Identification of Substrate Specificity Determinants for the Cell Cycle-regulated NIMA Protein Kinase*

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Kun Ping Lu†, Bruce E. Kemp‡, and Anthony R. Means§
From the Departments of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710 and the §St. Vincent’s Institute of Medical Research, 41 Victoria Parade, Fitzroy, Victoria 3065, Australia

NIMA is a cell cycle-regulated protein kinase required for the G2/M transition in the filamentous fungus Aspergillus nidulans. Previous biochemical characterization of the recombinant enzyme indicated that NIMA is a protein serine/threonine specific kinase with β-casein being the best substrate from the many proteins and peptides tested (Lu, K. P., Osmani, S. A., and Means, A. R. (1993) J. Biol. Chem. 268, 8769–8776). However, substrate specificity or physiologically relevant substrates for NIMA remained unknown. In search for a peptide substrate for this enzyme, we screened an assembled library of synthetic peptides that each contained a phosphorylation site for a known protein kinase and found an excellent peptide substrate for NIMA, phospholemman 42–72 (PLM(42–72)). NIMA kinase phosphorylated PLM(42–72) uniquely and stoichiometrically on Ser with a \( V_{\text{max}} \) of 1.4 μmol/min/mg and apparent \( K_m \) of 20.0 μM. These kinetic constants were about 10-fold higher and 3-fold lower than those for β-casein, respectively. A detailed analysis of substrate specificity determinants using synthetic peptide analogs of PLM(42–72) indicated that Phe-Arg-Xaa-Ser/Ihr represents the optimal primary sequence for NIMA kinase phosphorylation. Replacement of the Arg at P=2 with Ala resulted in a 6-fold increase in \( K_m \) and 2-fold decrease in \( V_{\text{max}} \) while substitution of the Phe at P=3 with Ala abolished NIMA phosphorylation. These results reveal the unique nature of substrate recognition by the NIMA kinase and should prove valuable in the search for biologically relevant NIMA substrates.

The NIMA kinase is the product of a cell cycle regulatory gene that was isolated by genetic complementation of a temperature-sensitive mutation in the nimA gene of Aspergillus nidulans (1). Cells carrying temperature-sensitive mutations in the nimA gene were specifically arrested in G2 at the restrictive temperature, but rapidly and synchronously entered mitosis when shifted to the permissive temperature. In contrast, overexpression of the nimA gene product induced premature mitotic arrest (1). These results indicate that NIMA plays a critical role in the progression of cells into mitosis. Examination of the amino acid sequence of NIMA deduced from the nimA cDNA suggested that the nimA gene product belonged to the family of SerThr protein kinases. Antibodies specific for NIMA precipitated a fungal protein, which phosphorylated β-casein in vitro. Using β-casein phosphorylation, the activity of NIMA was shown to fluctuate during the nuclear division cycle, peaking in late G2 and mitosis (2). To determine the biochemical properties of the protein kinase, we expressed NIMA in bacteria (3). Biochemical characterization of the purified recombinant enzyme revealed it to be a unique Ser/Thr-specific protein kinase, the activity of which was also regulated by Ser/Thr phosphorylation/dephosphorylation (3). However, nothing was known concerning NIMA substrate specificity or the identity of physiologically relevant substrates.

One strategy that can be used to search for relevant substrates of a novel protein kinase is to define the structural determinants that are required for phosphorylation of protein or peptide substrates. Of the many proteins and synthetic peptides utilized as substrates for well characterized Ser/Thr-specific protein kinases, β-casein was the best substrate for NIMA (3). However, we showed that under optimal assay conditions, NIMA phosphorylated β-casein on multiple residues and with a relatively low \( V_{\text{max}} \) of 156 nmol/min/mg (3). In this study, we have identified an excellent peptide substrate for NIMA, namely phospholemman 42–72 (PLM(42–72)), by screening an assembled library of 56 synthetic peptides representing the phosphorylation sites in many different proteins. NIMA kinase phosphorylated PLM(42–72) with a 10-fold higher \( V_{\text{max}} \) and a lower \( K_m \) than β-casein. After establishing that the unique residue phosphorylated by NIMA in this peptide (Ser) was different from those phosphorylated by cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC), we synthesized two series of peptide analogs to define the structural determinants required for NIMA kinase specificity. Our results reveal hitherto unknown unique sequence determinants required for phosphorylation of synthetic peptide substrates by the NIMA kinase. These results also demonstrate the utility of using a synthetic peptide screen to identify efficient substrates for new protein kinases and provide essential information to begin the search for the biological substrates for NIMA.

