Identification of the NIMA family kinases NEK6/7 as regulators of the p70 ribosomal S6 kinase

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Background: The p70 S6 kinase, like several other AGC family kinases, requires for activation the concurrent phosphorylation of a site on its activation loop and a site carboxyterminal to the catalytic domain, situated in a hydrophobic motif site FXXFS/TF/Y, e.g.,Thr412 in p70 S6 kinase (α1). Phosphorylation of the former site is catalyzed by PDK1, whereas the kinase responsible for the phosphorylation of the latter site is not known.

Results: The major protein kinase that is active on the p70 S6 kinase hydrophobic regulatory site, Thr412, was purified from rat liver and identified as the NIMA-related kinases NEK6 and NEK7. Recombinant NEK6 phosphorylates p70 S6 kinase at Thr412 and other sites and activates the p70 S6 kinase in vitro and in vivo, in a manner synergistic with PDK1. Kinase-inactive NEK6 interferes with insulin activation of p70 S6 kinase. The activity of recombinant NEK6 is dependent on its phosphorylation, but NEK6 activity is not regulated by PDK1 and is only modestly responsive to insulin and PI-3 kinase inhibitors.

Conclusion: NEK6 and probably NEK7 are novel candidate physiologic regulators of the p70 S6 kinase.

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Introduction

The p70 S6 kinase (p70S6K) is activated downstream of insulin and growth factor receptors through a complex sequence of multiple phosphorylations. Most steps in this activation mechanism have been elucidated in considerable, if not quite complete, detail, primarily through studies on the p70α isoform [1, 2]. The more recently identified p70β isoform shares with p70α all of the phosphorylation sites demonstrated thus far to be of regulatory significance; it is therefore likely that the mechanism of p70β activation will parallel that of p70α [3–5]. These kinases contain a centrally located kinase catalytic domain of the AGC subclass, immediately carboxyterminal to which is a segment of approximately 65 amino acids that is highly conserved (approximately 40% identity) among most AGC kinases, including the PKBs, PKCs, Rsk (N-terminal catalytic domain), and SGKs. The p70 sequences flanking these two domains, i.e., an N-terminal segment of 64–88 amino acids and a 104 amino acid carboxyterminal tail, are entirely unique to the p70 kinases; each flank contains important regulatory motifs. The initial step in p70 activation is the phosphorylation of a cluster of 4–5 (Ser/Thr)Pro motifs situated within a *psuedo*-substrate autoinhibitory domain in the carboxyterminal noncatalytic tail [6–8]; phosphorylation does not activate p70 catalytic function [9] but serves to dislodge the tail from the catalytic domain and thereby provides access to the activating kinases [10, 11]. Two sites of activating phosphorylation have been established with certainty; one is located in the p70 catalytic subdomain VIII on the "activation" loop (e.g., p70α1Thr252 [12]), and the other (e.g. p70α1Thr412) is located in the conserved segment carboxyterminal to the catalytic domain, in the sequence FXXFTY [13, 14]. Modification at either site alone gives significant activation; however, physiologic activation requires the simultaneous phosphorylation at both sites, which creates a strong, positively cooperative site-site interaction [15]. In addition, the phosphorylation of either of these two sites appears to promote the phosphorylation of the other [10, 11]. Considerable evidence indicates that PDK1 is responsible for the insulin/mitogen-stimulated phosphorylation of the p70 activation loop (e.g., p70Thr252). PDK1 phosphorylation catalyzes the direct preferential phosphorylation of this site in vitro. Cotransfection of p70 with PDK1 results in p70 activation, concomitant with increased phosphorylation at Thr252. Reciprocally, kinase-inactive ATP site mutants of PDK1 block serum/insulin-stimulated phosphorylation of the p70 activation loop [15, 16]. Embryonic stem (ES) cells lacking PDK1 show no IGF-1-stimulated phosphorylation of the p70 activation loop [17].

By contrast, the kinase responsible for the insulin/mitogen-stimulated phosphorylation of the p70 hydrophobic site (e.g., p70Thr412) is not known. This site can be phosphorylated in vitro by the mTOR kinase [18, 19], and its phosphorylation is concomitant with p70 activation. Subsequent phosphorylation of p70 by PDK1 gives a strongly synergistic increase in p70 activity [19]. Nevertheless, a Rapamycin-resistant p70 mutant (Δ2-46/

ΔCT104) exhibits unimpaired insulin-stimulated phosphorylation at the Thr412 site in vivo in the presence of Rapamycin at concentrations sufficient to fully inhibit the activity of the endogenous mTOR kinase [20]. The p70Thr412 site also undergoes phosphorylation in vivo on coexpression of p70 with PDK1 [21], and ES cells lacking PDK1 exhibit no IGF-1-stimulated phosphorylation at p70Thr412, although phosphorylation of the homologous site in PKBα (Ser473) is unimpaired [17]. PDK1 can phosphorylate p70Thr412 directly in vitro, although at a rate <5% the rate at which it phosphorylates p70Thr252. A fragment of the PKC-related kinase, PRK2, that encompasses the binding site for PDK1 inhibits PDK1-stimulated p70Thr412 phosphorylation in vitro, as well as insulin/mitogen-stimulated phosphorylation of p70Thr412 in vivo [21]. These data indicate that PDK1 is critical to p70Thr412 phosphorylation in vivo; however, whether this is because of the facilitating effect of Thr252 phosphorylation on Thr412 phosphorylation, the direct phosphorylation of Thr412 by PDK1, the activation of a Thr412 kinase by PDK1, or some other mechanism remains to be established.

