Molecular Basis for the Substrate Specificity of NIMA-related Kinase-6 (NEK6)

EVIDENCE THAT NEK6 DOES NOT PHOSPHORYLATE THE HYDROPHOBIC MOTIF OF RIBOSOMAL S6 PROTEIN KINASE AND SERUM- AND GLUCOCORTICOID-INDUCED PROTEIN KINASE IN VIVO

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The AGC family of protein kinases, which includes isoforms of protein kinase B (also known as Akt), ribosomal S6 protein kinase (S6K), and serum- and glucocorticoid-induced protein kinase (SGK) are activated in response to many extracellular signals and play key roles in regulating diverse cellular processes. They are activated by the phosphorylation of the T loop of their kinase domain by the 3-phosphoinositide-dependent protein kinase-1 and by phosphorylation of a residue located C-terminal to the kinase domain in a region termed the hydrophobic motif. Recent work has implicated the NIMA (never in mitosis, gene A)-related kinase-6 (NEK6) as the enzyme that phosphorylates the hydrophobic motif of S6K1 in vivo. Here we demonstrate that in addition to phosphorylating S6K1 and SGK1 at their hydrophobic motif, NEK6 also phosphorylates S6K1 at two other sites and phosphorylates SGK1 at one other site in vitro. Employing the Jerini pepSTAR method in combination with kinetic analysis of phosphorylation of variant peptides, we establish the key substrate specificity determinants for NEK6. Our analysis indicates that NEK6 has a strong preference for Leu 3 residues N-terminal to the site of phosphorylation. Its mutation to either Ile or Val severely reduced the efficiency with which NEK6-phosphorylated peptide substrates, and moreover, mutation of the equivalent Leu residue in S6K1 or SGK1 prevented phosphorylation of their hydrophobic motifs by NEK6 in vitro. However, these mutants of S6K1 or SGK1 still became phosphorylated at their hydrophobic motif following insulin-like growth factor-1 stimulation of transfected 293 cells. This study provides the first description of the basis for the substrate specificity of NEK6 and indicates that NEK6 is unlikely to be responsible for the IGF1-induced phosphorylation of the hydrophobic motif of S6K, SGK, and protein kinase B isoforms in vivo.

Stimulation of cells with growth factors and insulin leads to the activation of phosphatidylinositol 3-kinase and the production of the lipid second messenger PtdIns(3,4,5)P3 that triggers the activation of a number of downstream signaling pathways (1). One of these is a group of enzymes that belong to the AGC subfamily of protein kinases, which include isoforms of protein kinase B (PKB) (2, 3), p70 ribosomal S6 kinase (S6K) (4, 5) and serum-and glucocorticoid-induced protein kinase (SGK) (6). Phosphatidylinositol 3-kinase triggers the phosphorylation of PKB, S6K, and SGK isoforms at 2 residues, leading to their activation. One of these residues lies in the T-loop (also known as the activation loop) of the kinase domain (Thr308 in PKB, Thr265 in S6K1, and Thr229 in SGK1), and the other is located C-terminal to the catalytic domain, in a region termed the “hydrophobic motif” (Ser473 in PKB, Thr112 in S6K1, and Ser222 in SGK1). Phosphorylation of PKB at Thr308 is sufficient to activate PKB substantially, which is then further activated following its phosphorylation at Ser473. In contrast, S6K and SGK require phosphorylation of both their T-loop and hydrophobic motif residues to become activated significantly. PKB, S6K, and SGK isoforms are phosphorylated at their T-loop by the 3-phosphoinositide-dependent protein kinase-1 (PDK1) (7, 8). PDK1 is also an AGC family member and possesses a PtdIns(3,4,5)P3-binding Pleckstrin homology domain C-terminal to the catalytic domain. Following phosphatidylinositol 3-kinase activation, PDK1 and PKB are co-localized at the plasma membrane through their mutual interaction with PtdIns(3,4,5)P3. In addition to recruiting PKB to cell membranes, the binding of PtdIns(3,4,5)P3 to the Pleckstrin homology domain of PKB may induce a conformational change that enables PDK1 to phosphorylate Thr308 (reviewed in Ref. 9). In contrast, S6K and SGK do not interact with PtdIns(3,4,5)P3, since these enzymes do not possess a Pleckstrin homology domain; nor is the rate at which they are phosphorylated by PDK1 in vitro enhanced in the presence of PtdIns(3,4,5)P3. Recent evidence indicates that the phosphorylation of S6K and SGK at their hydrophobic motif plays a key role in promoting the interaction of these enzymes with PDK1, hence resulting in
their activation (10). This observation indicates that if phosphatidylinositol 3-kinase could trigger the activation protein kinase(s) that phosphorylates the hydrophobic motif of S6K and SGK, this would convert these enzymes into better substrates for PDK1.

Much recent work has therefore focused on identifying the protein kinases that phosphorylate S6K, SGK, and PKB at their hydrophobic motif. Recently, Avruch and colleagues purified a major activity from rat liver extracts that phosphorylates S6K1 at its hydrophobic motif and identified this enzyme as the NIMA (never in mitosis, gene A)-related kinase-6 (NEK6) (11). Overexpression of NEK6 in unstimulated cells, induced phosphorylation of S6K1 at Thr412, whereas a catalytically inactive mutant of NEK6 prevented insulin-mediated activation of S6K and its phosphorylation at Thr412. These observations suggested that NEK6 might phosphorylate the hydrophobic motif of S6K1 directly in vivo. The hydrophobic motifs of S6K, SGK, and PKB isoforms lie in a conserved Phe-Pro/Leu-Xaa-Phe-Ser/Thr-Tyr motif, where Xaa is any amino acid and the Ser/Thr is the phosphorylated residue. It is therefore possible that a common upstream protein kinase phosphorylates the hydrophobic motifs of all three enzymes. In this study, we investigate the molecular determinants of NEK6 substrate specificity and perform experiments that indicate that NEK6 is unlikely to phosphorylate the hydrophobic motif of PKB, S6K, and SGK in cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Protease inhibitor mixture tablets and sequencing grade trypsin were from Roche Molecular Biochemicals. Zwittergent 3-16, wortmannin, and rapamycin were from Calbiochem, and tissue culture reagents and insulin-like growth factor-1 (IGF1) and Microcystin-LR were from Invitrogen. Glutathione-Sepharose and [γ-32P]ATP were from Amersham Biosciences. The precast 4–12% BisTris gradient SDS-polyacrylamide gels were from Invitrogen.

