A Mitotic Cascade of NIMA Family Kinases

Nercc1/Nek9 ACTIVATES THE Nek6 AND Nek7 KINASES*

Received for publication, April 8, 2003, and in revised form, June 29, 2003
Published, JBC Papers in Press, July 2, 2003, DOI 10.1074/jbc.M303663200

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The NIMA family of protein kinases in humans is composed of 11 members that share an amino-terminal catalytic domain related to NIMA, an Aspergillus kinase involved in the control of several aspects of mitosis, and divergent carboxyl-terminal tails of varying length. Nek6 (314AA) and Nek7 (303AA), 76% identical, have little noncatalytic sequence but bind to the carboxyl-terminal noncatalytic tail of Nercc1/Nek9, a NIMA family protein kinase that is activated in mitosis. Microinjection of anti-Nercc1 antibodies leads to spindle abnormalities and prometaphase arrest or chromosome missegregation. Herein we show that Nek6 is increased in abundance and activity during mitosis; activation requires the phosphorylation of Ser206 on the Nek6 activation loop. This phosphorylation and the activity of recombinant Nek6 is stimulated by coexpression with an activated mutant of Nercc1. Moreover, Nercc1 catalyzes the direct phosphorylation of prokaryotic recombinant Nek6 at Ser206 in vitro concomitant with 20–25-fold activation of Nek6 activity; Nercc1 activates Nek7 in vitro in a similar manner. Nercc1/Nek9 is likely to be responsible for the activation of Nek6 during mitosis and probably participates in the regulation of Nek7 as well. These findings support the conclusion that Nercc1/Nek9 and Nek8 represent a novel cascade of mitotic NIMA family protein kinases whose combined function is important for mitotic progression.

The NIMA family of protein kinases is named after the Aspergillus nidulans protein kinase encoded by the nima gene (1). Mutation of nima (never in mitosis) arrests cells in G2 without interfering with p44/42 (activation) (2), suggesting that the NIMA protein has a central role in the G2/M transition. Moreover, if the G2 arrest of nima mutants is bypassed by additional mutations, the resulting mitotic cells show aberrant spindle and nuclear envelope organization (3, 4), pointing to functions of NIMA beyond the control of mitotic entry. NIMA can induce chromatin condensation and nuclear membrane breakdown in mammalian cells as it does in Aspergillus (3, 5, 6), suggesting that these functions are also regulated by protein kinases with similar specificity in vertebrate cells. Eleven protein kinases with a catalytic domain related to NIMA have been identified in the human genome (7), and a substantial fraction were first described very recently (8–12). The functions of these NIMA family kinases, mostly referred to as Neks, are largely unknown. The best characterized of these kinases, Nek2, has been implicated in the regulation of the centrosome (13); Nek1 and Nek8 mutations have been related to cystic kidney disease (14, 15); Nek6/7 have been suggested to phosphorylate and activate p70 S6 kinase (16); and Nek9/Nercc1 has been implicated in the control of mitotic spindle formation and chromosome segregation (10).

Nek6 together with its close homolog, Nek7, were purified from rat liver as the predominant kinases capable of phosphorylating in vitro the hydrophobic regulatory site (Thr172) of the p70 S6 kinase in vitro (16). Recombinant Nek6 polypeptide is recovered as an active protein kinase after transient expression in HEK293 cells and activates coexpressed p70 S6 kinase in vivo (as well as directly in vitro) in a manner synergistic with PDK1; nevertheless, recent evidence indicates that Nek6 (and by extension Nek7) is not a physiologic activator of p70 S6 kinase (17), and thus its roles in cell regulation are unknown.

To gain insight into Nek6/7 regulation and function, we sought to uncover the mechanism underlying the activation of the Nek6/7 kinases and to define the regulation of the endogenous enzymes. We find that Nercc1, another NIMA family kinase previously shown to bind Nek6, phosphorylates directly a critical site on the activation loop of both Nek6 and Nek7 and activates these kinases in vitro and in vivo. Moreover, like Nercc1, endogenous Nek6 is activated in mitosis. The ability of Nercc1 to directly activate Nek6 points to the likely operation of a cascade of NIMA-related mitotic protein kinases.

EXPERIMENTAL PROCEDURES

Materials—Expand HiFidelity DNA polymerase, sequencing grade trypsin, and CompleteED protease inhibitor mixture tablets were purchased from Roche Applied Science. Protein A- and G-Sepharose and GSH-Sepharose were from Amersham Pharmacia Biotech, Piscataway, NJ. Anti-Myc (9E10) monoclonal antibody, anti-cyclin B1 and rabbit anti-p70 polyclonal antibody (C-18) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-β-tubulin was from Zymed Laboratories Inc.. LipofectAMINE, pcDNA3.1-Myc/His6 mammalian expression vector, and all cell culture media except phosphate-free Dulbecco’s modified Eagle’s medium (ICN) was from Invitrogen. Cellulose TLC plates were bought from E.M. Science. Rabbit polyclonal sera raised against keyhole limpet hemocyanin-coupled peptide were generated at Cocalico Biologicals (Reamstown, PA). [32P]orthophosphate and [γ-32P]ATP were obtained from PerkinElmer Life Sciences and ICN.

—John J. Avruch, Massachusetts General Hospital and the Leukemia and Lymphoma Society. Supported in part by the Research to cure Leukemia Program of the St. Vincent’s Institute of Medical Research, Fitzroy, Victoria 3065, Australia, and ¶¶Cell Signaling Technologies, Beverly, Massachusetts 01915.

* This work was supported in part by National Institutes of Health Grant DK17776. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

††Supported in part by the Fund for Medical Discovery from Massachusetts General Hospital and the Leukemia and Lymphoma Society.

‡‡A National Health and Medical Research Council Fellow and supported by the Australian Research Council.

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represents phosphoserine); anti-phospho-Ser206).