We have partially purified the dominant p70Thr412 kinase present in extracts of HEK293 cells; an enzyme with essentially identical chromatographic behavior was more extensively purified from rat liver and was identified as a novel member of the NIMA family of kinases. Furthermore, analysis of ESTs revealed a second, closely related gene product. During the course of this work, Kandli et al [22] identified these sequences as murine ESTs and named them NEK6 and NEK7; we have adopted this nomenclature. The molecular structure and expression of NEK6 and NEK7, as well as a preliminary functional analysis of NEK6, are described in this report. The data indicate that NEK6 and NEK7, which are widely expressed, most highly in liver, are capable of phosphorylating and activating the p70S6K in vitro and in vivo in a manner that synergizes strongly with PDK1. The NEKs are thus candidate p70S6K-activating kinases.

Results

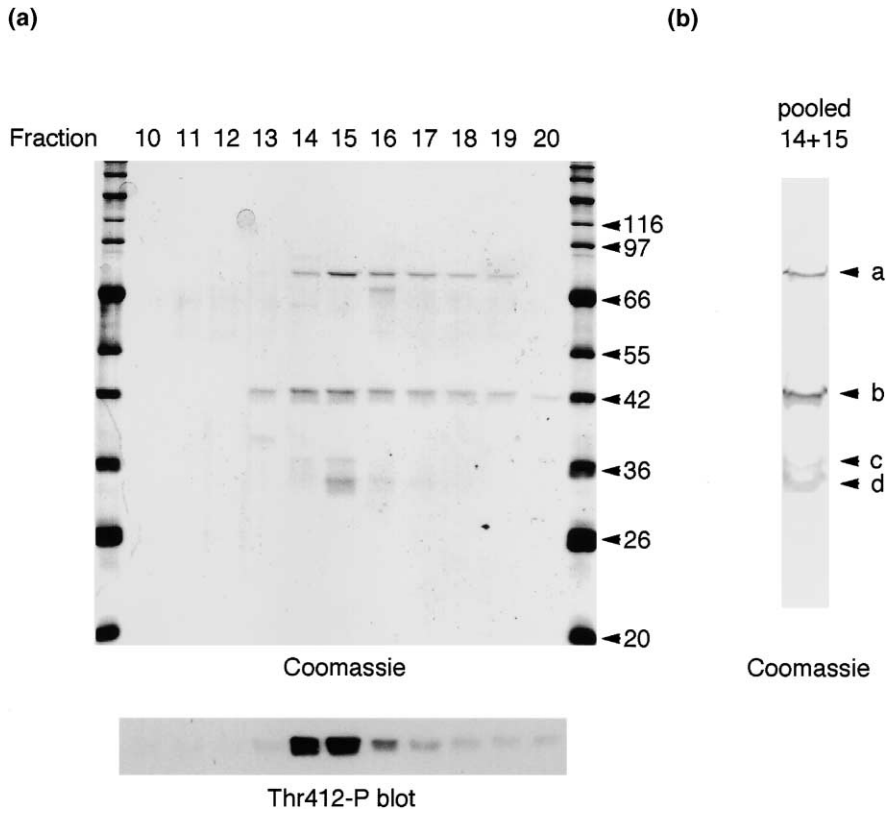
The p70S6K is phosphorylated at many sites in vivo, and it can be phosphorylated in vitro at these and other sites by numerous protein kinases. We sought to detect the subset of kinases capable of phosphorylating p70Thr412 so as to ascertain their identity and determine whether they participate in the physiologic regulation of p70S6K activity. To this end, we employed as a substrate a baculoviral recombinant p70ΔCT104 mutant, dephosphorylated at Thr412 in situ by the treatment of sf9 cells with Rapamycin (0.1 μM, 30 min) prior to extraction. The inactive p70ΔCT104 polypeptide was purified to >90% purity by sequential anion and cation exchange chromatography. The phosphorylation of p70Thr412 was detected by immunoblot with an anti-Thr412PO_4^- peptide antibody, previously employed for the characterization of p70α Thr412PO_4^- during transient expression in vivo [10]. The baculoviral recombinant p70ΔCT104 polypeptide substrate showed no ability to catalyze Thr412 phosphorylation during incubation in vitro with Mg^{++}ATP (Figures 1 and 3b).

