Properties and Regulation of the Cell Cycle-specific NIMA Protein Kinase of Aspergillus nidulans*

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NIMA is the protein product of the nimA gene of the filamentous fungus Aspergillus nidulans, required for progression of cells from G₂ into mitosis. The protein kinase activity of NIMA, assayed by phosphorylation of β-casein, varies during the nuclear division cycle, reaching a maximum in late G₂ and M. To investigate the biochemical properties of this cell cycle-regulated protein kinase, we have expressed nimA cDNA that encodes full-length NIMA in Escherichia coli as a fusion protein with glutathione S-transferase. Purified NIMA phosphorylated β-casein, with a Kₐ of 38 μM and Vₐₙₜ of 156 nmol/min/mg. NIMA also demonstrated a Kₐ of 69 μM for ATP. Both recombinant and cellular NIMA kinases behaved as oligomers on gel filtration chromatography, and their kinase activities were strongly inhibited by various salts. By using both protein and peptide substrates, NIMA demonstrated a serine/threonine-specific protein kinase activity. Cellular NIMA exists as a phosphoprotein, and bacterially expressed NIMA was also phosphorylated on multiple serine/threonine residues. Some of these phosphorylations appeared essential for NIMA activity as the enzyme could be dephosphorylated and inactivated in vitro by protein serine/threonine phosphatases. Use of a kinase-negative mutant of NIMA revealed that the NIMA enzyme undergoes autophosphorylation when expressed at high concentrations in bacteria. Taken together, these data suggest that cellular mechanisms may exist to regulate the phosphorylation state and activity of the NIMA protein kinase during the nuclear division cycle in A. nidulans.

Considerable progress was made in our understanding of the regulation of cell proliferation with the identification of a conserved serine/threonine protein kinase that regulates progression of many eukaryotic cells including the filamentous fungus Aspergillus nidulans, required for entry into mitosis. The enzyme apparently was present in very low amounts in cells, and the only available assay utilized an enzymatically active immune complex produced by peptide-specific antibodies. Antibodies specific for NIMA immunoprecipitated the protein from extracts of A. nidulans, which was found to phosphorylate β-casein, but not histone H1. Using β-casein phosphorylation, the activity of NIMA was found to fluctuate during the nuclear division cycle. The kinase activity was low in G₂ and S, but began to increase in G₂, reaching a plateau in late G₂ and mitosis (5). These results demonstrated that NIMA was a cell cycle-regulated protein kinase, different from p34*cdc2 and its related protein kinases, and was also required for entry into mitosis.

Whereas NIMA appeared to be critical for mitotic progression in A. nidulans, virtually nothing was known about the biochemical properties of the protein kinase. The enzyme was apparently present in very low amounts in cells, and the only available assay utilized an enzymatically active immune complex produced by peptide-specific antibodies (5). To better understand the properties of the NIMA kinase, we have expressed this enzyme in bacteria. The assays using purified NIMA were first optimized to yield a 100-fold increase in enzyme activity. Subsequent characterization of the NIMA kinase emphasized that it represents a charter member of a new family of serine/threonine protein kinases. Our studies further revealed that the active form of NIMA present both in A. nidulans and in Escherichia coli was phosphorylated on multiple serine/threonine residues and that this phosphorylation was essential for the protein kinase activity. These studies provide new evidence for a potential mechanism for the cell cycle regulation of NIMA kinase activity in A. nidulans.

**EXPERIMENTAL PROCEDURES**

Bacterial Expression, Purification, and Thrombin Cleavage of GST-NIMA—To introduce an NcoI site at the initiation codon of NIMA, a HindIII-EcoRV fragment of nimA cDNA cloned in pUC18 (pHO1) (6) was cloned into the vector Phagemid TH1. The insert of TH1 was removed as an XbaI-EcoRV fragment and cloned into M13 to produce TH2. Using the method of Kunkel (6), an NcoI site was introduced into TH2 using the mutagenic oligonucleotide GACGCCCACCATGGCAATC. The insert of TH2

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**The abbreviations used are: GST, glutathione S-transferase; CaM, calcium/calcmodulin-dependent protein kinase; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-1-thio-β-D-galactopyranoside; FPLC, fast protein liquid chromatography; MAP, microtubule-associated protein.
phosphoamino Acid and Phosphopeptide Analyses-To label A. nidulans