To be expressed in mammalian cells as a fusion protein with His 6 and complete protease inhibitor mixture (1 tablet/50 ml). Lysates were centrifuged at the initiator methionine by an optimum Kozak sequence.

This fragment was subcloned into pcDNA3.1 Myc/His 6, allowing Nek6 to be expressed in mammalian cells as a fusion protein with His 6 and Myc epitopes at the C terminus.

All site-directed mutant Nek6 variants in pcDNA3.1 Myc/His 6 were constructed by PCR-mediated overlap extension mutagenesis and subsequently subcloned into pcDNA3.1 Myc/His 6. All clones were verified by sequencing.

Generation of Rabbit Anti-Nek6 Antiserum—A synthetic peptide corresponding to the N-terminal sequence of mouse Nek6 (amino acids 2–15) conjugated to keyhole limpet hemocyanin was used to immunize rabbits in order to produce anti-total Nek6 antibodies. Phosphospecific antibodies were produced using peptides corresponding to Nek6 residues 194–204 (Cys-GRFFSSETpTAAH (where pT represents phosphothreonine); anti-phospho-Thr202) and 203 (Cys-GRFFSSETpTAAH (where pS represents phosphoserine); anti-phospho-Ser206).

Cell Culture, Expression, and Purification of Recombinant and Enogenous Proteins—Maintenance of HEK293 cells and H4-II-E-C3 cells was previously described (16). HELa cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% serum. HEK293 cells were transiently transfected using LipofectAMINE (according to the manufacturer’s protocol). Cells were lysed in lysis buffer or buffer A (20 mM Tris, pH 7.6, 2 mM EGTA, 1 mM EDTA, 5 mM MgCl 2, 20 mM β-glycerophosphate, 0.5% Triton X-100, 1 mM sodium orthovanadate, 1 mM dithiothreitol) supplemented with Calyculin A (50 nm) and Complete protease inhibitor mixture (1 tablet/ml). Lysates were centrifuged at 15,000 x g for 30 min at 4 °C.

To immunoprecipitate recombinant proteins, the corresponding antibodies prebound to protein A/G-Sepharose were incubated with the lysates at 4 °C. Immunocomplexes were washed and subsequently used.

GST-Nercc1 (732–979), Nek7 and Nek6 expression in the Escherichia coli strain BL21 DE3 pLys (Novagen) transformed with the pGEX KG expression vector was induced by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 16 h at 25 °C. The purification of this and other recombinant GST-tagged polypeptides from transfected mammalian cells was done using GSH beads using standard protocols.

In-gel Kinase Assay—In-gel kinase assay of Nek6 and Nek7 activity was according to Ref. 18. When indicated, 0.2 mg/ml MBP was included in the gel as a substrate.

MS Determination of P-sites—Gel bands were excised as digested with trypsin, and resultant peptides were extracted from the gel. An aliquot of peptide was loaded onto a fused silica (360 µm OD, 50 µm ID, PolyMicro) capillary column (18) (ODS) column with a pulled emitter tip (19). Peptides were HPLC gradient-eluted (0–100% B, where A = 0.1% trifluoroacetic acid and B = 70% CH 3CN and 0.1% trifluoroacetic acid) and analyzed by a LCQ DECA XP ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). The mass spectrometer was set to data-dependent mode to take MS/MS spectra of the top five most abundant m/z peaks in each MS scan. The MS/MS spectra were searched (Sequest; ThermoFinnigan, San Jose, CA) against a data base containing the protein in order to deduce the sequences. Potential post-translational modifications were also searched (STY = 80 (phosphorylation)).

An additional aliquot of each sample was subjected to immobilized metal affinity chromatography to enrich the sample for phosphorylated peptides. Each experiment was performed as previously described (20). Bacterial peptides were eluted onto a capillary immobilized metal affinity chromatography column. The column was washed to remove non-specific binding, and then the peptides were eluted onto a capillary 18 precolumn. The precolumn was washed with HPLC buffer and then connected to the analytical column described above. The peptides were eluted and analyzed as described above. Sequest peptide sequences were manually confirmed to ensure correct sequence identification.

Nek6 Kinase Assay—Immunoprecipitates of recombinant (anti-FLAG or anti-Myc) or endogenous Nek6 were first washed in the extraction buffer containing 0.5 M LiCl three times and twice in kinase buffer (50 mM MOPS, pH 7.4, 10 mM MgCl 2, 2 mM EGTA, 20 mM β-glycerophosphate). Kinase assays were performed in 30 µl of kinase buffer containing 1.5 µg of GST-p70 S6 kinase ΔCT104 (2522A) or MBP (0.2 mg/ml) and either radiolabeled ATP ([γ- 32P]ATP, 5 µCi/ml final concentration at 4000 cpm/ml). Reactions were incubated for 10 min at 30 °C and terminated by the addition of 4× SDS sample buffer. Kinase mixtures were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane, and Nek6 phosphotransferase activity was determined by excising Coomassie-stained bands corresponding to Nek6 and measuring radioactive content by Cerenkov scintillation counting.

Two-dimensional Tryptic Phosphopeptide Mapping—HEK293 cells expressing various Nek6-His 6/Myc constructs were incubated with phosphate-free Dulbecco’s modified Eagle’s medium containing 32P, for 4 h prior to harvest. The Myc immunoprecipitates were subjected to SDS-PAGE, fixation, and staining. The gel slice containing [ 32P]Nek6 was equilibrated in 50 mM ammonium bicarbonate buffer (pH 8.5), homogenized, and subjected to several rounds of tryptic digestion until at least 75% of initial [ 32P]Nek6 was extracted into the supernatant. The dried, salt-free digest was separated by thin layer electrophoresis at pH 1.9 followed by TLC as described previously (21). Plates were eluted using a PhosphoImager and assessed subsequently.