Extracts from HEK293 cells, added directly or after adsorption/elution on Fast Q-Sepharose, gave no detectable Thr412 phosphorylation; however, the Fast Q flow-through fraction catalyzed significant Thr412 phosphorylation. This activity was further purified by sequential adsorption/elution from S-Sepharose, Blue Agarose, and Heparin-Sepharose. The Thr412 kinase activity eluted at each of these steps in a single major peak; however, purification of HEK293 extracts beyond the Heparin-Sepharose step was limited by low recovery. Consequently, postmicrosomal extracts of rat liver were evaluated as an enzyme source and were observed to contain a major Thr412 kinase activity that exhibited identical chromatographic behavior to that seen with HEK293 extracts. Gel filtration of the Heparin-Sepharose eluate yielded a single major peak of Thr412 kinase activity at approximately 35 kDa. Subsequent fractionation of this peak on Mono Q anion exchange chromatography generally yielded two peaks. In each isolate, the peak that eluted at 15–35 mM NaCl contained three polypeptide bands (Figure 1); each was subjected to tryptic digestion and microcapillary RP-HPLC, and peptide sequence information was determined by electrospray ionization mass spectrometry. The spectra obtained from the tryptic digestion of the 35 kDa polypeptide contained four peptides that matched with the amino acid sequence of a human cDNA, AB026289; a subsequent report described the murine version of AB026289 as NEK6 [22]. Our mass spectroscopic analysis identified an additional five peptides that matched with amino acid sequences in murine expressed-sequence tag (est) AA102912, subsequently named NEK7. A full description of our database search and cDNA cloning of NEK6/7 cDNAs is provided in the Materials and methods and with this article online in the Supplementary material. Thus, the 35 kDa p70S6KThr412 kinase we purified from rat liver is probably a mixture of NEK6 and NEK7. Alternatively, this isolate may be entirely NEK7 in light of the fact that 3 of the 4 peptides attributed to NEK6 are identical in mass and/or sequence in NEK7, whereas 3 of the 5 peptides attributed to NEK7 are entirely unique to NEK7. The gene encoding human NEK6 is situated on chromosome 9, and that encoding NEK7 is on human chromosome 1; these two facts indicate that NEK6 and NEK7 are the products of separate genes. A *C. elegans* protein kinase (Z50873) is about 75% identical to both NEK6 and NEK7 (Figure 2).

NEK mRNA expression

cDNA probes reactive selectively with NEK6 or NEK7 were employed for probing a commercial blot containing

Figure 1



Mono Q fractionation of a p70S6KThr412 kinase activity from rat liver. The dominant Thr412 kinase activity in a rat liver extract was purified by sequential chromatography on Fast Q-Sepharose, SP-Sepharose, Reactive Blue 4 Agarose, Heparin-Sepharose HiTrap and Sephacryl S-200 HR gel filtration. **(a)** The activity peak from Sephacryl was applied to a Mono Q column, and Mono Q fractions were analyzed for p70Thr412 phosphorylation (anti Thr412PO_4^- immunoblot, lower panel) and protein content by Coomassie blue staining after SDS-PAGE (upper panel). Similar results were obtained in two other purifications carried through all steps. The fractions (14 and 15) containing the peak of Thr412 kinase activity were pooled and subjected to SDS-PAGE. **(b)** The polyacrylamide gel was stained with Coomassie blue, and candidate protein bands (a, b, and c/d) were excised, subjected to tryptic digestion, microcapillary RP-HPLC, and peptide sequence determination by electrospray ionization mass spectrometry.

mRNA extracted from mouse tissues (Figure 2). NEK6 and NEK7 are most highly expressed in liver. Significant signals for NEK6 are evident in brain and kidney; low-level expression is evident in all tissues on prolonged exposure (not shown). NEK7 mRNA is abundant in heart and kidney and is evident at lower levels in all other tissues examined.