Aspergillus cellular extract was applied to a Superose 6 FPLC column

antibodies (5) using "I-protein A (ICN) as described previously (11). The

buffer and separated by SDS-PAGE. To determine the kinetics of

versa in another series of reactions. The data were analyzed using a

constant concentration of ATP in one series of reactions and vice

by immunoprecipitation with the ANYRED NIMA-specific antibod-

ies as described by Osmani et

mapping of the labeled NIMA on glutathione-agarose beads

followed by labeling with 50 pCi/ml [32P]orthophosphate for 2 h at

32 °C and then for 3 h at 42 °C. Bacteria were labeled with [3P]

required for MgC12, with the greatest enzyme activity

involving in ATP binding, and its substitution to Met should

resulting GST-NIMA beads were stored on ice for up to 1 week in

ELISA, 2 mM benzamidine, 2 mM Na-p-tosyl-L-arginine methyl ester, 10 pg/ml N-tosyl-L-phenylalanine chloromethyl ketone, 10 μg/ml trypsin inhibitor, 50 pg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 2 mM dithiothreitol) and five times with PP buffer (50 mM Tris-HCl, pH 7.5, and 0.1-0.6% β-

mercaptoethanol). Phosphatase treatments were performed as de-scribed previously (14). Briefly, before the β-casein kinase assay, the

NIMA immunoprecipitates or purified GST-NIMA was incubated at

30 °C for 10 min (or the times indicated) with different concentrations of protein serine/threonine phosphatase 2A or 1 purified from rabbit skeletal muscle (15) or a bacterially expressed and purified glutathione S-transferase-tyrosine phosphatase 1B fusion protein containing the NHz-terminal 321 amino acids of tyrosine phosphatase 1B (16). 10.6 nM and 1 μM okadaic acid (Upstate Biotech Inc.) were used to inactivate protein serine/threonine phosphatases 2A and 1, respectively, whereas 1 mM sodium vanadate was used to inactivate tyrosine phosphatase 1B.

RESULTS

Expression and Purification of NIMA—To determine the biochemical properties and regulation of NIMA, we expressed NIMA in E. coli as a GST fusion protein employing the IPTG-inducible expression plasmid pGEX-2T (8). The expression system was chosen for two major reasons. It allows purification of the expressed proteins by a glutathione affinity column, and it has been shown that the GST vector protein does not have any detectable effect on biochemical properties and substrate specificity of a variety of enzymes including protein kinases and phosphatases (17, 18). When bacterial lysates were purified on a glutathione affinity column and visualized by SDS-PAGE, several proteins were apparent, including one with a molecular mass of 110 kDa, the expected size for the full-length GST-NIMA fusion protein. To further purify the fusion protein, the eluates from the glutathione affinity column were pooled and loaded onto a MonoQ FPLC column, followed by elution with a salt gradient (0-0.5 M). Using β-casein as a substrate, a major protein kinase peak was observed at a salt concentration of 0.26 M. Based on analysis of a Coomassie Blue-stained SDS-polyacrylamide gel, the 110-kDa GST-NIMA represented >90% of the total protein. The 110-kDa protein was confirmed to be NIMA by immunoblott analysis using the ANYRED peptide antibodies specific for NIMA (Fig. 1A). Lys-40 was predicted to be involved in ATP binding, and its substitution to Met should eliminate kinase activity (19). A mutant NIMA protein with a single amino acid substitution of Lys-40 with Met (K40M) was expressed in E. coli. Although the K40M mutant protein was expressed to a level similar to that of wild-type NIMA, the mutant protein did not show any casein kinase activity (Fig. 1A). These results confirm that Lys-40 is essential for NIMA kinase activity and reveal that there is no contaminating casein kinase activity in the purified GST-NIMA preparations.