Quantitative PCR—Total RNA was extracted using TRIzol (Invitrogen), further purified using the Qiagen (Chatsworth, CA) RNeasy total RNA isolation kit according to the instruction manual, and quantified using Ribogreen (Molecular Probes, Inc., Eugene, OR). After DNase I treatment, quantitative RT-PCR was performed in duplicate using the Brilliant One-Step quantitative RT-PCR kit (Stratagene, La Jolla, CA) containing SYBR Green I (1:30,000, Sigma), forward and reverse primers (25 ng each), and sample RNA (1 µg). The nucleotide sequences of the primers used were as follows: Nek6-F (5′-CGAGAAGAGAATGCG-CGGAG-3′) and Nek6-R (5′-TGGACCTTCTCCGACCGACT-3′) with Nek6-specific product size of 89 bp and TATA box-binding protein (5′-TGAGGCCTCTCTTGAAGCC-3′) and TBP-R (5′-GACGCTATGAGCAACTCA-3′) with a TBP-specific product size of 101 bp. The thermal cycling conditions comprised an initial RT reaction step at 48 °C for 30 min and 40 cycles at 95 °C for 15 s and 65 °C for 1 min. Accumulation of PCR product was monitored in real time (Mx4000; Stratagene) with appropriate controls. A standard curve was generated for each target, and the amount of each mRNA relative to total RNA was determined using the crossing threshold (Ct) method; the amount of Nek6 RNA was divided by the amount of TBP RNA.

RESULTS

Nek6 Activation Requires Phosphorylation of Ser206 in the Activation Loop—Overexpressed wild type FLAG-Nek6 migrates as a doublet on SDS-PAGE (Fig. 1A), whereas the kinase inactive mutant, FLAG-Nek6 (K74M/K75M) migrates as a single band corresponding in mobility to the more rapidly migrating band of wild type Nek6 (16). Only the slower moving Nek6 band exhibits autophosphorylation in a washed immunoprecipitate, and only this upper band catalyzes MBP phosphorylation after renaturation in an “in gel” kinase assay (Fig. 1A). Treatment of mammalian recombinant Nek6 with protein phosphatase 2A in vitro abolishes Nek6 kinase activity and eliminates the slower migrating band of Nek6 polypeptide on SDS-PAGE (16). These features indicate that the activity of recombinant Nek6 is dependent on Nek6 polypeptide phosphorylation, probably catalyzed by another protein kinase.

Wild type FLAG-tagged Nek6, immunopurified after transient expression in HEK293 cells, was subjected to SDS-PAGE, and each Coomassie Brilliant Blue-stained band of the closely spaced FLAG-Nek6 doublet (Fig. 1B) was excised and digested with trypsin, and the digests were analyzed by liquid chromatography/MS/MS in order to identify sites of phosphorylation. The inactive, more rapidly migrating band yielded a single phosphopeptide surrounding Ser206 in the activation loop, whereas the peptides derived from the slower moving, active Nek6 band exhibited phosphorylation at Ser206, Thr202, and...
Ser\textsuperscript{37}, with the peptide segment encompassing Thr\textsuperscript{202}/Ser\textsuperscript{206} yielding roughly equal amounts of peptides phosphorylated at Ser\textsuperscript{206} exclusively and those phosphorylated at both Ser\textsuperscript{206} and Thr\textsuperscript{202}. Peptides phosphorylated at Thr\textsuperscript{202} exclusively were not observed. We therefore prepared polyclonal anti-phosphopeptide antibodies specific for Nek6 phosphorylated at Ser\textsuperscript{206} or Ser\textsuperscript{206}/Thr\textsuperscript{202}.

**FIG. 1.** Active Nek6 is phosphorylated at Ser\textsuperscript{37}, Thr\textsuperscript{202}, and Ser\textsuperscript{206} in intact cells. A, in gel kinase assay of FLAG-Nek6 immunoprecipitates. FLAG-Nek6 was immunoprecipitated from HEK293 cells, washed with lysis buffer, and boiled in SDS sample buffer for 10 min. The sample was divided in two, and each aliquot was electrophoresed in a 10\% acrylamide SDS-PAGE gel polymerized without (upper panels) or with (lower panels) MBP. The in-gel kinase assay was carried out as described under “Experimental Procedures”; the gels were fixed, stained with Coomassie Brilliant Blue, and autoradiographed. Protein stain (left) and \(^{32}\)P autoradiography (right) are shown. B, recombinant FLAG-Nek6 is expressed as a doublet and binds endogenous Nercc1. FLAG-Nek6 polypeptide, transiently expressed in HEK293 cells, was immunoprecipitated with anti-FLAG, washed, and subjected to SDS-PAGE; the Coomassie Brilliant Blue-stained gel is shown. Bands a and b, each corresponding to FLAG-Nek6, were excised, digested in situ with trypsin, and analyzed by liquid chromatography/MS/MS. C, detection of phosphorylated Nek6 with phosphospecific antibodies. Left panel, the specificity of the antibodies raised against phosphopeptides containing either Nek6 phospho-Thr\textsuperscript{202} or Nek6 phospho-Ser\textsuperscript{206} was tested by immunoblot of Myc-Nek6 wild type, S206A, or T202A, immunoprecipitated after transient expression in HEK293 cells. Right, immunoblot using the different phosphospecific antibodies of a cell extract from HEK293 transfectected with FLAG-Nek6 wild type.
Fig. 2. A, effect of site-specific mutations on the activity of Nek6. The activity of Nek6 and of a variety of Nek6 site-specific mutants, each containing a C-terminal Myc/His
tag, was examined after transient expression in HEK293 cells and anti-Myc immunoprecipitation. Kinase assays were performed as described under "Experimental Procedures" using Mg\(^{2+}\)-[γ-\(^{32}\)P]ATP and purified recombinant GST-p70 S6 kinase ΔCT104 T252A polypeptide as the substrate. The reaction mixture was subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane, and the Coomassie Brilliant Blue-stained bands corresponding to GST-p70 S6 kinase ΔCT104 T252A were excised, and their \(^{32}\)P content was determined. Kinase activities of mutant Nek6 polypeptides are depicted by the histogram as the mean ± S.E. percentage of the activity of wild-type enzyme assayed in parallel from 3–5 observations for each Nek6 variant. A composite anti-Myc immunoblot is shown, normalized for the expression of the wild type Nek6, which was included in each experiment. B, two-dimensional tryptic phosphopeptide mapping of Nek6. 