EXPERIMENTAL PROCEDURES

Synthesis of Peptides—Peptides were synthesized and purified as described previously (4). All peptides were dissolved in distilled water at a concentration of 4 mg/ml and a series of dilutions was made using 0.25 mg/ml bovine serum albumin as a carrier. The precise concentration of each peptide was determined by amino acid analysis, which also established the predicted amino acid composition of all the synthesized peptides.

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† Present address: Molecular Biology and Virology Laboratory, The Salk Inst. for Biological Studies, P. O. Box 85860, San Diego, CA 92186-8600.

‡ To whom correspondence should be addressed: Dept. of Pharmacology, Duke University Medical Center, P. O. Box 3813, Durham, NC 27710. Tel.: 919-681-8290; Fax: 919-681-8461.

§ The abbreviations used are: PLM, phospholemman; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C, HPLC, high performance liquid chromatography.
was diluted prior to use in a buffer containing 50 mM HEPES, pH 7.5, bacterial expression system as described previously (3). Purified kinase was counted as described previously (3). Under these conditions, all kinase assay was repeated three to six times using at least two different preparations of NIMA kinase. Less than 10% standard deviation among duplicates within a given experiment were noted. The kinetic data were analyzed as described previously (5). Calmodulin-dependent protein kinase II and IV were assayed as described previously (6). Protein kinase A (PKA), protein kinase C (PKC), cyclin-dependent protein kinase Cdc2, and casein kinase I and II (Upstate Biotechnology Inc.) and assayed as described (7).

Peptide Phosphorylation—NIMA protein kinase was purified from a bacterial expression system as described previously (3). Purified kinase was diluted prior to use in a buffer containing 50 mM HEPES, pH 7.5, 0.5 mM MgCl2, 0.1 mM dithiothreitol. Phosphorylation of synthetic peptides was carried out in a 30-μl reaction containing 50 mM HEPES, pH 7.5, 50 μM MgCl2, 0.1 mM dithiothreitol, 100 μM or 300 μM [γ-32P]ATP (300–1000 counts/min/μmol), and 50–100 ng of NIMA. After incubation at 30 °C for 15 min, 25-μl aliquots were removed from the reaction mix and applied to phosphocellulose P81 filters. The filters were washed with 0.5% phosphoric acid, and the radioactivity was counted as described previously (3). Under these conditions, all kinase reactions were linear for at least 30 min. Every peptide phosphorylation assay was repeated three to six times using at least two different preparations of NIMA kinase. Less than 10% standard deviation among experiments using different kinase preparations and less than 5% standard deviation among duplicates within a given experiment were noted. The kinetic data were analyzed as described previously (5). Calmodulin-dependent protein kinase II and IV were assayed as described previously (6). Protein kinase A (PKA), protein kinase C (PKC), cyclin-dependent protein kinase Cdc2, and casein kinase I and II (Upstate Biotechnology Inc.) were assayed according to the manufacturer's conditions. Mitogen-activated protein kinase was kindly provided by Dr. Perry Blackshear (Dept. of Biochemistry, Duke University Medical Center) and assayed as described (7).