**Antibodies**—The antibody used to immunoblot S6K1 was raised in sheep against the His-S6K1 protein (12) and affinity-purified. The antibody used to immunoblot SGK1 was raised against the peptide LGFSYAPPTDSFL (corresponding to the C-terminal 13 residues of human SGK1 (14)) and is available from Upstate Biotechnology Inc.

**Phosphoamino acid analysis**—Phosphoamino acid analysis of 32P-labeled peptides and recombinant DNA procedures, including mutagenesis, were performed using standard protocols, and DNA constructs were verified by DNA sequencing. This was performed by the Sequence Service at the School of Life Sciences at the University of Dundee using DYEnamic ET terminator chemistry (Amerham Biosciences) on Applied Biosystems automated DNA sequencers. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard. Human kidney embryonic 293 cells were cultured on 10-cm-diameter dishes in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Phospholipid vesicles containing phosphatidylcholine, phosphatidylserine, and cholesterol were prepared by the following procedure. Cultured on 10-cm-diameter dishes in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Phospholipid vesicles containing phosphatidylcholine, phosphatidylserine, and cholesterol were prepared by the following procedure.

**Cloning of Human NEK6**—A full-length human NEK6 cDNA was obtained from the I.M.A.G.E. Consortium (17) (IMAGE clone ID 2458945, NCBI accession number A10952811), and the sequence of this clone was verified. This clone was used as a template to amplify the coding region of NEK6, incorporating an N-terminal FLAG tag using the following primers: 5′-ACTAGTTGCACATGCAGACTAACAGG-3′ and 5′-ACTAGTTGCACATGCAGACTAACAGG-3′. The resulting PCR fragments were ligated into the pcR2.1-Topo vector (Invitrogen), and the sequences were verified. SpeI-SpeI or EcoRI-EcoRI insert fragments from this intermediate vector were subcloned into the pEBG-2T or pCMV5 expression vectors, respectively, to create a catalytically inactive mutant of NEK6, Asp263 in subdomain VII of the kinase domain was mutated to Ala.

**Protein Expression and Purification**—All forms of S6K1 employed in this study are the long splice variant of rat S6K1 (accession number AAAA42104) (18). Full-length S6K1, S6K1CT (corresponding to a fragment of S6K comprising residues 1–421 lacking the C-terminal 104 residues) (18), S6K1CT(T412A), S6K1CT(L409I), S6K1CT(L419V), SGK1, SGK1NT, or PKBα (lacking the N-terminal 60 residues) (13), SGK1NT(S42A24), SGK1NT(L419I), SGK1NT(L419V), PKBα (16), the wild type NEK6, and kinase-dead NEK6(D183A) in the pEBG2T vector were used to express these enzymes with N-terminal glutathione S-transferase (GST) tag. The GST fusion proteins were expressed in human embryonic kidney 293 cells. For the expression of each construct, 40 10-cm-diameter dishes of 293 cells were cultured, and each dish was transfected with 10 μg of the pEBG-2T construct, using a modified calcium phosphate method (19). For PKBα, SGK, and kinase-dead NEK6 constructs, 24 h post-transfection, the cells were deprived of serum for 16 h and then lysed in 1 ml of ice-cold lysis buffer. The lysates were pooled and centrifuged at 4°C for 10 min at 13,000 × g, and the GST fusion proteins were purified by affinity chromatography on glutathione-Sepharose and eluted in buffer A containing 20 mM glutathione and 0.27 M sucrose, as described previously for PKBα (16). Wild type and kinase-dead NEK6 were expressed and purified in a similar manner except that the 293 cells were not deprived of serum. Typically between 0.3 and 0.5 mg of each GST fusion protein was obtained, and each protein was more than 90% homogeneous as judged by SDS-polyacrylamide gel electrophoresis (data not shown). Mitogen-activated protein kinase-activated protein kinase-2 was expressed as a GST fusion protein in Escherichia coli, purified, and activated by phosphorylation with extracellular signal-regulated kinase 2 as described previously (19).
Baculovirus Expression of NEK6—In order to generate transfer vectors for baculovirus production, the NEK6 cDNA SpeI-SpeI insert fragments derived from the pCR2.1-Topo vector were subcloned into the EcoRI site of pFastBAC-Hta vector (Invitrogen). The resulting construct encodes FLAG-NEK6 with an N-terminal hexahistidine tag and was then used to generate recombinant baculovirus using the Bac-to-Bac system (Invitrogen) following the manufacturer’s protocol. These baculoviruses were used to infect Spodoptera frugiperda 21 cells (1.5 × 10^6/ml) at a multiplicity of infection of 5. The infected cells were harvested 72 h postinfection, and His-tagged protein was purified as previously described for His-PDK1 (20); dialyzed into 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 150 mM NaCl, 50% glycerol, 0.05% Brij-35, 0.07% 2-mercaptoethanol, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride; and stored at −20 °C. NEK6 was purified with yields of ~5 mg/liter of infected cells and was greater than 85% homogeneous as judged by densitometric scanning of a Coomassie Blue-stained SDS-polyacrylamide gel.