Biological Properties of NIMA—Using β-casein as a substrate, assays were performed to establish the optimal reaction conditions for the recombinant NIMA kinase. NIMA kinase activity was linear for at least 40 min under the conditions used (Fig. 2A). NIMA kinase exhibited a broad concentration requirement for MgCl2, with the greatest enzyme activity occurring at concentrations >10 mM (Fig. 2B). MnCl2 could not substitute for MgCl2 in the NIMA kinase assay. In fact, MnCl2 (and also ZnCl2) strongly inhibited kinase activity even in the presence of 10 mM MgCl2 (Fig. 2B). ZnCl2 at concentrations of 0.1, 1, and 5 mM inhibited NIMA by 51, 87, and 98%, respectively. CaCl2, EGTA, or EDTA had no significant effect on activity at concentrations up to 1 mM (data
Regulation of NIMA Protein Kinase

A. Western analysis. Purified bacterially synthesized NIMA protein was separated by SDS-PAGE and stained either with Coomassie Blue (lane 1) or with the ANYRED peptide antibodies (lane 2) using 125I-protein A after transferring to filters. B. *in vitro* kinase activity. Assays were carried out as described under "Experimental Procedures" in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 100 μM [32P]ATP, 63.8 μM β-casein, and an equal amount of wild-type and mutant purified NIMA proteins as indicated in the *inset*. The values shown are the mean value from three different experiments.

not shown). Below pH 6, NIMA kinase was inactive. With an increase in pH, the protein kinase activity increased, became optimally active at pH 7.5, and remained maximally active at pH values up to 9.5 (Fig. 2C). NIMA could not effectively utilize GTP in the phosphotransferase assay. When GTP (up to 200 μM) was added to the standard assay, it did not alter the ability of NIMA to transfer 32P from ATP to casein (data not shown), indicating that NIMA preferred ATP as the phosphate donor. Kinetic analysis of recombinant NIMA (Fig. 3A) indicated *Kₘ* values of 69 μM for ATP and 38 μM for β-casein, with a *Vₘₐₓ* of 156 nmol/min/mg for the purified enzyme. These data indicated that the previous assay conditions using 2 μM ATP and 8.5 μM β-casein were clearly suboptimal (5). By modifying this assay to include 100 μM [32P]ATP and 63.6 μM β-casein, the NIMA kinase activity was increased >100-fold (Fig. 3B). Using these new assay conditions to assay the NIMA activity immunoprecipitated from *A. nidulans*, we observed a similar increase in kinase activity (data not shown). All subsequent experiments utilized the new assay conditions.

The apparent molecular mass of the recombinant and cellular NIMA proteins was determined by gel filtration chromatography. When purified recombinant GST-NIMA was fractionated by FPLC on a Superose 6 column, recombinant NIMA kinase activity was eluted just prior to ferritin with an apparent molecular mass of 430 kDa (data not shown). When NIMA activity in *A. nidulans* soluble extracts was similarly fractionated and assayed by immunoprecipitation of the column fractions with the ANYRED peptide antibodies, a molecular mass of ~300 kDa was observed (data not shown). Since the molecular masses of recombinant and cellular NIMA polypeptides on SDS-polyacrylamide gels are 110 and 85 kDa, respectively, these results suggest that NIMA exists as an oligomer, probably involving a complex of four catalytic subunits.

As shown in Fig. 4A, GST-NIMA kinase activity was progressively inhibited by increasing concentrations of LiBr, NaCl, or KCl. The data shown were obtained using the acidic protein substrate β-casein, but similar results were obtained using the basic polypeptide MBP or the small polypeptide phospholamban-(1-31) (data not shown), demonstrating that the salt effects were not due to the substrate used in the assay. Furthermore, similar effects of salts were also observed on NIMA cleaved from GST-NIMA (data not shown), indicating that the salt interactions were not mediated via GST. These inhibitory effects of salts were readily reversed (Fig. 4B).

Diluting the enzyme solutions to reduce LiBr or NaCl from 200 to 23.3 mM restored the full activity of NIMA to the level...
see with NIMA that was just subjected to 25 mM salt (Fig. 4A). Experiments carried out with NIMA immunoprecipitated from *A. nidulans* showed a similar sensitivity to salt (data not shown). These data indicate that the salts may interact preferentially with the enzyme and may be involved in disturbing the subunit interaction in the oligomeric form of NIMA that may be required for optimal kinase activity.