Analysis of Phosphorylated Residues—For phosphoamino acid and phosphopeptide analyses, peptides were phosphorylated in the presence of 50 μM [γ-32P]ATP. Phosphopeptides were separated from the free

### Table 1

Phosphorylation of synthetic peptides by NIMA kinase

| Serial no. | Peptide name | Peptide sequence | Relative phosphorylation |
|-----------|--------------|------------------|-------------------------|
| 61        | Phosphoenamid | CTNPQQRTGSEPQEEEETFPRSSRSLRRR  | 1235.4 |
| 50        | Phospholamban 1-31 | MKVYLLRSIAHRSTEMPOQAQRQNL  | 78.6 |
| 49        | Phospholamban 8-21 | TRSA1HRASTEMPOQAQRQNL  | 2.6 |
| 43        | ADR1 222-234 | LKKTLRAFSQAQ  | 67.3 |
| 42        | ADR1 222-234(R) | LKKTLRAFSQAQ  | 23.1 |
| 41        | ADR1 222-234(G) | LKKTLRAFSQAQ  | 22.1 |
| 36        | ADR1 222-234 | LKKTLRAFSQAQ  | 14.6 |
| 46        | ADR1 222-234(S) | LKKTLRAFSQAQ  | 10.3 |
| 47        | ADR1 222-234(G) | LKKTLRAFSQAQ  | 7.5 |
| 30        | ADR1 225-241 | LTRRAFSQAASQYAL  | 9.5 |
| 28        | PTHrP 13 | CRVSVDLRLRAV  | 40.1 |
| 57        | DARP 222-249 | PKTVRHGSFPTMPFRLS  | 27.7 |
| 60        | HMG-CoA reductase (661-876) | HLVSKHSMNRSKINL  | 20.4 |
| 51        | CaMKII (297-310) | RRRLKSGLITMLA  | 17.1 |
| 19        | GS1-10 | PLRKLSTVAA  | 11.1 |
| 32        | GS1-12 | PLRLTLSVAAK  | 10.6 |
| 41        | GS1-10 | PLRLTLSVAAK  | 0.0 |
| 21        | PKC 19-31S | HFKARGSLQMKNY  | 15.8 |
| 1        | MLC 11-23 | KPKPORATSNVFA  | 1.0 |
| 7        | MLC 11-23A  | AKPKPORATSNVFA  | 1.0 |
| 8        | MLC 11-23A  | AKPKPORATSNVFA  | 0.0 |
| 24        | MLC 11-23A  | KKSKRAGSTNVFA  | 0.6 |
| 22        | MLC 11-23G  | KKSKRAGSTNVFA  | 5.2 |
| 23        | MLC 11-23V  | KKSKRAGSTNVFA  | 0.6 |
| 25        | MLC 11-23G  | KKSKRAGSTNVFA  | 5.7 |
| 62        | MLC 11-23W  | KKSKRAGSTNVFA  | 8.9 |
| 13        | MLC 11-23A  | KKSKRAGSTNVFA  | 0.2 |
| 10        | mSMLC 1-17A  | PAAAKKRAAAGSHNSVS  | 4.6 |
| 5        | MLC 5-17  | KKKKAKKEGSHNSVS  | 0.3 |
| 26        | MLC 35-51  | RRLKQAIXTCLLA  | 2.0 |
| 55        | r-Acetyl-CoA-carboxylase (71-85)  | RRRHRMSSAMAGLHLV  | 8.0 |
| 54        | GP120 consensus (304-338) | CTRMNTRKRSHHIGFGAFYTTGEIGDIQAH  | 5.5 |
| 56        | mUPA 14-32A  | QNGUVASLYLFSR1C  | 4.6 |
| 57        | Phe hydroxylase (263-277) | HCTQQEMHGMRWT  | 4.5 |
| 45        | cGPK 72-83  | FRTKQPQASAEF  | 4.2 |
| 14        | K4PHOS. 1 | KKKKQTVSGVLDG  | 3.9 |
| 37        | cGPK (77-84) | LQGQSKRFET  | 3.4 |
| 35        | VIF 181-193 | CGHQBSTHMNGH  | 3.3 |
| 52        | cGPK 55-67 | PRTAQAQISLAEF  | 0.0 |
| 53        | cGPK 55-67A  | PRTAQAQISLAEF  | 0.0 |
| 17        | S6 229-239 | AKHRILASLA  | 2.7 |
| 24        | S6 229-239 | AKHRILASLA  | 2.7 |
| 38        | S6 229-239 | AKHRILASLA  | 1.8 |
| 56        | S6 kinase (723-733) | RRKVIPLATL  | 3.1 |
| 34        | S6 kinase (723-733) | RRKVIPLATL  | 2.6 |
| 36        | S6 kinase (723-733) | RRKVIPLATL  | 2.6 |
| 38        | S6 kinase (198-210) | KRSSEFEYHCVLUR  | 0.0 |
| 39        | JB analog PTHrP (1-34) | AT7AEPQFVQKVSGHRRKATVSRLSA  | 1.7 |
| 30        | Vinculin (105-126) | ROYLYDGRQYL  | 0.1 |
| 58        | A CoA-carboxylase (79-98) | MSGLHLVRQCDRRKQVDR  | 0.0 |
| 59        | Phosphate kinase A (1014-1023) | LRLSLTSTES  | 0.0 |
| 50        | Phosphate kinase A (10-19) | ARLRKLSDFG  | 0.0 |
radioactive ATP on an AG1-X8 ion-exchange column in the presence of 30% acetic acid as described previously (8). After repeated lyophilization in water, peptides were subjected to partial acid hydrolysis for phosphoamino acid analysis or repeated trypsin digestion for phosphopeptide analysis, followed by two-dimensional separation by thin layer chromatography as described previously (9).