Mapping the Sites on p70 S6K, SGK, and PKB Phosphorylated by NEK6—The wild and mutant AGC kinases (4 μg) were incubated with wild type NEK6 (2 μg in Fig. 2 and 1 μg in Fig. 1) in buffer A containing 10 mM magnesium acetate, 100 μM [γ-^32P]ATP (10^4 cpm/pmol), and 1 μM microcystin-LR in a total reaction volume of 30 μl. After the indicated times, the reactions were terminated by adding SDS to a final concentration of 1% (w/v) and dithiothreitol to 10 mM and heated at 100 °C for 1 min. After cooling, 4-vinylpyridine was added to a concentration of 50 mM, and the samples were left on a shaking platform for 30 min at room temperature in the dark to alkylate cysteine residues and then subjected to electrophoresis on a BisTris 4-12% polyacrylamide gel. The gels were stained with Coomassie Blue R250 and autoradiographed, and the bands corresponding to phosphorylated AGC kinases were excised and cut into smaller pieces. These were washed sequentially for 15 min on a vibrating platform with 1 ml of the following: water, a 1:1 mixture of water and acetonitrile, 0.1 M ammonium bicarbonate, a 1:1 mixture of 0.2 M ammonium bicarbonate and acetonitrile, and finally acetonitrile. The ^32P-labeled peptides that eluted at fractions 112 (termed peptide P_A1), 171 (termed peptide P_A2), 182 (termed peptide P_A3), 185 (termed peptide P_A4), 192 (termed peptide P_A5), and 203 (termed peptide P_A6) are indicated. No radioactivity was detected in fractions 1-100 and 220-240 (results not shown). Similar results were obtained in two separate experiments. Aliquots of peptides P_A1 (B), P_A2 (C), and P_A6 (D) were sequenced by Edman degradation using an Applied Biosystems 476A protein sequencer. ^32P radioactivity released after each cycle was measured in a separate experiment by solid-phase Edman degradation of the peptides coupled to a Sequelon-AA membrane (Milligen) as described previously (21). 2011 cpm (B), 3185 cpm (C), and 5753 cpm (D) were coupled to the membrane, and following the indicated cycles of Edman sequencing 972 cpm (B), 541 cpm (C), and 771 cpm (D) remained attached to the membrane. The ~3 Leu residues N-terminal to the phosphorylation sites are marked with an asterisk. The numbering of amino acid residues is based on the accession number AAA42104.
Identification of tryptic peptides isolated from S6K1 and SGK1 phosphorylated by NEK6 in vitro

The peptides isolated from Figs. 2 and 3 were analyzed on a PerSeptive Biosystems (Framingham, MA) Elite STR MALDI-TOF mass spectrometer in the linear and reflective mode, using 10 mg/ml α-cyanoanidinic acid as the matrix. Peptides P\textsubscript{A1}, P\textsubscript{A2}, P\textsubscript{A3}, and P\textsubscript{A4} were not present in sufficient amounts for their masses to be measured by MALDI-TOF mass spectrometry. For peptide P\textsubscript{B2}, the spectra were collected in the negative ion mode. The theoretical masses shown are for the monoisotopic masses. PO\textsubscript{4}, phosphate group.

| Peptide          | Residues   | Modification | Mass       |
|------------------|------------|--------------|------------|
| P\textsubscript{A} (S6K1 C T) | 359–421    | PO\textsubscript{4} (2) | 3890.00    |
| P\textsubscript{B} (S6K1 C T) | 359–421    | PO\textsubscript{4} (2) | 3810.10    |
| P\textsubscript{B} (SGK1)     | 368–383    | PO\textsubscript{4} (1) | 1817.77    |
| P\textsubscript{B} (SGK1)     | 415–441    | PO\textsubscript{4} (1) | 4928.40    |

**FIG. 3.** Mapping the sites on SGK1 phosphorylated by NEK6. A, full-length SGK1 (upper panel), SGK1 NT (middle panel), and SGK1 NT/S422A (lower panel) that had been phosphorylated in vitro as described under “Experimental Procedures” were digested with trypsin and chromatographed on a Vydac 218TP54 C\textsubscript{18} column as described for Fig. 1. Typically, >85% of the \textsuperscript{32}P radioactivity applied to the column was collected in the eluted fractions. The two major \textsuperscript{32}P-labeled peptides eluted at fractions 138 (termed peptide P\textsubscript{B1}, and 201 (termed peptide P\textsubscript{B2}) are indicated. No radioactivity was detected in fractions 1–100 and 220–240 (results not shown). Similar results were obtained in two separate experiments. Aliquots of peptide P\textsubscript{B1} and peptide P\textsubscript{B2} were digested together and following the indicated cycles of Edman sequencing 142 cpm (B) and 2383 cpm (C) remained attached to the membrane. The residue located 3 residues N-terminal to the phosphorylation site is marked with an asterisk.

**TABLE I**

**Identification of tryptic peptides isolated from S6K1 and SGK1 phosphorylated by NEK6 in vitro**

| Peptide          | Residues | Modification | Mass       |
|------------------|----------|--------------|------------|
| P\textsubscript{A} (S6K1 C T) | 359–421    | PO\textsubscript{4} (2) | 3890.00    |
| P\textsubscript{B} (S6K1 C T) | 359–421    | PO\textsubscript{4} (2) | 3810.10    |
| P\textsubscript{B} (SGK1)     | 368–383    | PO\textsubscript{4} (1) | 1817.77    |
| P\textsubscript{B} (SGK1)     | 415–441    | PO\textsubscript{4} (1) | 4928.40    |

platform for 10 min. The supernatant was removed, and the gel pieces were further washed for 10 min in 0.5 ml of 50 mM ammonium bicarbonate and 0.1% (by volume) trifluoroacetic acid. The combined supernatants containing >90% of the \textsuperscript{32}P radioactivity were chromatographed on a Vydac 218TP54 C\textsubscript{18} column as described for Fig. 1. Typically, >85% of the \textsuperscript{32}P radioactivity applied to the column was collected in the eluted fractions. The two major \textsuperscript{32}P-labeled peptides eluted at fractions 138 (termed peptide P\textsubscript{B1}, and 201 (termed peptide P\textsubscript{B2}) are indicated. No radioactivity was detected in fractions 1–100 and 220–240 (results not shown). Similar results were obtained in two separate experiments. Aliquots of peptide P\textsubscript{B1} and peptide P\textsubscript{B2} were digested together and following the indicated cycles of Edman sequencing 142 cpm (B) and 2383 cpm (C) remained attached to the membrane. The residue located 3 residues N-terminal to the phosphorylation site is marked with an asterisk.