Substrate Specificity of NIMA Kinase—Purified GST-NIMA kinase was first assayed for its ability to phosphorylate a variety of proteins known to be substrates for other protein kinases. Besides β-casein, NIMA could phosphorylate MAP2, phosvitin, and MBP, but only poorly phosphorylated histone H1 (Fig. 5A). IgG, poly(Glu:Tyr), tau protein, α and β tubulins, dynein, kinesin, myosin light chain, bovine serum albumin, MAP4, MAP1b, and GST were not substrates for NIMA under our assay conditions (Table I). With synthetic peptides as substrates, GST-NIMA phosphorylated the phospholamban-(1–31)-peptide and the [Ser3']protein kinase C-(19–31)-peptide substrate previously thought to be selective for protein kinase C, but NIMA failed to phosphorylate substrates for cAMP-dependent protein kinase (Kemptide), Ca2+/calmodulin-dependent kinase (GS10), S6 kinase (S6 kinase peptide), casein kinase I (D4), and casein kinase II (casein kinase II peptide) (Table I). A similar substrate specificity was obtained when NIMA was cleaved from the GST-NIMA fusion protein by thrombin and separated from GST as described under “Experimental Procedures” (data not shown). Phosphoamino acid analysis was performed on β-casein and MBP phosphorylated by NIMA. In contrast to most protein kinases that phosphorylate casein on serine residues, NIMA phosphorylated β-casein largely on threonines (threonine:serine = 5.5:1). In contrast, NIMA phosphorylated MBP exclusively on serines (Fig. 4B). MBP is also a substrate for other growth-associated protein kinases (e.g. extracellular regulated kinases 1 and 2), but these kinases phosphorylate MBP exclusively on threonine. NIMA had no detectable tyrosine kinase activity using either poly(Glu:Tyr) or various protein substrates. Furthermore, when bacteria containing the wild-type NIMA expression plasmid were induced with IPTG for different times and bacterial lysates were subjected to Western analysis using anti-phosphotyrosine antibody, we could detect no increase in immunoreactivity, in comparison to extracts from control bacteria containing the pGEX vector only (data not shown). These results confirm that NIMA is a serine/threonine-specific protein kinase.

Regulation of NIMA Protein Kinase—Many protein kinases are themselves subject to regulation by reversible protein phosphorylation. To examine the potential role of protein phosphorylation, we first demonstrated that NIMA exists as a phosphoprotein in vivo. *A. nidulans* cells were labeled with
[32P]phosphate, followed by immunoprecipitation of a soluble cellular extract with ANYRED peptide antibodies specific for NIMA. At the nimT23 and nimA5 G2 arrest points when NIMA activity is maximal (5), two 32P-labeled proteins with molecular masses between 80 and 85 kDa were immunoprecipitated (Fig. 6A). These two proteins were not detected if the immune serum was preabsorbed with the antigenic peptide coupled to Affi-Gel (Fig. 6A), demonstrating the specificity of the immunoprecipitation procedure. To determine the importance of this phosphorylation in the regulation of kinase activity, NIMA, immunoprecipitated from the nimT23 G2-arrested cells where NIMA was phosphorylated, was incubated with either protein serine/threonine phosphatase 2A or tyrosine phosphatase 1B, and the kinase activity was analyzed. Protein serine/threonine phosphatase 2A, but not tyrosine phosphatase 1B, rapidly decreased NIMA activity. Furthermore, this effect of protein serine/threonine phosphatase 2A was inhibited by 16.6 nm okadaic acid, a potent protein serine/threonine phosphatase inhibitor (Fig. 6B). These results indicate that serine/threonine phosphorylation of NIMA, immunoprecipitated from A. nidulans, may regulate NIMA protein kinase activity.