Thr\(^{202}\) (Fig. 1C, left). An immunoblot of transiently expressed FLAG-Nek6 indicates that detectable immunoreactivity with either antibody was evident only in the upper, more slowly moving band of Nek6 polypeptide (Fig. 1C, right). It is likely that the detection by MS-MS of phospho-Ser\(^{206}\) in the bottom, faster Nek6 band reflects trace contamination by the upper band, a view supported by the finding that mutation of Ser\(^{206}\) to Asp results in a slowing of the Nek6 polypeptide on SDS-PAGE (Fig. 2A). Transiently expressed mutant, inactive Nek6 (K74M/K75M) yielded no phosphopeptides on MS-MS analysis of tryptic digests; the tryptic peptide encompassing Ser\(^{206}\)/Thr\(^{202}\) was identified only in its unphosphorylated state.

The activity of a variety of Nek6 site-specific mutations was characterized (Fig. 2A). Conversion of Ser\(^{206}\) to Ala reduced Nek6 activity by 98%, and S206D mutant, although upshifted in mobility on SDS-PAGE, exhibited only ∼5–10% of wild-type activity. Conversion of Thr\(^{202}\) to Ala or Cys or conversion of the adjacent Thr residues at 201 and 202 both to Ala reduced Nek6 activity by 75–80%, whereas conversion of the nearby pair of Ser at 198 and 199 to Ala had no effect on Nek6 activity. Introduction of a Thr\(^{202}\) → Glu mutation into wild-type Nek6 increases the apparent specific activity by ∼20%; however, introduction of Thr\(^{202}\) → Glu into the Ser\(^{206}\) → Asp background increases the low activity of this mutant by ∼3-fold, so that the double mutant (S206D/T202E) exhibits ∼20% of wild-type activity. These results indicate that Nek6 activation is absolutely dependent on Ser\(^{206}\) phosphorylation and support the likelihood that phosphorylation at Thr\(^{202}\), although not indispensable or of primary importance, further augments Nek6 activity.

The primary role of Nek6 Ser\(^{206}\) phosphorylation is further supported by the findings on two-dimensional \(^{32}\)P-tryptic peptide maps of transiently expressed wild-type and mutant Nek6 polypeptides (Fig. 2B), which demonstrate that phosphorylation of Ser\(^{206}\) is necessary for the phosphorylation of other Nek6 sites. The \(^{32}\)P incorporation in vivo into Nek6 (S206A) is reduced by >80% as compared with wild-type Nek6. Moreover, whereas two-dimensional \(^{32}\)P-tryptic peptide maps of \[^{32}\text{P}\]Nek6 exhibit a single dominant \(^{32}\)P-peptide, two minor \(^{32}\)P-peptides, and several trace \(^{32}\)P-peptides, maps of \[^{32}\text{P}\]Nek6 (S206A) lack all three of the characteristic \(^{32}\)P-peptides and exhibit only a faint background of the multiple trace, presumably nonspecific, \(^{32}\)P-peptides. The overall \(^{32}\)P incorporation into the Nek6 (T201A/T202A) mutant is also substantially reduced from wild-type; however, the \(^{32}\)P-tryptic peptide map of this variant contains each of the three predominant \(^{32}\)P-peptides seen in digests of wild-type Nek6, in a proportion similar to that seen in the wild-type Nek6. We interpret this pattern to indicate that the mutation of Thr\(^{202}\) affects Nek6 activity primarily by reducing the extent of Ser\(^{206}\) phosphorylation and secondarily by the loss of its own phosphorylation. The persist-
ence of the three major $^{32}$P-peptides in the T201A/T202A mutant suggests the occurrence in this mutant of phosphorylation at other sites situated on the same tryptic peptide (i.e. Ser$^{198}$ or Ser$^{199}$).

In summary, Nek6 activity requires phosphorylation of Ser$^{206}$ on the activation loop. Ser$^{206}$ phosphorylation also appears to facilitate Thr$^{202}$ phosphorylation, which may increase catalytic activity somewhat further. As to the mechanism by which these phosphorylations are accomplished, in vivo, the absence of detectable phosphorylation in the Nek6 ATP site mutant (K74M/K75M) suggests that either Ser$^{206}$ phosphorylation is catalyzed by intramolecular autophosphorylation or, alternatively, Nek6 (K74M/K75M) interacts poorly with an upstream kinase. The evidence presented next strongly supports the latter explanation, at least in mammalian cells.