**Phosphopeptide Sequence Analysis**—Peptides were analyzed by MALDI-TOF mass spectrometry on a PerSeptive Biosystems Elite-STR mass spectrometer using α-cyanoanidinic acid as the matrix. Spectra were obtained in both the linear and reflector mode. Edman sequencing on an Applied Biosystems 494A sequenator also confirmed the sequence identity of certain peptides. The site of phosphorylation of all of the peptides was determined by solid-phase Edman degradation, and the \textsuperscript{32}P radioactivity released after each was measured as described in the legend to Fig. 1. 2251 cpm (B) and 5379 cpm (C) were coupled to the membrane, and following the indicated cycles of Edman sequencing 142 cpm (B) and 2383 cpm (C) remained attached to the membrane. The residue located 3 residues N-terminal to the phosphorylation site is marked with an asterisk.

**Phosphopeptide Residues Modification**—Phosphopeptide sequence analysis

**Immunoblotting**—Immunoblotting with the anti-phospho-Thr\textsuperscript{413} S6K phosphospecific antibodies (1:2000 dilution of stock antibody), anti-S6K1 (0.2 μg/ml), and anti-SGK1 (0.2 μg/ml) was performed overnight at 4 °C in 50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 0.5% (by volume) Tween 20 (TBS-Tween) containing 10% (by mass) skimmed milk. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reagent (Amerham Biosciences).

**Array Production**—For substrate identification, peptide arrays termed Jerini pepSTAR were employed. Peptides for the arrays were synthesized on cellulose membranes in a parallel manner using SPOT technology (22, 23). Following side chain deprotection, the solid phase bound peptides were transferred into 96-well microtiter filtration plates (Millipore Corp., Bedford, MA) and treated with 0.1% (by volume) triethylamine for 1 h in order to cleave the peptides from the cellulose. After filtration, the triethylamine solution was removed by lyophilization and treated with 75% (by volume) Me\textsubscript{2}SO in aqueous acetate buffer (0.2 M, pH 4). The peptides were deposited to aldehyde functionalized glass slides (Quantifilm, Jena, Germany) using a noncontact piezoelectric driven pipetting system as described by the manufacturer (GESIM, Großerkmansendorf, Germany). For each peptide spotted onto the glass slide, four droplets of 0.5 ml were sequentially deposited. Each spot was separated by 0.7 mm and possessed a diameter of 550 μm. Finally, the peptides were covalently immobilized to the glass slide surface via an N-terminal spacer element, composed of a moiety that is able to react chemoselectively with the aldehyde groups at the glass slides and a β-alanine spacing unit. Two types of pepSTAR arrays were employed in
**NEK6 Substrate Specificity**

**FIG. 4.** Identification of a NEK6 peptide substrate using the Jerini pepSTAR method. A Jerini pepSTAR chip containing peptide sequences derived from 710 phosphorylation sites found in different human proteins was incubated with NEK6 and [γ-32P]ATP as described under “Experimental Procedures.” The chip was phosphorimaged, and the position of the major phosphorylated peptides is marked by a dotted circle. The amino acid sequences of the peptides found to be phosphorylated are indicated. The Ser or Thr residues, which are located 3 residues C-terminal to a Leu or Phe, which are potential sites of NEK6 cated 3 residues C-terminal to a Leu or Phe, which are potential sites of NEK6 phosphorylation, are indicated in boldface type and underlined. A representative result of two separate experiments in which three triplicate chips were analyzed is shown. NR-1, glutamate (N-methyl-D-aspartate) receptor subunit (1: Rbr-2, retinoblastoma-like protein 2; CK-6F, keratin type II cytoskeletal 6F protein; CAC, cell adhesion kinase; MEKK5, mitogen-activated protein kinase kinase kinase-5; PLEK, leukocyte tyrosine kinase receptor precursor.

Peptide Array Assay—Two peptide chips were layered face to face to each other, with a polypropylene spacer of 0.2-mm thickness placed at both ends. The resulting space between them was filled with a 0.3-ml solution containing 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 30 mM MgCl₂, 4 mM dithiothreitol, 2 mM EGTA, 100 mM [γ-32P]ATP (500 cpm/pmol), and 3 μg of His-NEK6. After a 60-min incubation at 30 °C, the peptide microarrays were washed three times, alternating between water and 0.1 M phosphoric acid. The phosphorylated peptides were detected following phosphorimaging on a FLA-3000 phosphor imager (Fuji, Japan) with a resolution of 50 μm. The evaluation of the data was performed using ArrayPro software (Media Cybernetics, Silver Spring, MD).

**Peptide Synthesis**—Peptides were synthesized using fluorenylmethoxycarbonyl-protected amino acids on rink amide resin (24). A 20% solution of piperidine in dimethylformamide was used for deprotection of resin-bound fluorenylmethoxycarbonyl-conjugated peptides. Final deprotection and resin cleavage was performed using 98% (by volume) trifluoroacetic acid for 1 h. Peptides were precipitated in methyl tert-butyl ether. Following filtration, peptides were lyophilized and dissolved in 30% acetonitrile in water. Peptides were then purified using a preparative high pressure liquid chromatography (Merck; column: 25 × 250 mm, LiChrosorb, 7 μm, RP18, Merck) and aqueous acetonitrile containing 0.1% trifluoroacetic acid. The purity and identity of all peptides was checked by high pressure liquid chromatography and mass spectrometry, respectively. In all cases, purity exceeded 95%.

**NEK6 Peptide Kinase Assay**—A 40-μl reaction mixture was prepared containing 200 ng of His-NEK6 in 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1 mM N-ethylmaleimide, and 0.5 μM microcystin-LR, and the indicated concentration of peptide substrate. After incubation at 30 °C for 5 min, the reaction was initiated by the addition of 10 μl of 50 mM magnesium acetate and 0.5 mM [γ-32P]ATP (500 cpm/pmol). After incubation for 5–15 min at 30 °C, incorporation of [32P]phosphate into each peptide substrate was determined using P81 phosphocellulose paper, as described previously (25). Time course reactions were performed in order to ensure that the rate of phosphorylation was linear with time. Kinetic data were analyzed according to the Michaelis-Menten relationship by nonlinear regression using the computer program GraphPad Prism (GraphPad Software Inc., San Diego, CA).