Recombinant NEK6 phosphorylates p70S6KΔCT104 at Thr412

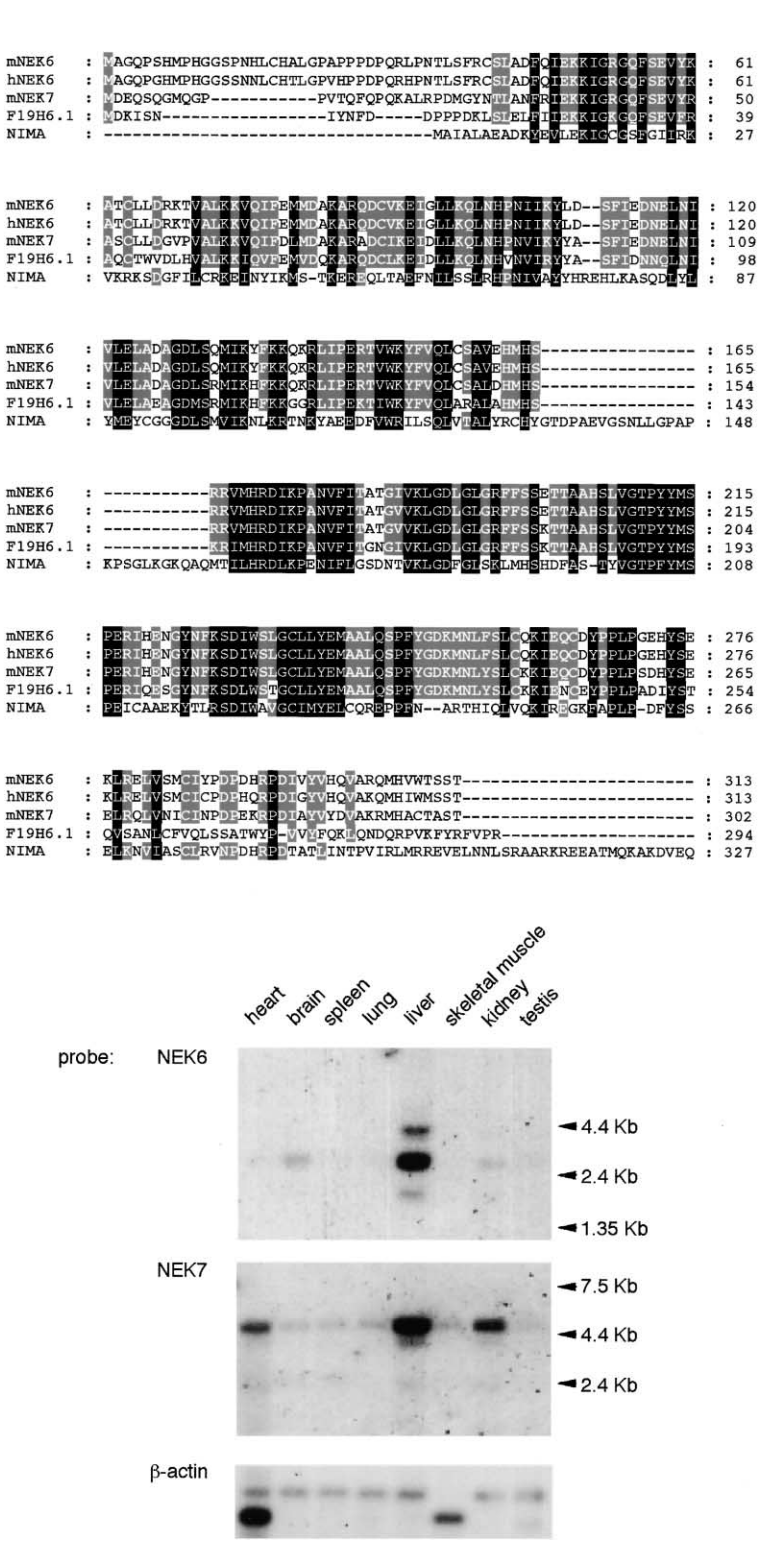
The NEK6 and NEK7 cDNAs were transiently expressed in HEK293 cells, either as a fusion downstream of GST or with an N-terminal FLAG epitope, and examined for their ability to phosphorylate the p70S6K. In vitro, GST-p70S6KΔCT104 purified from Rapamycin-treated HEK293 cells exhibits a slight autophosphorylation, which is abolished by mutation of the p70S6K ATP binding site (K123M). NEK6 catalyzes a time- and concentration-dependent phosphorylation of GST-p70S6KΔCT104 (wild-type and K123M), whereas GST is not phosphorylated by NEK6 (Figure 3a,c). A comparable amount of recombinant NEK7 polypeptide, by contrast, phosphorylates GST-p70S6KΔCT104 at 25% of the rate of catalysis by NEK6 (Supplementary material); further experiments therefore focused on the characterization of recombinant NEK6. Mutation of the NEK6 ATP binding site (K74 K75 to MM) abolished NEK6-catalyzed p70S6K phosphorylation (Figure 3c). As shown by anti-Thr412PO_4^- immunoblotting, NEK6-catalyzed phosphorylation of p70S6KΔCT104 (wild-type and K123M) occurs in part on Thr412, and this component is abolished by mutation of p70Thr412 to Ala (Figure 3b,c). Nevertheless, this mutation diminishes overall NEK6-catalyzed p70S6KΔCT104 phosphorylation by only 20%-30% (Figure 3c). Thus, NEK6 corresponds to the p70S6KThr412 kinase purified from liver; however, NEK6 also catalyzes substantial phosphorylation of p70S6K at sites other than Thr412 that are yet to be identified.

Activation of p70S6KΔCT104 by NEK6 in vitro and in intact cells: interaction with PDK1

We next examined the effect of NEK6 on p70S6K activity in the presence and absence of PDK1. Phosphorylation of mammalian recombinant GST-p70SK6ΔCT104 by NEK6 resulted in a time-dependent increase in p70S6K activity; this increase reached approximately 3-fold at an overall incorporation of 0.4 mol PO_4^-/mol p70S6K (Figure 4a,b; lane 3 versus lane 1). In parallel, PDK1 catalyzed the incorporation of approximately 0.15 mol PO_4^-/mol p70S6K (Figure 4a; lane 5 versus lane 1) accompanied by a very slight (approximately 1.5-fold) increase in p70S6K activity (Figure 4b; lane 5 versus lane 1). However, if the