Nercc1 Binds, Phosphorylates, and Activates Nek6 and Nek7

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**Fig. 2—continued**

Overexpression of wild type Nek6 with the constitutively activated Nercc1 mutant, Nercc1 (Δ347–732) (10), results in an
**Fig. 3. Nercc1 binds and activates Nek6 in vivo.** A, Nek6 binding to Nercc1. Vectors encoding wild type or kinase-inactive (K74M/K75M) GST-Nek6 were cotransfected in HEK293 cells with wild type or mutant versions of FLAG-Nercc1. Extracts were incubated with GSH-agarose; aliquots of the cell extracts and of the SDS eluates from well washed beads were subjected to SDS-PAGE and immunoblot using the antibodies indicated. B, cotransfection with constitutively active Nercc1 increases the activity of coexpressed Nek6 activity. HEK293 cells were transfected with Nek6 Myc-His6 alone or in combination with increasing amounts of FLAG-Nercc1 (Δ347–732) and deprived of serum 24 h prior to harvest. The kinase activity of recombinant Nek6 immunoprecipitated from cell lysates was measured using GST-p70 S6 kinase CT104 (T252A) as substrate; the latter is not a substrate for Nercc1. C, a constitutively active Nercc1 causes phosphorylation of the Nek6 (K74M/K75M) activation loop in vivo. HEK293 cells were transfected with GST-Nek6 (K74M/K75M) alone or together with FLAG-Nercc1 (Δ347–732) or FLAG-Nercc1 (Δ347–732, K81M). GST-Nek6 (K74M/K75M) was isolated by adsorption to GSH-agarose; each eluate from the washed beads was divided in three and subjected to immunoblot using an antibody specific for total Nek6 polypeptide (top panel) or the anti-Nek6 phosphospecific antibodies, anti-phospho-Thr202 (second from top) and anti-phospho-Ser206 (third from top); an immunoblot of the extracts using anti-FLAG antibody is shown in the bottom panel.
observable upshift in the electrophoretic mobility of Nek6 (Fig. 3, B and A, lane 3 from left) and in a 2-fold increase in the specific activity of coexpressed Nek6 (Fig. 3B). Thus, active Nerc1 is able to induce Nek6 phosphorylation and activation in vitro; the modest stimulation of Nek6 activity by Nerc1 (Δ347–732) probably reflects the already high basal activity of recombinant overexpressed Nek6. Nerc1 (Δ347–732) also slows the electrophoretic mobility of the coexpressed inactive Nek6 mutant, K74M/K75M (Fig. 3C). Trypsin digests of Nek6 (K74M/K75M) coexpressed with Nerc1 (Δ347–732) exhibited on liquid chromatography/MS/MS considerable phosphorylation at Ser206 and small amounts of peptide phosphorylated at both Ser206 and Thr202, these findings were subsequently confirmed on anti-phosphopeptide immunoblots (Fig. 3C). The ability of coexpressed Nerc1 (Δ347–732) to promote these phosphorylations of Nek6 (K74M/K75M) requires an active Nerc1 catalytic domain. Thus, coexpression of Nek6 with active Nerc1 results in phosphorylation at Ser206 and a modest increase in the already substantial activity of coexpressed recombinant Nek6. We next sought to determine whether Nerc1 could directly phosphorylate Nek6. Immuno-purified, recombinant wild type Nerc1 activated in vitro by autophosphorylation does catalyze the phosphorylation of a mammalian recombinant Nek6 (K74M/K75M) substrate at Ser206 (Fig. 4A). This modification occurs, however, with a rather low efficiency, consistent with the very poor binding of Nek6 (K74M/K75M) to Nerc1. Notably, all of the Ser206-phosphorylated Nek6 (K74M/K75M) polypeptide co-precipitates with Nerc1 (Fig. 4B), suggesting that the “stable” binding of Nek6 to Nerc1 is critical for Nerc1-catalyzed Nek6 phosphorylation.

We therefore sought to use wild type Nek6 as substrate. Inasmuch as Nek6 overexpressed in mammalian cells shows high levels of Ser206 phosphorylation and kinase activity, we utilized bacterial recombinant GST-Nek6 and GST-Nek7. Surprisingly, GST-Nek6, which expresses poorly in bacteria and is predominantly insoluble, exhibits varying extents of Ser206 phosphorylation and spontaneous kinase activity depending on the batch (data not shown). In contrast, bacterial recombinant GST-Nek7 never showed activation loop (Ser195) phosphorylation or significant kinase activity when purified from bacteria. We examined “inactive” preparations of bacterial recombinant GST-Nek6 and GST-Nek7 for their ability to be phosphorylated and activated by Nerc1 (Fig. 4C). A computer search using the putative motif for Nek6 phosphorylation specificity (17) identified several candidate Nek6 substrates; a GST fusion of the extreme carboxyl terminus of Cdc16, which contains several LXXS motifs, was readily phosphorylated by Nek6 and Nek7 but not significantly phosphorylated by active Nerc1.2 GST-Nek6 and GST-Nek7 were incubated with Mg2+/ATP in the presence or absence of active Nerc1; after 30 min, GST-Cdc16-CT and tracer [γ-32P]ATP were added for an additional 30 min. As shown in Fig. 4C, Nerc1 catalyzes the phosphorylation of Nek6 (Ser206) and the equivalent site on Nek7 (Ser195), resulting in a 20–25-fold activation of Nek6/7 kinase activity. In contrast, Nek7, which is devoid of basal phosphorylation at Ser195 exhibits no ability to catalyze autophosphorylation at Ser195 during this in vitro incubation; Nek6 exhibits a low level of basal phosphorylation at Ser206 which does not change during the incubation with Mg2+/ATP. A similar experiment using active Nerc1 and GST-Nek7 was analyzed using an in-gel kinase assay with MBP as substrate (Fig. 4D); an upshift in Nek7 mobility is evident after Nerc1-catalyzed Nek7 phosphorylation, and the “activated” MBP kinase activity co-migrates entirely with the upshifted band of Nek7.