**S6K1 and SGK1 Activity Assay**—For the data shown in Figs. 7 and 8, 50 μg of 293 cell lysate were used to affinity-purify on glutathione-Sepharose the wild type and mutant forms of GST-S6K1 or GST-SGK1. The lysates were incubated at 4 °C for 1 h on a shaking platform with 10 μl of glutathione-Sepharose beads. The beads were washed twice with 1 ml of lysate buffer containing 0.5 M NaCl and twice with 1 ml of Buffer A. The assay (50-μl total volume) in addition to the washed glutathione-Sepharose beads contained 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 20 mM glutathione, 2.5 μM protein kinase A inhibitor (TMYADPIASGRGRRNAHID), 10 mM magnesium...
acetate, 0.1 mM $[^{32}P]$ATP (500 cpm/pmol), and 30 nM Crosstide (GRPRTSSFAEG) as the S6K1/SGK substrate. The assays were carried out for 15 min at 30°C, with the assay tubes being agitated continuously, and then terminated and analyzed as described previously for extracellular signal-regulated kinase 2 kinase assays (25). 1 milliunit of activity is the amount of enzyme that catalyzes the phosphorylation of 1 pmol of Crosstide in 1 min.

RESULTS
Phosphorylation S6K1, SGK1, and PKB by NEK6 in Vitro — We first tested the initial rate at which NEK6 phosphorylates S6K1, SGK1, and PKB (Fig. 1). As reported previously (11), wild type NEK6, but not a catalytically inactive NEK6 mutant, phosphorylated S6K1. We were able to phosphorylate S6K1 to a maximal stoichiometry of 0.5 mol of phosphate/mol of protein. SGK1 was phosphorylated at about half of the initial rate of S6K1, and the stoichiometry reached after 40 min of incubation was 0.3 mol of phosphate/mol of protein. In comparison, the initial rate at which PKB was phosphorylated by NEK6 was very poor, and phosphorylation of PKB was not affected by the inclusion of lipid vesicles containing PtdIns(3,4,5)P3 (Fig. 1). However, in a parallel experiment, PKB was still phosphorylated to a stoichiometry of 0.7 mol of phosphate/mol of protein (data not shown) by 50 units/ml mitogen-activated protein kinase-activated protein kinase-2, which phosphorylates PKB exclusively at Ser473 (26).

Mapping the Residues in S6K1 Phosphorylated by NEK6 — Full-length S6K1, S6K1 CT, or S6K1 CT(T412A) (Fig. 2A) was phosphorylated with NEK6, digested with trypsin, and chromatographed on a C18 column. For the full-length S6K1 and S6K1 CT, two major 32P-labeled peptides (PA4 and PA6) and four more minor peptides (PA1, PA2, PA3, and PA5) were recovered (Fig. 2A). A combination of MALDI-TOF mass spectrometry and solid phase and gas phase Edman sequencing was used to identify the sites of phosphorylation in these proteins. Peptide PA4 was identified by solid phase and gas phase Edman sequencing (Fig. 2B) as the phosphopeptide comprising residues 20–71 phosphorylated at Ser53. Peptide PA5 comprised residues 389–422 of full-length S6K1 or 389–421 of the S6K1 CT, which terminates at this residue. Peptide PA5 was phosphorylated at two major sites, Thr403 and Thr412 (the hy-
drophobic motif site) (Fig. 2C and Table I). Consistent with this analysis, Peptide PA5 was not present when S6K1/CT(T412A) was phosphorylated with NEK6 (Fig. 2A, lower panel). Peptide PA6 comprises the same peptide as PA5 except that it was phosphorylated at Thr403 but not Thr412 (Fig. 2D and Table I). In addition, the possibility that PA5 and PA6 are also phosphorylated at low stoichiometry at Ser394 and Ser398 cannot be ruled out (Fig. 2, C and D).

Mapping the Residues in SGK1 Phosphorylated by NEK6—Full-length SGK1, SGK1/NT, and SGK1/NT(S422A) (Fig. 3A) were phosphorylated with NEK6, digested with trypsin, and chromatographed on a Vydac 218TP54 C18 column as described in the legend for Fig 1. The position of peptide P_A5 containing the hydrophobic motif site is marked with an asterisk, and the position of the Ser377- and Ser422-containing 32P-labeled peptides is also indicated. In a parallel experiment, 0.5 μg of GST fusion of wild type S6K1/CT or the indicated mutants were phosphorylated with 0.2 μg of NEK6 and immunoblotted with an antibody that recognizes the phosphorylated hydrophobic motif of S6K1 (termed T412-P) or an anti-S6K1 antibody raised against the S6K1 protein. B, as above except that GST fusion proteins of wild type SGK1/NT and the indicated mutants replaced S6K1/CT. The position of the Ser377- and Ser422-containing 32P-labeled peptides is indicated. For phosphoimmunoblotting of the hydrophobic motif of SGK1(S422-P), we employed the T412-P S6K1 antibody, which cross-reacts with the phosphorylated Ser422 (see “Results”).

As mentioned above, PKBα was poorly phosphorylated by NEK6. Consistent with this, only three minor 32P-labeled peptides were recovered, which were not present in sufficient amounts to enable their identification (data not shown).

Analysis of the Substrate Specificity of NEK6 Employing the Jerini pepSTAR Method—Peptide chips containing 710 different peptides derived from annotated phosphorylation sites in human proteins were incubated with NEK6 and [γ-32P]ATP as described under “Experimental Procedures.” In Fig. 4, we show a representative result that was obtained and list the sequences of the major peptides that were identified as substrates.