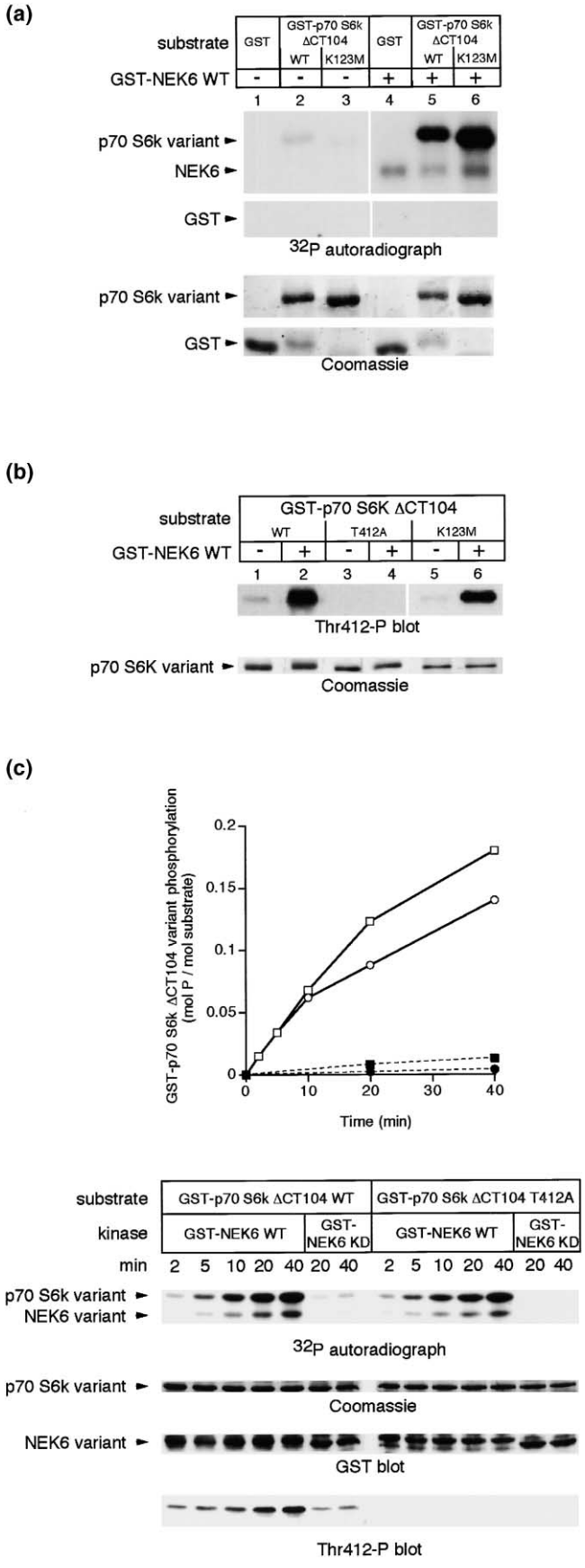
Figure 2

Amino acid sequence and expression of NIMA family kinases (NEK) 6 and 7. **(a)** Alignment of predicted amino acid sequence of murine and human NEK6 and murine NEK7 (derived from overlapping est sequences and PCR from cDNA libraries; see Materials and methods) with *C. elegans* F19H6a.1 and *A. nidulans* NIMA. Amino acid number starting from the initiator methionine is indicated. Conserved residues are shaded in black, and similar amino acids are in gray. The 11 subdomains of a protein kinase are indicated (I-XI). **(b)** Expression of NEK6 and NEK7 mRNA in mouse tissues.



p70S6KΔCT104 polypeptide is first phosphorylated by NEK6 to a stoichiometry of approximately 0.3 mol PO_4^-/mol p70S6K (Figure 4a; lane 4), the addition of PDK1 for 30 min then gives an activation that is nearly 2-fold greater than what would be observed if incubation were continued with NEK6 alone (Figure 4b; compare lane 4

