultures shifted to restrictive temperature and then returned to permissive temperature. Fig. 10 shows that 3 h after shift to restrictive temperature (42°C), the nimA1 mutant, LPW2, accumulated NIMA and NIMX CDC2 kinase to levels above that in asynchronous cultures. Return to permissive temperature induced these cells to synchronously enter mitosis, and resulted in a severalfold increase in NIMA activity but no significant increase in NIMX CDC2 activity (Fig. 10 C). These results are in agreement with Pu et al. (1995), and they demonstrate that nimA1 blocks progression into mitosis but does not prevent full activation of NIMX CDC2. The nimA1, sonA1 double mutant, LPW29, did not accumulate NIMA or NIMX CDC2 activity above that of LPW2 either before or after a shift to 42°C (Fig. 10, A and B, compare first and second panels). Based on the peak of mitotic cells after the return to permissive temperature, ~25% of the LPW29 cells had accumulated in late G2 during incubation at 42°C, consistent with previous data showing that suppression of nimA1 by sonA1 is not complete. The wild-type (GR5) and sonA1 single mutant (LPW16) were essentially identical under these conditions (Fig. 10, A and B, third and fourth panels).

Given that sonA1 did not cause an increase in the levels of NIMA or NIMX CDC2 activity, and that SONA is related to the GLE2/RAE1 nucleocytoplasmic transporter, we considered the possibility that sonA1 may suppress the NIME Cyclin B localization defect of nimA1 mutants. We constructed nimA1, sonA1 double mutants and nimA5, sonA1 double mutants which expressed HA-NIME Cyclin B as their only NIME Cyclin B. Where nimA1 cells cultured at restrictive temperature for nimA1 arrested in G2 with HA-NIME Cyclin B staining throughout the cell, nimA1, sonA1 double mutants continued to divide and showed nuclear-specific HA-NIME Cyclin B staining (Fig. 11). The percentage of nimA1, sonA1 double mutants showing nuclear HA-NIME Cyclin B staining was 33% (n = 100) compared with 51% (n = 102) for wild-type cells. These results are consistent with the fact that sonA1 only partially suppresses the growth and nuclear division phenotype of nimA1 mutants (see Figs. 6 and 9). The nimA5, sonA1 double mutant incubated at restrictive temperature arrested in G2 and did not accumulate nuclear-specific HA-NIME Cyclin B (data not shown), demonstrating that the ability of sonA1 to suppress the NIME Cyclin B localization defect of nimA1 mutants was allele specific.

Discussion

Inactivation of the NIMA kinase causes a specific cell cycle arrest in G2 without preventing activation of the H1 kinase activity of NIMX CDC2/NIME Cyclin B. NIMA is, there-
fore, required for mitotic initiation by a mechanism other than activation of NIMX CDC2 as an H1 kinase. Although NIMA itself is probably required for normal mitosis independently of NIMX CDC2 (O’Connell et al., 1994; Lu and Hunter, 1995; Pu and Osmani, 1995), some mechanism must be in place to prevent activated NIMX CDC2 from inappropriately inducing mitosis in the absence of NIMA function. One possibility is that NIMA affects the mitosis-promoting activity of NIMX CDC2 at some level other than activation of enzyme activity. Two lines of evidence presented here, one genetic, the other cytological, lend support to such a hypothesis and indicate a role for NIMA in the nuclear localization of NIMX CDC2/NIME Cyclin B.

The NIMX CDC2/NIME Cyclin B Complex Localizes to the Nucleus in a NIMA-dependent Manner

We have shown that the major A. nidulans B-type cyclin, NIME Cyclin B, localized to the chromatin, nucleolar, and SPB regions of the nucleus in a NIMA-dependent manner. NIME Cyclin B localization to the nucleus in S and G2 parallels its accumulation (Ye et al., 1995, 1996), similar in many respects to localization of the S. pombe G2 cyclin, p63cdc2 (Booher et al., 1989; Alfa et al., 1990, 1991; Gal-
The localization of NIMX CDC2 to the nucleus correlated with that of NIME Cyclin B and was also dependent on NIMA function, suggesting that localization of NIMX CDC2/NIME Cyclin B complex itself is perturbed in nimA mutants.