Inasmuch as the binding of Nek6 to Nerc1 appeared to be critical for the Nerc1-catalyzed phosphorylation of Nek6 in vitro (i.e. see Fig. 4B), we examined the effect of overexpressing the Nerc1 noncatalytic tail on the activity of coexpressed Nek6. A FLAG-tagged polypeptide consisting of Nerc1 amino acids 347–979 strongly inhibits the activity of coexpressed GST-Nek6 (Fig. 5A), concomitant with decreased Nek6 phosphorylation at Ser206 and Thr202 (Fig. 5B) (i.e. decreased Nek6 activation). Interestingly, the noncatalytic tail of Nerc1 (amino acids 732–979) is also able to directly inhibit the enzymatic activity of preactivated, wild type Nek6 in vitro (Fig. 5C). Nek6 phosphorylates the recombinant GST-Nerc1 (732–979) polypeptide; however, substantial inhibition of the phosphorylation of the Nek6 substrate (i.e. p70 S6 kinase ΔCT104 in this experiment) is evident at GST Nerc1 (Δ732–979) concentrations far below those of the p70 S6 kinase ΔCT104 polypeptide, and the Nek6-catalyzed phosphorylation of GST Nerc1 (732–979) actually diminishes as the concentration of GST Nerc1 (732–979) is increased. These features suggest the occurrence of a noncompetitive mode of inhibition of Nek6 catalytic activity by GST Nerc1 (732–979). Thus, the binding of Nek6 to the Nerc1 noncatalytic tail may serve not only to facilitate Nek6 activation by active Nerc1 but possibly to restrict the catalytic activity of Nek6 upon activation by Nerc1 and perhaps direct it to specific targets.

Nek6, Like Nerc1, Is Activated in Mitosis—We next examined the regulation of endogenous Nek6 activity in vivo. An affinity-purified anti-Nek6 peptide antibody raised against the amino-terminal murine Nek6 peptide (AGGPSSHPHMGGSPN Cys-keyhole limpet hemocyanin) gave highly specific immunoblots and was capable of modest immunoprecipitation of the endogenous Nek6 polypeptide, although at very low efficiency; thus far we have been unable to produce useful antibodies reactive with endogenous Nek7. Nek6 immunoreactivity is evident in several commonly used mammalian cell lines including COS7 cells (Fig. 6A). We chose to examine first the regulation of Nek6 in the rat hepatoma line H4IIEC, inasmuch as Nek6 mRNA shows highest abundance in liver (16); moreover, Nek6 had been isolated as a candidate p70 S6 kinase-Thr412 kinase, and we previously characterized insulin regulation of endogenous p70 S6 kinase in this cell line (22). Insulin generates a rapid, 15-fold activation of endogenous p70 S6 kinase in serum-deprived H4 cells; under these conditions, however, no change occurs in the basal activity of immunoprecipitated endogenous Nek6 (Fig. 6B). The activity of p70 S6 kinase in insulin-stimulated H4 cells is inhibited progressively by increasing concentrations of wortmannin (IC50 ~30 nM; Fig. 6C) or rapamycin (IC50 ~2 nM; Fig. 6D); these inhibitors of phosphatidylinositols 3-kinase and mTOR (respectively) do not alter significantly the activity of Nek6. Taken together, these results indicate that Nek6 activity is not rapidly regulated by signal transduction pathways downstream of the insulin receptor, and it is therefore unlikely that Nek6 functions as an activating p70 S6 kinase in response to activation of receptor tyrosine kinases. The specific activity of Nek6 in H4 cells is, however, sensitive to serum withdrawal and declines progressively by 80% over 48 h (Fig. 6E). This decline in Nek6 activity, however, is much slower and less severe than that observed for the p70 S6 kinase.

The fall in Nek6 activity as H4 cells are brought to quiescence by serum withdrawal together with the previous observation that Nerc1 activity increases at mitosis (10), led us to examine the abundance and activity of Nek6 in cells arrested in mitosis by nocodazole, as compared with nonmitotic cells. It is evident (Fig. 6F) that the relative abundance of Nek6 is increased by 3–4-fold in H4 cells arrested in mitosis as compared...
with the amount in the nonmitotic cells; moreover, Nek6 mobility on SDS-PAGE is retarded in mitotic cells, and Nek6 kinase activity increases in parallel to its abundance. Similar results were obtained with human U2OS cells (Fig. 7A), suggesting that endogenous Nek6, like Nercc1, is activated in mitosis in different cell lines. Mitotic HeLa cells also show an increase in the abundance of the Nek6 polypeptide and a marked increase in its phosphorylation at Ser206 and Thr202 (Fig. 7B); we were unable, however, to immunoprecipitate endogenous HeLa Nek6, precluding direct assay of its activity in this cell line. RT-PCR shows that the abundance of Nek6 mRNA (relative to that of TATA-box-binding protein) was 3-fold higher in mitotic than in exponentially growing HeLa cells (Fig. 7C), pointing to at least one mechanism for the increase in Nek6 protein content in mitotic cells. Thus, endogenous Nek6, like Nercc1, is increased in abundance and activated in mitosis.