For phosphopeptide sequencing, peptides were phosphorylated in the presence of 500 μM unlabeled ATP containing a trace amount of [γ-32P]-ATP. The phosphorylated peptides were separated from unphosphorylated peptides by reversed-phase HPLC using a gradient of 20–41% acetonitrile (v/v) in 0.1% trifluoroacetic acid (v/v) with a flow rate of 1 ml/min. The purified phosphorylated peptides were digested overnight at 37 °C in 100 μl of 0.1 M NH₄HCO₃ containing about 2% (w/v) protease V8 (Boehringer Mannheim). Following lyophilization, peptides were again separated by reversed-phase HPLC using 5–20% acetonitrile (v/v). The single radioactive peak was sequenced by Dr. Richard Cook (Baylor College of Medicine) using an Applied Biosystems Inc. 470A gas-phase sequenator and a 120A phenylthiohydantoin amino acid analyzer.

RESULTS AND DISCUSSION

Screening the Assembled Peptide Library—To identify peptide substrates of the NIMA kinase, we used 56 synthetic peptides representing sites identified in a wide range of proteins that are phosphorylated by many different protein kinases (10). As shown in Table I, dramatic differences in the NIMA activity toward these peptides were observed. Most of the peptides were not phosphorylated by NIMA. Only eight peptides were phosphorylated at a rate above 20% of that observed using β-casein as a substrate. However, a single peptide PLM(42–72) was observed to be a better substrate than β-casein. NIMA phosphorylated this peptide at a rate that was more than 10-fold faster than β-casein.