Since the NIMA concentration in A. nidulans appears to be very low (5) and an effective purification procedure for cellular NIMA remains to be established, the only approach available to obtain NIMA is through immunoprecipitation. This procedure makes it difficult to determine the phosphorylation state of the enzyme and does not exclude the involvement of exogenous kinases or regulatory proteins in NIMA regulation. Therefore, we have examined the phosphorylation of NIMA using the purified recombinant enzyme. Since NIMA was active when purified from E. coli, we first determined whether bacterially synthesized NIMA was also phosphorylated. Bacteria containing the wild-type GST-NIMA expression plasmid were labeled with [32P]phosphate, and NIMA was purified using glutathione-agarose. As shown in Fig. 7A, GST-NIMA was also labeled with 32P, indicating that the enzyme was already phosphorylated in the bacteria. In initial experiments, two proteins were noted and were similar to those observed in A. nidulans (Fig. 6A). However, the amount of the lower molecular mass NIMA varied with the extraction conditions and was completely eliminated by the addition of multiple protease inhibitors as shown in Fig. 7A. These data suggest that the smaller form of NIMA may be generated by proteolysis. Fig. 7A also reveals that incubation of 32P-labeled GST-NIMA with protein serine/threonine phosphatase 1 completely removed all protein-bound phosphate, indicating that recombinant NIMA is largely phosphorylated on serine/threonine residues.

Fig. 8 demonstrates that phosphorylation of purified recombinant NIMA was also required for the protein kinase activity. Incubations with either protein serine/threonine phosphatase 2A or 1 decreased the NIMA kinase activity in both a concentration- and time-dependent manner (Fig. 8, A and B, respectively). Furthermore, okadaic acid completely blocked the effect of the protein phosphatases at concentrations of 16.6 nm and 1 μM, which inhibit the respective phosphatases (Fig. 8). These phosphatase treatments did not decrease or degrade the recombinant NIMA protein as shown by silver staining (data not shown). In contrast, incubation with protein tyrosine phosphatase 1B had no effect on the NIMA kinase activity, even at 100-fold higher concentrations than the protein serine/threonine phosphatases (Fig. 8). These results strongly support the idea that NIMA is phosphorylated on only serine/threonine residues and that this phosphorylation is mandatory for NIMA kinase activity.

As phosphorylation of NIMA occurred in bacteria, we next questioned whether this was due to autophosphorylation or...
phosphorylation by bacterial kinases. To address this question, we labeled bacteria containing the kinase-negative K40M mutant NIMA cDNA with \(^{32}\text{P}\) phosphate in parallel with bacteria containing the wild-type NIMA cDNA. Although the mutant protein was expressed at levels similar to those of wild-type NIMA (Fig. 1B), the mutant protein was not phosphorylated in metabolically labeled bacteria (Fig. 7A). These data strongly support that NIMA protein phosphorylation in E. coli reflects the autophosphorylation activity of the NIMA kinase. Phosphoamino acid analysis of NIMA phosphorylated in bacteria revealed phosphoserine and phosphothreonine at a ratio of 4:1, but no phosphotyrosine (Fig. 7B). The enzyme was phosphorylated at multiple sites since phosphopeptide analysis revealed several phosphopeptides (Fig. 7C). These results suggest that recombinant NIMA undergoes autophosphorylation at multiple sites when expressed in bacteria and that one or more of these phosphorylation events result in activation of the enzyme.

**DISCUSSION**

In this report, we have expressed the A. nidulans NIMA kinase in bacteria. Initial characterization of the enzyme led to the development of a new assay with a 100-fold increase in sensitivity. This assay was used to show that the NIMA enzyme will phosphorylate a number of protein and peptide substrates on serine/threonine residues. However, other analog peptide substrates for several known protein kinases such as CAMP-dependent kinase, Ca\(^{2+}\)/calmodulin-dependent kinase, casein kinases I and II, and S6 kinases were not phosphorylated. This spectrum of substrates for NIMA is considerably different from that of any other protein kinases that have been characterized to date. Finally, we demonstrated that the active NIMA kinase is a phosphoprotein both in A. nidulans and in bacteria and that selected protein serine/threonine phosphatases completely dephosphorylate NIMA and thereby inhibit its kinase activity. These studies have identified a new regulatory mechanism for NIMA involving reversible phosphorylation on serine/threonine residues.