The peptide GLAKSFGSPNRAY derived from the T-loop of cyclin-dependent protein kinase-7 (CDK7) appeared to be the peptide phosphorylated most efficiently by NEK6 (Fig. 4) and was therefore selected for further analysis. In cells, CDK7 is activated by phosphorylation of the Ser residue corresponding to the second serine in the GLAKSFGSPNRAY (27). In contrast, NEK6 phosphorylates the first serine of this peptide, since its mutation to Ala abolishes NEK6 phosphorylation, while mutation of the second serine to Ala has no major effect (Fig. 5A).
FIG. 7. Activation and hydrophobic motif phosphorylation of −3 Leu mutants of S6K1 in IGF1-stimulated 293 cells. A, GST-S6K1ΔCT wild type or the indicated mutants were expressed by transient transfection in 293 cells. 24 h after transfection, cells were serum-starved for 16 h and pretreated for 30 min with (+) or without (−) 100 nM rapamycin and then either left unstimulated or stimulated with 50 ng/ml IGF1 for 40 min. GST-S6K1 proteins were affinity-purified on glutathione-Sepharose and assayed as described under “Experimental Procedures.” The activities are presented as means ± S.D. for three separate experiments. Cell lysates (20 μg) were also immunoblotted with either the T412-P or the anti-S6K1 antibody. B, 293 cells transiently transfected as above and either left unstimulated or stimulated with 50 ng/ml IGF1 for the indicated times. Cell lysates (20 μg) were immunoblotted with either the T412-P or the anti-S6K1 antibody. Similar results were obtained in two separate experiments.

In Table II, we align the sequences encompassing Ser53, Ser103, and Thr312 of S6K1, Ser177 and Ser422 of SGK1, and the CDK7 T-loop peptide, which are phosphorylated by NEK6. A striking feature of this alignment is that, apart from the Ser177 residue in SGK1, all of the other phosphorylated residues possess a Leu residue located 3 residues N-terminal to the site of phosphorylation by NEK6. Moreover, several (but not all) of the peptides identified in the Jerini pepSTAR screen as NEK6 substrates (Fig. 4) also possessed a Leu residue 3 residues N-terminal to a potential Ser or Thr residue phosphorylation site. This Leu is hereafter termed the −3 Leu. We also performed a Jerini pepSTAR screen with a Chip containing peptides derived from histone H1 and MBP (data not shown). This screen revealed one major phosphorylated peptide, namely GKRGGLSLSRFSW, which is derived from MBP and also possesses a −3 Leu N-terminal to the likely NEK6 phosphorylation site (underlined).

To determine the importance of the −3 Leu residue in enabling NEK6 to phosphorylate the CDK7 T-loop peptide, we mutated it to either Ile or Val and found that this severely reduced the efficiency at which NEK6 could phosphorylate these peptides. Kinetic analysis indicates that mutation of the −3 Leu reduces the apparent Vmax by over 10-fold without altering the apparent Km significantly (Fig. 5A).

We next generated another Jerini pepSTAR chip (termed the “substitution chip”), in which each residue of the peptide GLAKSFGSPNRAY was mutated to every other proteinogenic amino acid residue and then retested for NEK6 phosphorylation (Fig. 5B). This analysis provided a qualitative indication of the overall substrate specificity requirements of NEK6. It confirmed that the first Ser in the peptide was the major phosphorylation site and suggests that Leu as well as Phe, Trp, or Tyr are the preferred residues at the −3 position. Relevant to this observation, a NEK6-phosphorylated peptide, FGMSRNLYAGDYY, isolated from the Jerini pepSTAR screen of the 710 peptide chip (Fig. 4) possesses a Phe 3 residues N-terminal to a Ser residue (underlined). The screen performed with the peptide variants of GLAKSFGSPNRAY also indicates that NEK6 has a strong preference for an aromatic residue at the −4- and +1-positions and does not tolerate a Pro residue at the +1-position, demonstrating that NEK6 is not a proline-directed kinase (Fig. 5B).

To further investigate the substrate specificity requirements of NEK6, we next studied the kinetic parameters at which NEK6 phosphorylated variants of the GLAKSFGSPNRAY peptide. Changing the −4 Gly in GLAKSFGSPNRAY to Phe increased the apparent Vmax −3-fold without affecting the apparent Km (Fig. 5C). Changing the −1 Lys in GLAKSFGSPNRAY to a Phe residue, which is found at the equivalent position of the hydrophobic motif of S6K1 and SGK1, reduced the apparent Vmax value at which NEK6 phosphorylated this peptide almost 4-fold. Although the results presented in Fig. 5B indicate that the residue at the −1-position does not influence NEK6 substrate specificity greatly, the kinetic data indicate that a Phe at this position is unfavorable. Consistent with a strong preference of NEK6 for an aromatic residue at the +1-position observed in the substitution screen (Fig. 5B), changing the +1 Phe in GLAKSFGSPNRAY to either Gly or Ala decreased the apparent Vmax −7-fold and moderately increased Km, whereas changing the +1 Phe to Trp moderately increased the Vmax and reduced the Km (Fig. 5C). NIMA was reported to have strong preference for an Arg residue at the −2
position (28, 29); however, the substitution Jerini pepSTAR chip screen performed did not indicate that an Arg was significantly preferred at the −2-position for NEK6 (Fig. 5B). Consistent with this, changing the −2 Ala in GLAKSGFGSPNRAY to Arg had no major effect on the catalytic efficacy at which this peptide was phosphorylated by NEK6 (Fig. 5C). Interestingly, the substitution performed by Jerini pepSTAR chip screen indicated that changing the +8 Tyr of GLAKSGFGSPNRAY to an acidic residue was unfavorable for its phosphorylation by NEK6 (Fig. 5B). We therefore studied the kinetic parameters by which NEK6 phosphorylated the peptide in which the +8 Tyr of GLAKSGFGSPNRAY was changed to Glu and found that the $V_{\text{max}}$ was reduced 2-fold and that the $K_m$ was increased 2-fold, confirming that an acidic residue at this quite distant position from the site of phosphorylation is not favored.