Identification of NIMA, PKA, and PKC Phosphorylation Sites on PLM(42–72)—When the peptide PLM(42–72) was incubated with NIMA for 1 h, about 0.8 mol of phosphate was incorporated per mol of the peptide, suggesting that PLM(42–72) was phosphorylated on a single residue. To identify the NIMA phosphorylation site on PLM(42–72), phosphoamino acid analysis, phosphopeptide isolation, and sequencing were carried out. Phosphoamino acid analysis indicated that NIMA exclusively phosphorylated a serine residue (Fig. 1B). There are 3 serines in the peptide PLM(42–72) at positions 62, 63, and 68 (Fig. 1A). To distinguish whether NIMA phosphorylated Ser62, Ser63, and/or Ser68, phosphopeptide analysis was undertaken. The phosphorylated synthetic peptide was cleaved exhaustively with trypsin, and the digest was separated by thin layer chromatography according to the protocol of Boyle et al. (9). A single phosphorylated peptide, peptide 1 (Fig. 1C), was detected, indicating that NIMA could phosphorylate either Ser62, Ser63, or Ser68, but not both, since trypsin cleaved at arginines located between these serines. To identify the phosphorylated residue(s), peptide PLM(42–72) was phosphorylated by NIMA in the presence of ATP containing a trace amount of [γ-32P]-ATP; the phosphorylated product was purified by reversed-phase HPLC, and digested repeatedly with protease V8 under conditions where the protease cleaves peptides specifically at the COOH-terminal side of Arg or Glu residues, respectively. A, phosphoamino acid analysis. PLM(42–72) was phosphorylated by the different protein kinases as indicated and phosphorylated peptides were isolated and subjected to acid hydrolysis, followed by two-dimensional separation on thin layer chromatography plates. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. c, phosphopeptide analysis. The phosphorylated peptides generated by the enzymes shown at the top of each panel were exhaustively digested with trypsin and then subjected to two-dimensional separation on thin layer chromatography plates separately (left panel) or in combination (right panel). The ratios of phosphopeptide radioactivity loaded on the thin layer chromatography plates containing the combined samples were 3:1 for NIMA/PKA, NIMA/PKC, and PKA/PKC.

residues, as did NIMA (Fig. 1B). Phosphopeptide analysis indicated that a single tryptic peptide, peptide 2, was phosphorylated by PKA and that the mobility of this peptide was different from that phosphorylated by NIMA, as determined by mixing experiments (Fig. 1C). These results indicate that PKA predominantly phosphorylated Ser68, which contains the appropriate consensus sequence RXXS for phosphorylation by PKA. Two tryptic peptides (phosphopeptides 1 and 2) were phosphorylated by PKC at a ratio of approximately 4:1 (Fig. 1C). In phosphopeptide-mixing experiments, these two peptides comigrated with peptides 1 and 2 which were independently phosphorylated by NIMA and PKA, respectively. Given the specificity requirements of PKC, it seems reasonable that it phosphorylates Ser63 (peptide 1) and Ser68 (peptide 2) where peptide 2 contains the most favored site which corresponds to
NIMA Kinase Specificity

**Table II**

*Kinetics of phosphorylation of phospholemman peptides by NIMA*

Peptide phosphorylation was described as described under "Experimental Procedures." Kinetic constants were estimated as previously described (5).

| Peptide     | Sequence                  | $K_m$ (μM) | $V_{max}$ (μmol/min/mg) | $V_{max}/K_m$ x 10^{-3} |
|-------------|---------------------------|------------|-------------------------|--------------------------|
| PLM(42-72)  | CKFNQQGTEGPEDEGTSSRSSLR    | 20.0       | 1.4                     | 71                       |
| PLM(43-72)  | KFNQQGTEGPEDEGTSSRSSLR    | 17.5       | 1.4                     | 80                       |
| PLM(49-72)  | RTGPEGDEGTSSRSSLR         | 19.0       | 1.4                     | 76                       |
| PLM(54-72)  | DEUTFSRSSLR               | 20.3       | 1.5                     | 75                       |
| PLM(58-72)  | GTPRSSRSSLR               | 44.6       | 1.6                     | 36                       |
| PLM(60-72)  | FSSRSSLR                  | 110.6      | 0.9                     | 8                        |
| PLM(65-72)  | IRSSLR                    | 81.6       | 1.7                     | 20                       |
| PLM(58-70)  | GTPRSSIRLST               | 85.3       | 1.3                     | 16                       |
| PLM(58-67)  | GTPRSSIRL                 | 68.5       | 1.1                     | 17                       |

**Table III**

*The role of specific amino acids in peptide phosphorylation by NIMA*

Kinetic constants were determined as described under "Experimental Procedures."