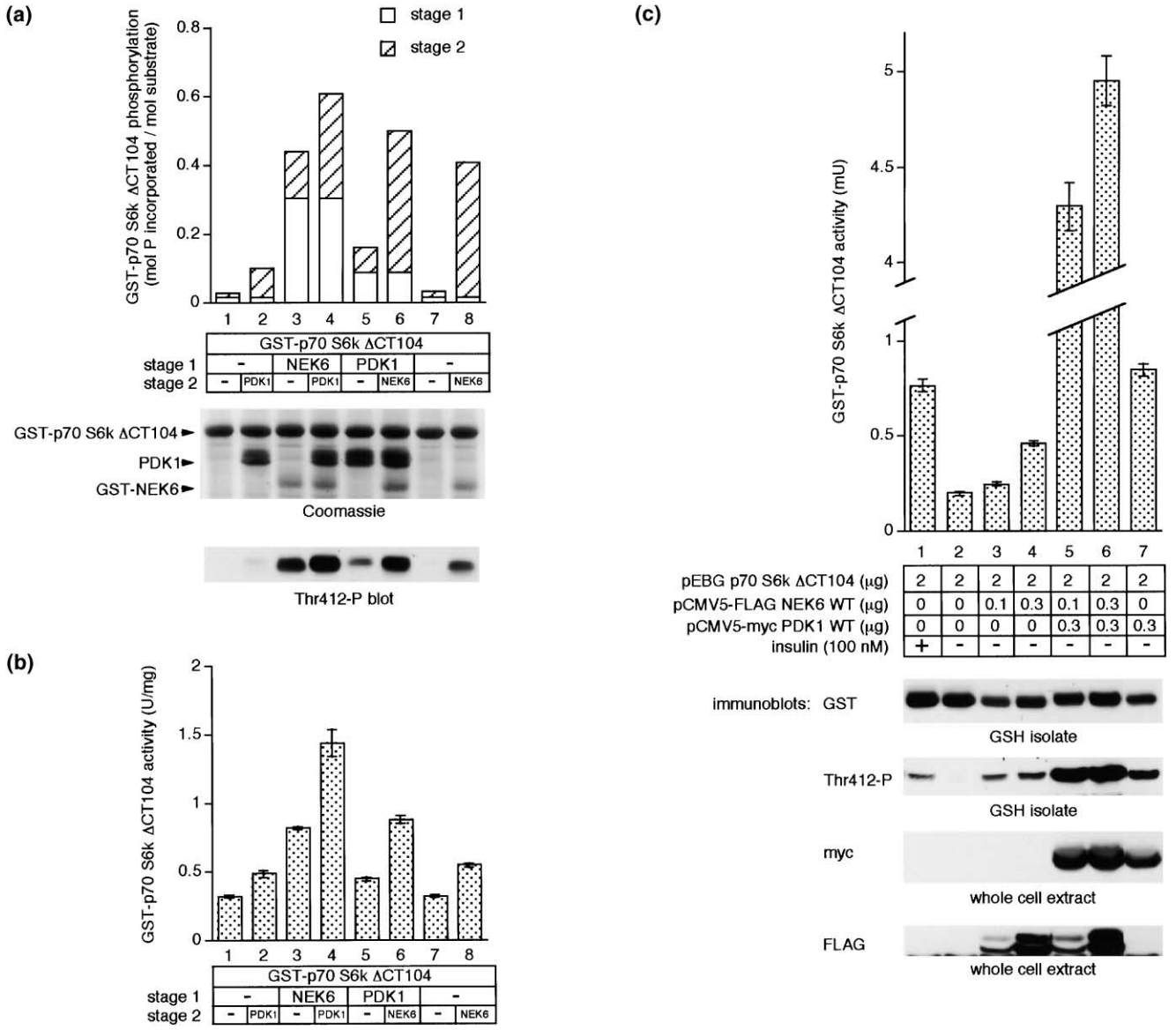
Figure 3



to lane 3). The increase in p70S6K activity engendered by the addition of PDK1 after prior NEK6-catalyzed phosphorylation is substantially greater than the sum of the effects of PDK1 alone (Figure 4b; lane 2 − lane 1) plus NEK6 alone (Figure 4b; lane 3 − lane 1). This synergistic effect is attributable to at least two phenomena; first, the extent of the PDK1-catalyzed phosphorylation of p70S6KΔCT104 is increased nearly 3-fold by the prior NEK6-catalyzed p70 phosphorylation (Figure 4a; lane 2 − lane 1 = 0.05 mol PO_4^-/mol p70S6K, PDK1 alone; lane 4 − lane 3 = 0.17 mol PO_4^-/mol p70S6K, PDK1 after NEK6). This enhancement in the overall PDK1-catalyzed phosphorylation of p70S6K, however, is not sufficient per se to account for the concomitant increase in p70 activity. Simply prolonging the incubation of PDK1 with p70S6K also enables the incorporation of 0.15 mol PO_4^-/mol p70S6K (e.g., Figure 4a, lane 5); the resultant increase in p70 activity (Figure 4b; lane 5 − lane 1) is far less than that achieved by a comparable extent of PDK1-catalyzed p70S6 phosphorylation occurring concomitantly with NEK6 (Figure 4b; lane 4 − lane 3). The phosphorylation of Thr412 when NEK6 and PDK1 act concomitantly may be relatively greater than the sum of the phosphorylation that occurs when either acts alone (compare Thr412PO_4^- in Figure 4a, lane 4 to that in lane 3 plus lane 2); however, we have only qualitative data on this point. It is also likely that the previously demonstrated, positively cooperative effect of the concurrent phosphorylation of Thr252 and Thr412 on p70S6K activity [15] makes an important contribution to the synergistic effect of NEK6 and PDK1 on p70S6K activity. Notably, the synergism between NEK6 and PDK1 on p70S6K activity is evident primarily when NEK6 phosphorylation of p70 precedes PDK1 phosphorylation of p70 (Figure 4b; compare lane 4 to lane 2) rather than the reverse order (Figure 4b; lane 6 versus lane 8), although the low extent of PDK1-catalyzed phosphorylation in the latter conditions tempers this conclusion. Thus, when purified polypeptides were used in vitro, phosphor-

Recombinant NEK6 phosphorylates p70S6KΔCT104 at Thr412. **(a)** NEK6 phosphorylates p70S6K. GST, GST-p70S6KΔCT104 (WT), or the ATP site mutant GST-p70S6KΔCT104 (K123M) were incubated with Mg^{2+} [γ-^{32}P]ATP (0.1 mM) for 15 min at 30°C in the absence (-) or presence (+) of GST-NEK6 (WT). An autoradiograph is shown in the upper two panels. **(b)** NEK6 phosphorylates p70S6KThr412. Incubation as in (a), except for the additional presence of GST-p70S6KΔCT104 (Thr412Ala). An anti-Thr412PO_4^- immunoblot is shown. **(c)** Time course of the NEK6-catalyzed phosphorylation of p70S6KΔCT104 and p70S6KΔCT104 (Thr412Ala). GST-p70S6KΔCT104 (squares) or GST-p70S6KΔCT104 (Thr412Ala) (circles) were incubated as in (a) with either GST-NEK6 (open symbols) or the ATP site mutant GST-NEK6 (K74M, K75M) (closed symbols); the reactions were terminated at the times indicated (minutes). ^{32}P incorporation (mol PO_4^-/mol polypeptide) into the p70S6K variants is shown quantitatively and by autoradiography. The bottom panel shows the time course of Thr412 phosphorylation by an αThr412PO_4^- immunoblot.