Effect of Mutation of the −3 Leu on the Phosphorylation of the Hydrophobic Motif of S6K1 and SGK1—In order to assess whether the −3 Leu was also required for the phosphorylation of protein substrates by NEK6, we mutated this residue in the hydrophobic motif of S6K1 or SGK1 to either Ile or Val and tested how this affected phosphorylation by NEK6. The S6K1(L409I) and S6K1(L409V) mutants were not detectably phosphorylated at their hydrophobic motif by NEK6, as judged by either tryptic phosphopeptide mapping or by using a hydrophobic motif phosphospecific antibody (Fig. 6A). As expected, however, the S6K1(L409I) and S6K1(L409V) mutants were phosphorylated to the same extent as wild type S6K1 at Ser$^{422}$ and Ser$^{405}$. Similarly, mutant forms of SGK1 in which the −3 Leu residues were mutated (SGK1[L419I] and SGK1[L419V]) were not detectably phosphorylated at their hydrophobic motif by NEK6 under conditions where NEK6 still phosphorylated Ser$^{377}$ to the same extent as wild type SGK1 (Fig. 6B).

NEK6 Is Not Rate-limiting for the Phosphorylation of the Hydrophobic Motif of S6K1 and SGK1 in Vivo—We next tested whether the −3 Leu hydrophobic motif mutants of S6K1 (Fig. 7) and SGK1 (Fig. 8), when expressed in 293 cells, became phosphorylated at their hydrophobic motifs and became activated in response to IGF1. In Fig. 7A, we demonstrate that a 40-min IGF1 stimulation induced an ~5-fold activation of wild type S6K1, S6K1(L409I), and SGK1(L409V). Activation was accompanied by a similar phosphorylation of these enzymes at their hydrophobic motif (Thr$^{112}$). Moreover, rapamycin, a known inhibitor of S6K activation (4), prevented IGF1-mediated hydrophobic motif phosphorylation and activation of wild type S6K1, S6K1(L409I), and SGK1(L409V). As expected, a mutant form of S6K1 in which the hydrophobic motif phosphorylation site was changed to Ala (S6K1[T412A]) was not activated by IGF1 and not recognized by the S6K hydrophobic motif phosphospecific antibody (Fig. 7A). We also compared the rate of phosphorylation of wild type S6K1 and S6K1(L409I) at Thr$^{112}$ in IGF1-stimulated cells and found both the wild type and mutant S6K1 to be phosphorylated at the same rate (Fig. 7B).

Stimulation of 293 cells with IGF1 for 10 min induced a ~5-fold activation of wild type SGK1, SGK1(L419I), and SGK1(L419V), which was accompanied by a similar phosphorylation of Ser$^{422}$ in their hydrophobic motif (Fig. 8A). The phosphatidylinositol 3-kinase inhibitor wortmannin prevented the IGF1-induced activation and hydrophobic motif phosphorylation of wild type SGK1, mutant SGK1(L419I), and SGK1(L419V). In order to detect the hydrophobic motif phosphorylation of SGK1, we employed a commercial phosphospecific antibody raised against the S6K1 hydrophobic motif, which we found to also cross-react with SGK1 phosphorylated
at its hydrophobic motif. Consistent with this antibody recognizing Ser422 of SGK1 specifically, a mutant SGK1(S422A) was not recognized by this antibody in IGF1-stimulated cells (Fig. 8A). Wild type SGK1 and mutant SGK1(L419I) were phosphorylated at the same rate at Ser422 in IGF1-stimulated cells (Fig. 8B).

**DISCUSSION**

Entrance into mitosis in *Aspergillus nidulans* requires the activation of the NIMA serine/threonine kinase, whereas exit from mitosis requires its proteolytic destruction (30, 31). To date, eight different NIMA-related kinases have been reported in mammals (NEK1 to -8), but the functions of these enzymes are poorly understood, and, to our knowledge, none have thus far been shown to perform mitotic functions related to those of NIMA in *A. nidulans*. NEK6 was first identified by Kandil et al. (32) searching for novel mammalian NIMA-related family members. It possesses 313 amino acids and is most closely related to NEK7 (32). Although one study indicated that the mRNA for NEK6 is widely expressed in human tissues (32), Avruch and colleagues (11) reported that NEK6 mRNA levels were vastly higher in mouse liver than other tissues investigated. This may explain why these authors purified NEK6 as the major biochemical activity detectable in rat liver extracts that phosphorylates the hydrophobic motif of S6K1.

NEK6 was originally reported to phosphorylate S6K1 (T412A) to ~80% of the level observed with wild type S6K1 (11), indicating that NEK6 phosphorylates other residues in S6K1. Analysis of the peptides phosphorylated by NEK6 (Fig. 2), performed in the present study has confirmed this and identified two novel sites on S6K1 (Ser53 and Ser403) as major sites of NEK6 phosphorylation. Ser422 is located N-terminal to the kinase domain, Ser403 is immediately C-terminal to the kinase domain, and neither residue has previously been reported to be phosphorylated. Following phosphorylation of S6K1 by NEK6, the peptide containing the hydrophobic motif residue (peptide P3, Fig. 2), is a minor 32P-labeled peptide and is also phosphorylated at Ser485. In contrast, we demonstrate for the first time that NEK6 phosphorylates SGK1 efficiently at its hydrophobic motif in *vivo*, and peptide-mapping analysis indicates that this is the major site of phosphorylation (Fig. 3). Ser477 is a more minor site of phosphorylation located in the kinase domain of SGK1, which has not been reported to undergo phosphorylation previously.

One of the central findings in this study was the observation that mutating the Leu located 3 residues N-terminal to the hydrophobic motif of S6K1 and SGK1 prevented NEK6 from phosphorylating these enzymes at their hydrophobic motifs (Fig. 6). We exploited this finding to demonstrate that mutants of S6K1 and SGK1, in which the -3 Leu N-terminal to the hydrophobic motif phosphorylation site is changed to either Ile or Val, are as efficiently phosphorylated at their hydrophobic motifs and activated by IGF1 just as efficiently and at the same rate as the wild type enzymes (Figs. 7 and 8). These results strongly indicate that NEK6 itself is not rate-limiting for the hydrophobic motif phosphorylation of S6K1 and SGK1 in cells under conditions we have examined so far. We have not analyzed the substrate specificity requirements of NEK7, which was also reported to phosphorylate S6K1 at its hydrophobic motif, albeit at a much lower rate than NEK6 (11). However, since this isoform possesses 87% identity to NEK6 in the predicted kinase domain, it is likely to have substrate specificity requirements similar to those of NEK6.