We noted no obvious nuclear NIME Cyclin B staining in metaphase cells, even though we have looked at well over a thousand cells from cultures synchronously entering mitosis. We did observe nuclear staining very early in mitosis, before significant SPB separation. These results differ somewhat from cyclin B localization to the mitotic apparatus at metaphase in S. pombe and mammalian cells (for examples see Alfa et al., 1990; Pines and Hunter, 1991; Gallant and Nigg, 1992; Jackman et al., 1995), however, the significance of this difference is not clear. Either we cannot detect whatever NIME Cyclin B is present in metaphase nuclei or NIME Cyclin B is lost from the nucleus before metaphase. We are currently investigating NIME Cyclin B localization in cells overexpressing NIME Cyclin B and in cells blocked in mitosis by drugs or by cell cycle mutations to clarify this issue.

**NIMA and SONA in the Nucleocytoplasmic Transport of NIME Cyclin B**

The mechanism by which NIMA functions to promote nuclear NIME Cyclin B localization may be indicated by the identification of sonA as an allele-specific suppressor of nimA. SONA shows high sequence similarity to RAE1 of S. pombe (Brown et al., 1995), GLE2 of S. cerevisiae (Murphy et al., 1996), and the mammalian protein, MRNP41 (Kraemer and Blobel, 1997), all of which have been implicated in nucleocytoplasmic transport. The nuclear mRNA export defect in rael" and gle2" mutants, and the association of MRNP41 with mRNA indicate an important role for these proteins in mRNA export. However, none of
these proteins contain RNA binding motifs, suggesting that their interaction with mRNA is indirect. Furthermore, GLE2 and MRNP41 localize predominantly to nuclear pore complexes, gle2− mutations derange nuclear pore complex structure, and GLE2 interacts with SRP1 (importin α) in a two-hybrid assay, suggesting that these proteins play a more general role in nucleocytoplasmic transport (Murphy et al., 1996; Kraemer and Blobel, 1997). The localization of SONA to the nuclear periphery (refer to Fig. 8) is consistent with a role for SONA in nucleocytoplasmic transport.

Given that proper NIME Cyclin B localization is dependent on NIMA function (refer to Fig. 4), and that the sonA1 mutation suppresses the mitotic and NIME Cyclin B localization defect in nimA1 mutants without causing an increase in NIMA or NIMXCDC2 activity (refer to Fig. 10), we propose that NIMA and SONA are involved in the nucleocytoplasmic transport of NIME Cyclin B. Two models consistent with our results (Fig. 12) propose that nuclear localization of NIME Cyclin B is a function of its rate of import into the nucleus and its rate of export into the cytoplasm (Fig. 12). These models predict that localization of NIME Cyclin B to the SPB is dependent on its accumulation in the nucleus, as if NIME Cyclin B accumulates at the nucleoplasmic surface of the SPB. This prediction is consistent with the fact that the SPB is tightly associated with the nucleus throughout the cell cycle in A. nidulans (Oakley and Morris, 1983).

SONA is proposed to play a positive role in nuclear export, based on the mRNA export defects of rael1/gle2 mutants, although we cannot formerly exclude a role for SONA in nuclear import. NIMA is proposed to oppose SONA function, either by facilitating nuclear import (Fig. 12 A), or by antagonizing export (Fig. 12 B). In either scenario, loss of NIMA function results in a net decrease in nuclear NIME Cyclin B, which can be offset by loss of SONA function.

These models are not meant to exclude additional functions for NIMA beyond that in the nucleocytoplasmic transport of NIMXCDC2/NIME Cyclin B. The finding that gain of function mutations in nimA can induce abnormal mitosis in the absence of CDC2 function (O’Connell et al., 1994; Lu and Hunter, 1995; Pu and Osmani, 1995) clearly indicates that NIMA has additional roles in promoting mitosis.