Figure 4



NEK6 activates p70S6KΔCT104 in vitro and in vivo and synergizes with PDK1. **(a)** Phosphorylation of p70S6KΔCT104 by NEK6 in the absence and presence of PDK1. In the first stage of a three-stage incubation, dephosphorylated GST-p70S6KΔCT104 is incubated in the presence of Mg ATP for 30 min, alone (open bars, lanes 1 and 2, 7 and 8) or in the presence of GST-NEK6 (open bars, lanes 3 and 4) or baculoviral PDK1 (open bars, lanes 5 and 6). In the second stage, the vehicle (hatched bars, lanes 1 and 3) or PDK1 (hatched bars, lanes 2 and 4) was added to GST-p70S6KΔCT104 that had been previously incubated alone (lanes 1 and 2) or with NEK6 (lanes 3 and 4). In parallel, the vehicle (hatched bar, lanes 5 and 7) or NEK6 (hatched bars, lanes 6 and 8) was added to GST-p70S6KΔCT104 that in Stage 1 had received the vehicle (lanes 7 and 8) or PDK1 (lanes 5 and 6). The reaction was continued for a further 30 min and was terminated by the addition of SDS electrophoresis buffer. The incorporation of ^{32}P during the first stage (open bars) and a second stage (hatched bars) is shown in the upper graph, a Coomassie blue stain of the incubation mixtures after SDS-PAGE is shown in the middle panel, and an αThr412PO_4^- immunoblot of the transfer is shown in the lower panel. **(b)** Activation of p70S6KΔCT104 by NEK6 in the absence or presence of PDK1. After the completion of steps 1 and 2 described in (a), the activity of GST-p70S6KΔCT104 was assayed as the incorporation of ^{32}P into the peptide substrate KKRNRTLTV (see Materials and methods). The error bars indicate ± one standard deviation of triplicate measurements from a single experiment; this experiment is representative of four replicates. **(c)** Activation of p70S6KΔCT104 by NEK6 in intact cells. The reporter plasmid pEBG p70S6KΔCT104 (2 μg) was coexpressed in CHO-IR cells with pCMV5 FLAG vector alone (lanes 1 and 2), pCMV5 Myc-PDK1 alone (0.3 μg, lane 7), or pCMV5 FLAG-NEK6 (0.1 μg, lanes 3 and 5 or 0.3 μg, lanes 4 and 6), alone (lanes 3 and 4) or with (lanes 5 and 6) pCMV5 Myc-PDK1 (0.3 μg). After serum starvation for 16 hr, cells were lysed and the activity of GST-p70S6KΔCT104 in GSH-Sepharose isolates was measured; the cell extracts were immunoblotted for Myc and FLAG, and the GSH isolates were immunoblotted for GST and Thr412PO_4^-, as indicated. Similar results were obtained in three experiments.

ylation of p70S6K ΔCT104 by NEK6 alone substantially activated p70S6K; moreover, NEK6 phosphorylation promoted PDK1-catalyzed p70S6 phosphorylation, and concomitant phosphorylation of p70 by NEK6 and PDK1 provides a synergistic activation of the p70S6K. NEK6 can also phosphorylate GST-PKB in vitro in a Ptd Ins 3,4,5P_3-independent manner, at a rate approximately 20% that of GST-p70S6KΔCT104 (Supplementary material).

The ability of NEK6 to activate p70S6KΔCT104 during transient expression was examined next. A GST-p70S6KΔCT104 fusion protein, expressed in CHO cells that stably overexpress a recombinant human insulin receptor (IR), is activated about 3-fold by insulin. This increase in S6K activity is concomitant with increased phosphorylation at p70S6KThr412 (Figure 4c; lanes 1 and 2). Coexpression of p70S6KΔCT104 with NEK6 results in a dose-dependent increase in p70S6K activity. This increase is approximately 2-fold at 0.3 μg NEK6 DNA and is concomitant with an increase in p70Thr412 phosphorylation (Figure 4c; lanes 3 and 4). Higher amounts of NEK6 DNA result in substantial inhibition of the expression of the recombinant p70S6K polypeptide; such inhibition precludes analysis. Coexpression with PDK1 also activates GST-p70S6KΔCT104, with 0.3 μg PDK1 DNA providing a 3-fold activation similar to that elicited by insulin (Figure 4c; lane 7). Although PDK1 is a rather weak Thr412 kinase in vitro (Figure 4a; lanes 2 and 5 versus lanes 8 and 3), the coexpression of p70S6K with PDK1 in vivo results in robust phosphorylation at Thr412PO_4^- (Figure 4c; lane 7 versus lane 2). The concomitant expression of NEK6 and PDK1 increases p70S6K activity in a strongly cooperative fashion, more than 10-fold greater than the sum of the effects of NEK6 and PDK1 expressed individually with p70S6ΔCT104 (Figure 4c; compare lanes 5 and 6 with lanes 4 and 7). This vigorous activation is accompanied by a clear-cut synergism in the phosphorylation at Thr412. Thus, NEK6 alone activates p70S6K in vivo as well as in vitro and acts synergistically with PDK1 both in the activation of p70S6K and in the phosphorylation at p70S6KThr412.

PDK1 and NEK6 do not alter each other's activity in vitro or in vivo

The clear-cut synergistic effect of NEK6 and PDK1 on p70S6K activity, especially as observed during coexpression in vivo, led us to inquire as to whether NEK6 activates PDK1 or vice versa; this does not appear to be the case. Under conditions in vitro where PDK1 catalyzes significant (Ptd Ins (3,4,5)P_3-dependent) phosphorylation of a recombinant GST-PKB fusion protein, PDK1 catalyzes negligible ^{32}P incorporation into NEK6 [in the absence of Ptd Ins (3,4,5)P_3] and does not alter NEK6 activity, measured as the phosphorylation of GST-p70S6KΔCT104 (Thr252Ala) (Figure 5a). Similarly, coexpression of increasing amounts of Myc-PDK1 with FLAG-NEK6 in HEK293 cells does not alter the ability of FLAG-NEK6 to catalyze MBP phosphorylation in vitro (Figure 5c). Thus, PDK1 does not regulate NEK6 activity, either directly in vitro or by coexpression in vivo. Reciprocally, the incubation in vitro of active GST NEK6 with baculoviral recombinant PDK1 results in negligible PDK1 phosphorylation, although GST-p70S6KΔCT104 is phosphorylated vigorously by GST NEK6 in a parallel incubation (Figure 5b); moreover, PDK1 activity toward GST PKB [in the presence of Ptd Ins (3,4,5)P_3] is unaltered by NEK6. Similarly, the coexpression of increasing amounts of FLAG-NEK6 with Myc-PDK1 in HEK293 cells does not alter PDK1 activity toward GST PKB measured in vitro. Thus, there is no crossregulation evident between PDK1 and NEK6, and this finding indicates that their synergistic activation of p70S6K is mediated at the level of p70S6K itself.