**DISCUSSION**

Recombinant Nek6 transiently expressed in mammalian cells exhibits substantial spontaneous activity, which is abol-
Nercc1 noncatalytic domain inhibits Nek6 activation in vivo and activity in vitro. A, Nek6 activity is inhibited by overexpression of a Nercc1 variant lacking the N-terminal kinase domain in vivo. GST-Nek6 was coexpressed with increasing quantities of FLAG-Nercc1-(347–979). The kinase activity of GST-Nek6 was measured using MBP as substrate. B, Nercc1-(347–979) suppresses Nek6 activation loop phosphorylation in vivo. GST-Nek6 was cotransfected in HEK293 cells with increasing amounts of FLAG-Nercc1-(347–979), and immunoblots of the extracts were carried out using the indicated antibodies. C, Nercc1-(732–979) inhibits Nek6 activity in vitro. Transiently expressed FLAG-Nek6 was incubated for 1 h at 4 °C alone or with increasing quantities of either purified prokaryotic recombinant GST (lanes 6–8) or prokaryotic recombinant GST-Nercc1-(732–979) (lanes 2–5). Nek6 activity was assayed using p70 S6 kinase ΔCT104 as substrate. Upper panel, mean ± S.E. percentage of control Nek6 activity. Lower panels, 32P autoradiography, Coomassie stain of the GST-Nercc1-(732–979) or GST proteins and Western blot of the FLAG-Nek6.
Fig. 6. Regulation of endogenous Nek6 activity. A, anti-Nek6 immunoblot of cell extracts of PTK, HEK293, COS7, H4-II-E-C3, CHO-IR, and NIH3T3 cells, normalized for total protein. B, C, and D, Nek6 activity endogenous to H4-II-E-C3 hepatocytes is not altered by agents that modify p70 S6 kinase activity. Endogenous Nek6 polypeptide was immunoprecipitated from lysates of rat H4-II-E-C3 hepatoma cells following prior treatment for varying times (min) with 100 nM insulin (B) or insulin plus increasing concentrations of either wortmannin (C) or rapamycin (D) for 30 min. Nek6 kinase activity was measured using GST p70 S6 kinase ΔCT104 (T252A) as substrate (see "Experimental Procedures").
ished by treatment with protein phosphatase, pointing to the likelihood that Nek6 phosphorylation is at least required for the expression of Nek6 kinase activity. The MS/MS analysis of recombinant Nek6 revealed three sites of phosphorylation, two on the activation loop at Ser<sup>206</sup> and Thr<sup>202</sup> as well as Ser<sup>207</sup>. Elimination of Ser<sup>207</sup> did not alter activity (Fig. 2A); however, mutation of Ser<sup>206</sup> essentially abolishes activity as well as phosphorylation at other sites, whereas replacement of Thr<sup>202</sup> reduces but does not eliminate Nek6 activity and phosphorylation. Thus, the activity of recombinant Nek6 requires phosphorylation at Ser<sup>206</sup>; phosphorylation at Thr<sup>202</sup>, which was never observed without concomitant Ser<sup>206</sup> phosphorylation, probably augments activity somewhat further but is of secondary importance. The occurrence of Ser<sup>206</sup> phosphorylation on endogenous Nek6 concomitant with its activation during mitosis (Fig. 7, A and B) provides evidence that this modification is critical to the physiologic regulation of Nek6.

Several lines of evidence point strongly to the likelihood that another NIMA family kinase, Nercc1, is responsible for Nek6 Ser<sup>206</sup> phosphorylation. 1) Incubation of recombinant Nek6 with Mg<sup>2+</sup>-ATP in vitro, with or without prior phosphatase treatment, fails to increase catalytic activity, suggesting a requirement for an upstream kinase. 2) Activated Nercc1 directly catalyzes the phosphorylation of Nek6 Ser<sup>206</sup> (and of the equivalent site, Ser<sup>199</sup>, in Nek7) and generates a robust activation of wild type Nek6<sub>7</sub> in vitro. 3) Coexpression of Nek6 with an active mutant of Nercc1 increases the already substantial activity of recombinant Nek6 by about 2-fold. More significantly, the Nek6 (K74M/K75M) mutant, which is intrinsically inactive and greatly impaired in its ability to bind recombinant and endogenous Nercc1, lacks detectable phosphorylation when transiently expressed; if, however, Nek6 (K74M/K75M) is coexpressed with the constitutively active Nercc1 mutant, ∆347–732, the Nek6 (K74M/K75M) polypeptide undergoes considerable phosphorylation at Ser<sup>206</sup>. The ability of Nek6 to induce phosphorylation of the Nek6 (K74M/K75M) activation loop and increase the activity of coexpressed wild type Nek6 requires an active Nercc1 catalytic domain in vivo. 4) Nercc1 is activated during mitosis in parallel with an increase in Nek6 protein (due, at least in part, to an increase in Nek6 mRNA), Ser<sup>206</sup> phosphorylation, and activity. 5) Recombinant Nek6 binds to endogenous Nercc1 specifically and in a detergent-resistant manner. Despite this tight binding, however, only a small portion of the transiently expressed recombinant Nek6 polypeptide comigrates with endogenous Nerc1 on gel filtration (data not shown). Moreover, we have not detected endogenous Nek6 in immunoprecipitates of endogenous Nercc1, whether prepared from nonsynchronized, cycling cells or mitotic, nocodazole-arrested cells. These results reflect in part the lack of a robust anti-Nek6 antibody but also indicate that important determinants of the Nek6/Nercc1 interaction remain to be identified. Nevertheless, the data presented strongly point to the likelihood that Nercc1 is the immediate upstream activator of Nek6 during mitosis.

The occasional recovery of prokaryotic recombinant Nek6 as an active kinase phosphorylated on Ser<sup>206</sup> indicates that Ser<sup>206</sup> phosphorylation can also be catalyzed through autophosphorylation (at least during overexpression in E. coli) probably in trans, much as occurs with prokaryotic expression of the kinase A catalytic subunit. Mammalian recombinant Nek6 is quite highly expressed after transient transfection in HEK293 cells, so that it is conceivable that, as during expression in E. coli, autophosphorylation accounts for the spontaneous activation in vivo of transiently expressed Nek6. Whatever the mechanism for activation of overexpressed mammalian recombinant Nek6, the results discussed above and the inability of Nek6 to autophosphorylate in vitro argue strongly that the mitotic activation of endogenous Nek6 is mediated by another protein kinase. We have not observed any increase in Ser<sup>206</sup> phosphorylation or in the activity of mammalian or bacterial recombinant Nek6 or Nek7 on incubation in vitro with Mg<sup>2+</sup>-ATP, either with or without prior protein phosphatase treatment. This is in strong contrast to the behavior of kinases for whom autophosphorylation is important in a physiologic context such as, for example, calmodulin kinase II (23), the insulin receptor kinase (24), and several Src family kinases (25, 26); these enzymes show substantial autophosphorylation/autocatalysis in vitro even in the absence of their “activating” ligands. Moreover, most kinases regulated through autophosphorylation are dimers or higher oligomers, and Nek6/Nek7 were recovered on gel filtration at around 35 kDa (16).