The only other protein kinase demonstrated to phosphorylate the hydrophobic motif of S6K1 in *vivo* is the mammalian target of rapamycin (mTOR) (33–35). However, a rapamycin-insensitive mutant of S6K1 lacking the N-terminal 46 residues is still phosphorylated at its hydrophobic motif in IGF1- and rapamycin-treated cells under conditions where mTOR is inactive (36). This observation indicates that a protein kinase(s) distinct from mTOR may phosphorylate S6K1 at this residue in *vivo*. It will be interesting to investigate whether mTOR or other protein kinases that phosphorylate the hydrophobic motif of S6K1, can phosphorylate S6K1(L409I) and S6K1(L409V) as efficiently as wild type S6K1.

The present study is the first report of a protein kinase (NEK6) capable of phosphorylating the hydrophobic motif of SGK1, although our data suggest that NEK6 may not mediate this reaction in cells. Nevertheless, the phosphorylation of the hydrophobic motif of SGK1 in *vivo*, coupled with the phosphorylation of the T-loop with PDK1, may be a useful way of generating fully active wild type SGK1.

In this study, we describe the use of a technology termed the Jerini pepSTAR method that employs peptide arrays that have been immobilized on glass chips to investigate the substrate specificity determinants of protein kinases. Using this methodology, in the first round of screening, around 1000 peptides (including the MBP and histone peptides) were initially tested as potential substrates for NEK6 in just a few hours. About 10 peptides were observed to become phosphorylated significantly by NEK6 in this analysis. We synthesized one of these peptides derived from the T-loop of the CDK7 protein kinase that appeared to be the most efficient substrate and found that it was phosphorylated in *vivo* with an apparent $K_m$ of 325 μm and a $V_{max}$ of 80 units/mg. Using this substrate, we were able to demonstrate that mutation of the -3 Leu to Ile or Val severely reduced the efficacy at which the peptide was phosphorylated by NEK6 and established that this was due to a reduction in the apparent $V_{max}$ but not the $K_m$ (Fig. 5A). This mutation is therefore affecting the rate of catalysis rather than substrate binding.

In order to learn more about the substrate specificity requirements of NEK6, we generated a secondary NEK6 peptide chip, termed a Jerini pepSTAR substitution chip, containing all combinations of amino acids at each position of the CDK7 T-loop peptide. The results from such an experiment provide an overall qualitative picture of the substrate specificity determinants of NEK6. The results indicate that NEK6 prefers an aromatic residue at the -4-position and +1-position, as is found in the hydrophobic motif of S6K1 and SGK, together with the -3 Leu (Fig. 5B). These findings were confirmed more quantitatively by performing kinetic analysis on variant forms of the CDK7 NEK6 peptide substrate (Fig. 5C). These findings presumably explain why NEK6 is capable of phosphorylating the hydrophobic motifs of S6K1 and SGK1 in *vivo*. The hydrophobic motif of PKBα, like those of S6K1 and SGK1, possesses an aromatic residue at the -4- and +1-position. However, we found that PKBα, in comparison with S6K1 and SGK1, is phosphorylated very poorly by NEK6 (Fig. 1) and were unable to demonstrate phosphorylation of Ser477 (data not shown). PKBα possesses a Pro residue rather than the preferred Leu at residue -3 to Ser477, and the data generated by the Jerini pepSTAR method indicate that Pro is poorly tolerated at this position (Fig. 5B). Therefore, the presence of Pro at this position in PKBα is likely to account for the inability of NEK6 to phosphorylate PKBα efficiently at its hydrophobic motif.

The Jerini pepSTAR method is clearly a very useful way of rapidly characterizing the substrate specificity requirements of protein kinases as well as permitting the facile development of optimal peptide substrates for these enzymes. A major challenge is to identify physiological substrates for

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2 J. M. Lizcano, unpublished results.
NEK6 as well as other NEK family members. The description of the substrate specificity requirements of NEK6 described here may help to identify potential substrates for this enzyme in the same way that substrates for other protein kinases have been found through a knowledge of the substrate specificity determinants of these enzymes (reviewed in Ref. 37). The substrate specificity of NIMA was investigated previously (28, 29) and was found to have a strong preference for a Phe residue at the −3 position, although a Leu or Met were well tolerated (29). In contrast to NEK6, NIMA does not appear to have a marked preference for an aromatic residue at the −4 or +1 position (29). Furthermore, NIMA has a marked preference for an Arg residue at the −2 position (28, 29), but our analysis indicates that NEK6 does not possess such a preference (Fig. 5, B and C). Based on these observations, the key feature of the substrate specificities of the NIMA/NEK family of protein kinases, may be a marked preference for a large hydrophobic residue at the −3 position, although the preference of each family member for other residues may vary. It will be critical to establish in the future how mutation of the −3 hydrophobic residue affects the phosphorylation of substrates for NEK6 and other NEK family members to determine whether such proteins are physiological substrates.

Acknowledgments—We thank the Sequencing Service (School of Life Sciences, University of Dundee, Scotland) as well as Jane Leitch for preparation of antibodies and for DNA sequencing, respectively, and Jane Leitch for assistance for a large hydrophobic residue at the −3 position. The substrate specificity of NIMA was investigated previously (28, 29) and was found to have a strong preference for a Phe residue at the −3 position, although a Leu or Met were well tolerated (29). In contrast to NEK6, NIMA does not appear to have a marked preference for an aromatic residue at the −4 or +1 position (29). Furthermore, NIMA has a marked preference for an Arg residue at the −2 position (28, 29), but our analysis indicates that NEK6 does not possess such a preference (Fig. 5, B and C). Based on these observations, the key feature of the substrate specificities of the NIMA/NEK family of protein kinases, may be a marked preference for a large hydrophobic residue at the −3 position, although the preference of each family member for other residues may vary. It will be critical to establish in the future how mutation of the −3 hydrophobic residue affects the phosphorylation of substrates for NEK6 and other NEK family members to determine whether such proteins are physiological substrates.

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