| Peptide     | Sequence                  | $K_m$ (μM) | $V_{max}$ (μmol/min/mg) | $V_{max}/K_m$ x 10^{-5} |
|-------------|---------------------------|------------|-------------------------|--------------------------|
| PLM(58-72)  | GTFRS SIRLST              | 44.8       | 1.7                     | 38                       |
| PLM(58-72A) | GTFRS A SIRLST            | 94.9       | 2.0                     | 21                       |
| PLM(58-72A) | GTFRS A SIRLASSR          | 70.8       | 1.9                     | 27                       |
| PLM(58-72A) | GTFRS A SIRLASSR          | 260.1      | 1.1                     | 4                        |
| PLM(58-72A) | GTFRS ASIRLASSR           | 185.7      | 1.4                     | 9                        |
| PLM(58-72A) | GTFRS ASIRLASSR           | 152.0      | 1.5                     | 10                       |
| PLM(58-72A) | GTFRS ASIRLASSR           | 106.0      | 1.7                     | 16                       |
| PLM(58-72A) | GTFRS A SIRLASSR          | 260.1      | 1.1                     | 21                       |
| PLM(58-72A) | GTFRS A SIRLASSR          | 70.8       | 1.9                     | 27                       |
| PLM(58-72A) | GTFRS A SIRLASSR          | 260.1      | 1.1                     | 4                        |

**Phosphorylation of Peptides in Which Individual Residue(s) Are Changed to Alanine**—To identify the amino-terminal specificity determinants in substrates for NIMA kinase, we investigated the role of individual residues in the minimal peptide substrate PLM(58-72). A series of PLM(58-72) analogs was synthesized in which a single or multiple residues were substituted with alanines. As mentioned above, this approach allowed the identification of the functionally important amino acids independent of the potentially confounding effects of peptide length variation. The kinetic constants for phosphorylation of these peptides by NIMA are shown in Table III. Substitution of Ser68 with Ala gave a peptide that was not phosphorylated by NIMA, while substitution of Ser69 and Thr69 or Ser69 and Thr69 with alanines had little effect on the overall kinetics of peptide phosphorylation by NIMA. These results confirmed that NIMA phosphorylated a single residue, Ser68. When Arg65, Arg66, or Arg70 were replaced singly or collectively with Ala, the kinetic constants were only slightly altered. However, replacing Arg65 with Ala increased the apparent $K_m$ about 6-fold and also reduced $V_{max}$, resulting in a much poorer peptide substrate and indicated that the Arg at position -2 from the phosphorylated Ser(P-2) may be important for substrate recognition. The most striking finding of these studies was that the substitution of Phe60 with Ala resulted in a near complete inhibition of peptide phosphorylation by NIMA. This indicates that a Phe at P-3 is absolutely required for substrate phosphorylation and may represent a distinguishing feature of NIMA substrate recognition when compared to other known protein kinases.

In support of this unique requirement for NIMA substrates, we compared phosphorylation of two peptide substrates of NIMA as potential substrates for several other Ser/Thr protein kinases. As shown in Table IV, only NIMA and PKC could phosphorylate PLM(58-72)A60,68,69. Substitution of Phe60 with Ala (PLM(58-72)A60,68,69) reduced peptide phosphorylation by NIMA approximately 20-fold whereas this change had only a modest but opposite effect on phosphorylation by PKC increasing it by 1.5-fold. These data reveal that even though NIMA
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TABLE IV
Phosphorylation of selected PLM peptides by different protein kinases

Peptide phosphorylation was determined as described under "Experimental Procedures." The amounts (ng) of kinases used are: NIMA, 20; PKC, 5; PKA, 280; (MAP) kinase, 200; casein kinase I, 250; casein kinase II, 25; calmodulin (CaM)-dependent kinase II, 217; CaM-dependent kinase IV, 259, and cyclin-dependent CDC2, 250.