The NIMA enzyme also exhibits other properties that set it apart from other protein kinases that have sequence similarities. Based on the computer-assisted comparisons of Hanks and Quinn (20), the most closely related known enzyme is Pim1. One similarity between these two enzymes is sensitivity to salt (21). Whereas our data support the possibility that NIMA is an oligomer, no such information is available for Pim1. However, even if both enzymes share an oligomeric structure, the similarities of properties come to an

**Table I**

Phosphorylation of in vitro substrates for NIMA

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(\beta)-Casein phosphorylation</th>
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<tr>
<td>(\beta)-Casein</td>
<td>100</td>
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<tr>
<td>Phosvitin</td>
<td>49.3</td>
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<tr>
<td>MAP2</td>
<td>40.4</td>
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<tr>
<td>MBP</td>
<td>26.5</td>
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<tr>
<td>Histone H1</td>
<td>43.3</td>
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<tr>
<td>Tau protein</td>
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<tr>
<td>(\alpha)-Tubulin</td>
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<tr>
<td>(\beta)-Tubulin</td>
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<tr>
<td>Dynemin</td>
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<tr>
<td>Kinesin</td>
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<tr>
<td>MLC(^a)</td>
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</tr>
<tr>
<td>BSA</td>
<td></td>
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<tr>
<td>MAP4</td>
<td></td>
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<tr>
<td>MAP1b</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>Phospholamban-(1-3')</td>
<td>78.6</td>
</tr>
<tr>
<td>[Ser(^{32})]PKC-(19-31)</td>
<td>15.7</td>
</tr>
<tr>
<td>S6 kinase peptide</td>
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<tr>
<td>Kemptide</td>
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<td>GS10 peptide</td>
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<td>D4 peptide</td>
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<tr>
<td>Casein kinase II peptide</td>
<td></td>
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<tr>
<td>Poly(Glu:Tyr)</td>
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\(^a\) MLC, smooth muscle myosin light chain; BSA, bovine serum albumin; PKC, protein kinase C.

![Fig. 6](image-url)

*In vivo* NIMA \(^{32}\text{P}\) labeling and effect of protein phosphatases on activity of A. nidulans NIMA. A, *in vivo* \(^{32}\text{P}\) phosphate labeling of NIMA. Conidia from the nimT23 and nimA5 strains were grown at the permissive temperature for 20 h and then at the restrictive temperature for 3 h and labeled for the last 5 h with \(^{32}\text{P}\) phosphate. Lysates were prepared and subjected to immunoprecipitation with the ANYRED NIMA-specific peptide antibody in the presence or absence of the antigenic ANYRED peptide as described (5). The precipitates were solubilized in SDS sample buffer and separated on SDS-10% acrylamide gels with prestaining molecular markers. B, effects of phosphatases. NIMA was immunoprecipitated from G\(_2\)-arrested cells and incubated with 1.5 \(\mu\)l of protein serine/threonine phosphatase 2A (PP2A) or tyrosine phosphatase 1B (PTP-1B) for 10 min in the presence or absence of okadaic acid (OK) in a total volume of 20 \(\mu\)l. After okadaic acid or sodium vanadate was used to inhibit protein serine/threonine phosphatase 2A or tyrosine phosphatase 1B, respectively, \(\beta\)-casein kinase activities were assayed as described under “Experimental Procedures.” The specific activities of protein serine/threonine phosphatases 2A and 1 and tyrosine phosphatase 1B were 1.2, 10.0, and 100 units/ml, respectively.
Regulation of NIMA Protein Kinase

A. autoposphorylation of recombinant NIMA in E. coli. Bacteria containing the wild-type (WT) or K40M mutant NIMA expression plasmid were labeled with \([32P]\)phosphate for 12 h, and the expressed proteins were purified by glutathione-agarose chromatography. The purified proteins were separated by SDS-PAGE with or without protein serine/threonine phosphatase 1 (PPI) treatment. B, phosphoamino acid analysis. C, phosphopeptide analysis. Autophosphorylation of \([32P]\)-labeled NIMA was repeatedly digested with trypsin and subjected to two-dimensional separation on thin-layer chromatography plates as described.

Two cDNA clones have been recently isolated from yeast and mouse that encode proteins containing a conserved kinase domain similar to that of NIMA at the predicted amino acid level. The yeast kinase gene was cloned by three different expression libraries using anti-phosphotyrosine antibodies, is a dual specificity kinase. It can phosphorylate IgG (stronger than casein), histone H1, and poly(Glu:Tyr) as well as both serine/threonine and tyrosine residues in bacterial proteins. Our own results reveal NIMA to be specific for serine/threonine residues.