We have proposed a specific (although not necessarily physical) interaction between NIMA and SONA in controlling nuclear NIME Cyclin B levels because of the allele-specific suppression of nimA1 (and not nimA5) by sonA1. The mechanism underlying this allele-specific interaction may be explained by quantitative differences in NIMA kinase activity in nimA1 versus nimA5 mutants. For example, extracts from cells arrested in G2 by the nimA1 mutation contain residual NIMA kinase activity (refer to Fig 10; Pu et al., 1995), whereas equivalent extracts from nimA5 cells contain only trace NIMA kinase levels (Ye et al., 1995). Accordingly, the nimA5 mutation may cause too severe a defect in the nuclear accumulation of NIME Cyclin B for the sonA1 mutation to suppress. Alternatively, the allele-specific nimA1/sonA1 interaction may be due to a direct interaction between NIMA and SONA. In this regard, we note that SONA contains three consensus NIMA phosphorylation sites (Lu et al., 1994) (refer to Fig. 7; FGAT at 5–8, FYKT at 198–201, and FNRT at 31–316). Regardless of the underlying mechanism, the allele-specific interaction between nimA and sonA indicates a specific interaction and demonstrates that inactivation of sonA does not simply bypass the need for nimA in the promotion of mitosis.

**Nucleocytoplasmic Transport and Regulation of Mitosis**

This study establishes a role for NIMA in the nucleocytoplasmic transport of NIMXCDC2/NIME Cyclin B. One implication of this finding is that nucleocytoplasmic transport and regulation of mitosis are intimately linked. Such transport is particularly relevant to closed mitosis, in which the nuclear envelope remains intact, as occurs in many fungi including A. nidulans, S. pombe, and S. cerevisiae. At the very least,
tubulin from disintegrated cytoplasmic microtubules probably needs to be rapidly imported into nuclei to form the intranuclear spindle. Regulators of mitosis may also undergo nuclear or cytoplasmic transport at mitosis, as was proposed for the p107wee1 regulator, p70 nim1 in *S. pombe* (Wu and Russell, 1997). A causal relationship between transport and mitosis is also supported by the G2/M arrest due to a mutation in the *S. cerevisiae* SRP1 (importin α) gene (Loeb et al., 1995) and by the finding that the *S. pombe rae1* mutation causes a G2 arrest (Brown et al., 1995).

Another significant implication of this work is the identification of a mechanism by which NIMX<sup>CDC2</sup>/NIME<sub>Cyclin B</sub> function is coordinated with that of NIMA. The requirement for the function of both kinases for the initiation of mitosis can now be explained by a model in which each kinase independently promotes some events of mitosis while also being sensitive to the function of the other. In this model, NIMA is not fully functional until it is hyperphosphorylated by NIMX<sup>CDC2</sup>/NIME<sub>Cyclin B</sub> and NIMX<sup>CDC2</sup>/NIME<sub>Cyclin B</sub> is not properly localized unless NIMA is functional. Given the finding of NIMA-like functions in other organisms (O’Connell et al., 1994; Fry and Nigg, 1995; Gallant et al., 1995; Pu et al., 1995; Pu and Osmani, 1995; Lu and Hunter, 1995), and the evolutionary conservation of SONA/GLE2/RAE1/MNRP41 (refer to Fig. 7; Brown et al., 1995; Murphy et al., 1996; Kraemer and Blobel, 1997), the analysis of NIMA and SONA interactions in *A. nidulans* should serve as an important model for the elucidation of fundamental mechanisms coordinating nucleocytoplasmic transport and mitosis.

Finally, it is very interesting that mutation of *rae1* of *S. pombe* results in a G2 arrest without preventing full activation of p34<sub>cdc2</sub> (Brown et al., 1995; Whalen et al., 1997). Perhaps mutation of *rae1* prevents correct localization of p34<sub>cdc2</sub>/p63<sub>cdc13</sub>, which would further indicate the conserved nature of this level of mitotic regulation.

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**Figure 11.** The *sonA1* mutation suppresses the nuclear NIME<sub>Cyclin B</sub> localization defect of *nimA1*. Cells of the *nimA1* strain, SFC403-19, and the *nimA1*, *sonA1* double mutant, SFC444-1, were cultured, fixed, and then prepared for immunocytochemistry as described in Materials and Methods. *Left*, strain identity; *top*, images corresponding to Ab or DAPI staining; *top row arrows*, (*nimA1* single mutant) position of nuclei in cells showing general cytoplasmic staining; *bottom row arrows*, position of example nuclei showing NIME<sub>Cyclin B</sub> staining. Bar, 10 μm.

**Figure 12.** Model for NIMA and SONA function in the nucleocytoplasmic transport of NIME<sub>Cyclin B</sub>.
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