Designation of Nercc1 as the immediate upstream activator of Nek6 does not preclude the existence of other activation mechanisms or additional proximate upstream kinase activators of Nek6/Nek7; however, we have no evidence for their existence. Nek6 is not subject to rapid regulation downstream of receptor tyrosine kinases (e.g. Fig. 6.), and although Nek6 is capable of activating the p70 S6 kinase, directly in vitro and by overexpression in vivo (16), the present data, together with the findings of Alessi and co-workers (17), eliminate the possibility that Nek6 is the element recruited by insulin for this function. Furthermore, p70 S6 kinase activity declines progressively during M phase, when Nek6 activity is highest, and increases during the transition back to G<sub>1</sub> (27), when Nek6 activity is declining.

To our knowledge, the activation of Nek6 by Nercc1 is the first direct identification of the participation of a NIMA family kinase in a protein kinase cascade. NIMA itself appears to be regulated both at the level of polypeptide abundance, with highest levels in M phase, and by phosphorylation (1), since NIMA is completely deactivated by phosphatase 2A in vitro (28) or by mutation of Ser<sup>199</sup> in the activation loop (29). As to the mechanism of NIMA activation and phosphorylation, NIMA extracted from A. nidulans is an oligomer, as is prokaryotic recombinant NIMA; both are autophosphorylated in vivo and already active when extracted. Nevertheless, once dephosphorylated in vitro by protein phosphatase, NIMA is capable of catalyzing reactivation in vitro upon incubation with Mg<sup>2+</sup>-ATP. NIMA is apparently unphosphorylated in S phase but undergoes phosphorylation and partial activation in G<sub>2</sub>; on entry to M, NIMA undergoes hyperphosphorylation and further activation dependent on p34<sup>cdc2</sup> (30). This in vivo phosphorylation generates MPM-2 epitopes on NIMA, and phosphorylation by Cdc2 in vitro also generates MPM-2 epitopes. Nevertheless,
direct activation of NIMA by Cdc2 has not been observed in vitro, and other kinases, including Polo (31) and CK2 (32), have been reported to generate MPM-2 epitopes. Among the metazoan NIMA family, Nek2 has been characterized most fully thus far (13). The Nek2 polypeptide is an obligate dimer that is most abundant during S/G2 (33). Deletion of the leucine zipper abolishes dimerization and drastically decreases kinase activity and autophosphorylation, which occurs on the C-terminal noncatalytic domain (34). The high basal activity of prokaryotic recombinant Nek2 is greatly diminished by treatment with phosphatase 1 in vitro. Interestingly, native Nek2 (but not Nek2B) forms a stable complex with phosphatase 1 catalytic subunit in vitro, and cross-regulation of these two polypeptides may occur (35). Nek2 is also activated in murine spermatocytes during the meiotic G2/M transition in a manner sensitive to the mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor U0126. The addition of activated recombinant Rsk2 to spermatocyte extracts increases the activity of immunoprecipitated endogenous Nek2. Moreover, Rsk2 can phosphorylate in vitro a recombinant catalytic do-

Fig. 7. Nek6 in mitosis. A, Nek6 abundance and activity increase in mitotic U2OS cells. Mitotic and nonmitotic cells were obtained as in Fig. 6D. Nek6 protein kinase activity was determined after immunoprecipitation using immunopurified Nek6 antibodies and GST-p70 S6 kinase 355–525 as a substrate. Mock immunoprecipitations with normal IgG were performed in parallel as a control (not shown). The relative Nek6 activity (mean ± S.E., n = 3 experiments) and protein content in the extracts is shown. B, Nek6 protein levels are increased, and Nek6 Thr202 and Ser206 are phosphorylated in mitotic HeLa cells. Extracts prepared from HeLa cells exponentially growing (Exp.) or arrested in mitosis by 500 ng/ml nocodazole and isolated by mitotic shake off (M) were subjected to SDS-PAGE and immunoblot using the indicated antibodies. C, Nek6 mRNA expression is increased in mitotic HeLa cells. RT-PCR quantification of Nek6 mRNA levels from exponentially growing and mitotic HeLa cells (nocodazole arrested in mitosis and isolated as above) was carried out as described under “Experimental Procedures.” Nek6 RNA amount relative to TBP RNA amount is shown. Average ± S.E. of three different experiments is shown.
main fragment of Nek2. Thus, in addition to activation through autophosphorylation, Nek2 may be regulated through the mitogen-activated protein kinase cascade by Rsk (36).

The mechanism of Nerc1 activation during mitosis is not yet known; however, Nerc1 activated during mitosis can be deactivated by phosphatase. Unlike Nek6, Nerc1 is capable of autoactivation on incubation with Mg²⁺-ATP in vitro (10). Our working model is that Nerc1 is maintained in an inactive state in vivo through binding to an endogenous inhibitor, and this interaction is abrogated during mitosis, whereupon Nerc1 undergoes autophosphorylation and activation. Nevertheless, the fraction of Nerc1 activated during M and the cellular localization of active Nerc1 are both unknown. Nerc1 is a cytoplasmic protein that binds the Ran GTPase (preferring Ran GDP over Ran GTP) through both its catalytic and RCC1 domains. Whether and how these properties contribute to Nerc1 activation in vivo is currently under study. The ability of anti-Nerc1 antibodies to interfere with mitotic progression when injected into cells in prophase provides strong evidence that Nerc1 has substrates whose function are required for mitotic progression. The present results identify Nek6 and probably Nek7 as candidate physiologic Nerc1 substrates. The specific mitotic targets of Nek6 and Nek7 as well as the identity of other Nerc1 substrates remain to be discovered.

Acknowledgments—We thank Yensou Lin for GST-p70 S6 kinase Ct production and J. Prendable for preparation of the manuscript.

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