Several protein kinases involved in cell cycle regulation are also subject to control by protein phoshorylation/dephosphorylation. Examples are p34<sup>cdc2</sup>, which is phosphorylated on both tyrosine and threonine residues (26-31), and MAP kinases, which also undergo dual regulatory phosphorylation events (32, 33). Each of these enzymes is involved in a cascade of reactions involving a series of protein kinases and phosphatases. Bacterially expressed MAP kinase becomes auto-phosphorylated on both threonine and tyrosine residues in vitro, which activates the enzyme (34). Dephosphorylation of either residue results in inactivation (33). Yet in the cell, MAP kinase is phosphorylated by specific MAP kinase kinases (35-39), and the sequence of the first member of this class of enzymes was recently reported by Crews et al. (40). Thus, some parallels can be made as to how MAP kinase and NIMA may be activated. We have shown NIMA to be a phosphoprotein and serine/threonine phosphorylation to be required for activity both in A. nidulans and when expressed in bacteria. In bacteria, NIMA is clearly autophosphorylated exclusively on multiple serine/threonine residues. We suspect that these activating intermolecular modifications are due to the high concentration of the enzyme. When NIMA is dephosphorylated, it becomes inactive. Preincubation of the enzyme under phosphorylation conditions results in a slow rate of autophosphorylation, but in this case, no kinase activity is recovered (data not shown). Even when active NIMA is used to phosphorylate the dephosphorylated enzyme, activity of the latter is not restored (data not shown). However, we estimate that the enzyme concentration in the in vitro reaction is at least 100 times lower than that in bacteria. Since the concentration of NIMA in A. nidulans appears to be very low (5), it is unlikely that autoprophosphorylation is responsible for activation of the kinase in G<sub>1</sub>. Therefore, NIMA may well also be an intermediary component in a cascade of protein kinase and phosphatase reactions that are required for progression from G<sub>1</sub> to mitosis.

Very little information is available concerning how NIMA is regulated in the cell, and nothing is known regarding physiologically relative substrates. Lu et al. (11, 41) created a strain of A. nidulans that is conditional for expression of the Ca<sup>2+</sup>-binding protein calmodulin. At low levels of calmodulin, cells become blocked in G<sub>0</sub>, and NIMA is inactive. When the expression of calmodulin is induced, NIMA is active, and cells progress into mitosis (41). We have shown that bacterially expressed NIMA neither binds nor is activated by Ca<sup>2+</sup>/calmodulin (data not shown). Thus, it seems likely that Ca<sup>2+</sup>/calmodulin may regulate the activity of an enzyme that phosphorylates NIMA. In A. nidulans, the only candidate enzyme that has been purified and whose gene has been cloned is a homolog of the multifunctional Ca<sup>2+</sup>/calmodulin-dependent protein kinase also known as CaM kinase II (42). Bartelt et al. (43) have shown that A. nidulans CaM kinase has proper-
ties very similar to those of vertebrate CaM kinase II, and several studies have implicated the involvement of the latter enzyme in the initiation of mitosis in other species (44, 45). In preliminary studies, we have found that dephosphorylated NIMA is a substrate for CaM kinase purified from A. nidulans or rat brain and that this phosphorylation results in the reappearance of kinase activity (data not shown). Since the genes for CaM kinase and NIMA are available, it should be possible to utilize the genetics of A. nidulans to confirm if these enzymes are components of the same regulatory pathway required for mitosis.

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REFERENCES


FIG. 8. Effects of phosphatases on activity of recombinant NIMA. Purified recombinant NIMA was incubated with various amounts of different phosphatases for 10 min (A) or with 1.5 µl of various phosphatases for the times indicated (B) in the presence or absence of okadaic acid (OK) in a total volume of 20 µl. After okadaic acid or sodium vanadate was used to inhibit protein serine/threonine phosphatases or tyrosine phosphatase, respectively, β-casein kinase activities were assayed as described under “Experimental Procedures.” The specific activities of the phosphatases were the same as those described for Fig. 7. PP2A and PP1, protein serine/threonine phosphatases 2A and 1, respectively; PTP-1B, tyrosine phosphatase 1B.