| Peptide | Protein kinase | Phosphorylation |
|---------|----------------|-----------------|
| 60GGTRASIRLARARRR<sup>72</sup> (PLM(58-72) A<sup>62,68,69</sup>) | NIMA | 100.0 ± 1.9 |
|         | PKC            | 60.9 ± 4.4      |
|         | PKA            | 9.9 ± 1.9       |
|         | MAP kinase     | 1.0 ± 0.2       |
|         | Casein kinase I| <0.5            |
|         | Casein kinase II| <0.5           |
|         | CaM-dependent kinase II| <0.5 |
|         | CaM-dependent IV| <0.5           |
|         | Cyclin-dependent CDC2| <0.5 |
| 60GGTARSSIRLARARRR<sup>72</sup> (PLM(58-72) A<sup>60,68,69</sup>) | NIMA | 4.5 ± 0.1 |
|         | PKC            | 89.6 ± 3.8      |

and PKC can both phosphorylate Ser<sup>63</sup>, there is a distinctly different requirement for the amino acid at P-3 for these protein kinases. To our knowledge, NIMA is the first protein kinase to be characterized that requires a Phe at P-3 of the phosphorylation site. PKA has a requirement for an aromatic residue at the P-11 position for high affinity binding of the protein kinase I peptide (12), and Colbran et al. (13) have shown that Phe at the P+4 position can act as a negative determinant for PKA.

We have not attempted to model the NIMA structure (1), but inspection of the NIMA catalytic domain sequence in the light of the recognition requirements and three-dimensional structure of PKA (14, 15) reveals some similarities and some important differences between these two enzymes. Both share Glu residues analogous to Glu<sup>170</sup> and Glu<sup>230</sup> of the PKA that are important for recognition of an Arg at the P-2 position relative to the serine that is phosphorylated (P0). On the other hand Glu<sup>127</sup> which is required for P-3 Arg recognition and Glu<sup>203</sup> which is required for P-6 Arg recognition in PKA are replaced in NIMA by Asp and Phe, respectively. These differences favor the view that P-3 and P-6 basic residue determinants are not important for NIMA. The aromatic pocket in the PKA structure contains Tyr<sup>238</sup> and Phe<sup>209</sup> which are required for high affinity binding of protein kinase inhibitor at the P-11 position. As these residues are replaced by Glu and Asn in NIMA, respectively, we surmise that NIMA substrates do not have a similar requirement to PKA for this aromatic pocket. However, a second hydrophobic pocket in the PKA structure that is utilized for recognition of the P+1 position, namely Leu<sup>198</sup>, Pro<sup>202</sup>, and Leu<sup>209</sup> is reasonably conserved in NIMA by Tyr, Pro, and Met, respectively. The differences in the substrate recognition residues between the two enzymes are consistent with the observation that substrates for PKA, such as Kemptide are poor substrates for NIMA. The likely recognition site for the Phe at P-3 is not known although inspection of the NIMA sequence reveals that in the sequence corresponding to the region in the PKA structure between the β strand 9 and the F-α helix, SHDFASTYGFTPFPMSPEIC, 3 aromatic residues are present that may provide a recognition pocket for Phe<sup>60</sup> in the PLM(42-72) peptide.

Whereas the NIMA protein kinase is critical for mitotic progression in A. nidulans (1, 2, 16, 17), it is not known whether homologs of this enzyme exist in other organisms. Even in the fungal system the physiological relevant substrates for NIMA remain to be identified. The pair of peptides developed and characterized in this report (Table IV) should be useful agents in both searches. On the one hand enzyme activities can be identified that recognize PLM(58-72)<sup>62,68,69</sup> but will not phosphorylate PLM(58-72)<sup>60,68,69</sup>. On the other hand, one can now search for potential substrates that contain a Phe at P-3 of the phosphorylated residue. In such instances it is also possible that PLM(58-72)<sup>62,68,69</sup> in which the Ser<sup>63</sup> is also converted to a nonphosphorylatable residue might serve as a competitive inhibitor of NIMA-like protein kinases.

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