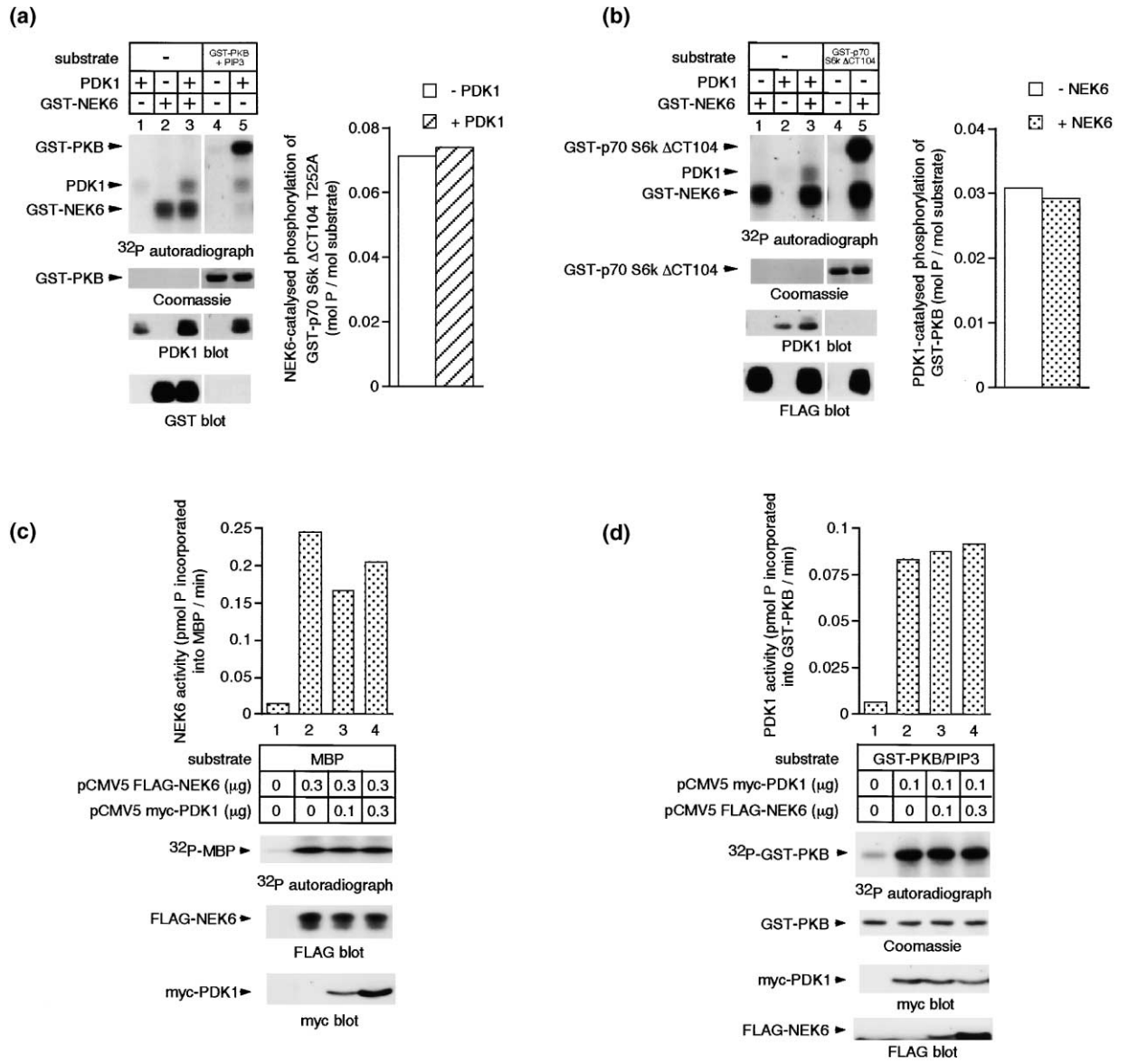
A kinase-deficient NEK6 interferes with insulin-stimulated p70S6KΔCT104 activation and Thr412 phosphorylation

The ability of NEK6 to activate p70S6K, in vitro and in vivo, in a cooperative fashion with PDK1 is consistent with a role for NEK6 in the physiologic regulation of p70S6K. To test further this possibility, we examined whether a kinase-deficient NEK6 could interfere with the ability of insulin to activate the p70S6K. NEK6 (K74M, K75M) was expressed in CHO-IR cells with GST-p70S6KΔCT104. Increasing amounts of inactive NEK6 progressively repressed expression of the p70S6K; however, at levels of kinase-inactive NEK6 that did not affect the expression of p70S6K reporter polypeptide, we observed an approximately 50% decrease in both the basal and insulin-stimulated activity of p70S6KΔCT104. This decrease was concomitant with a decrease in phosphorylation at Thr412 (Figure 6). Thus, a kinase-inactive NEK6 can interfere with the ability of insulin to phosphorylate and activate the p70S6K.

NEK6 activity requires serine/threonine phosphorylation and is sensitive to inhibitors of PI 3-kinase

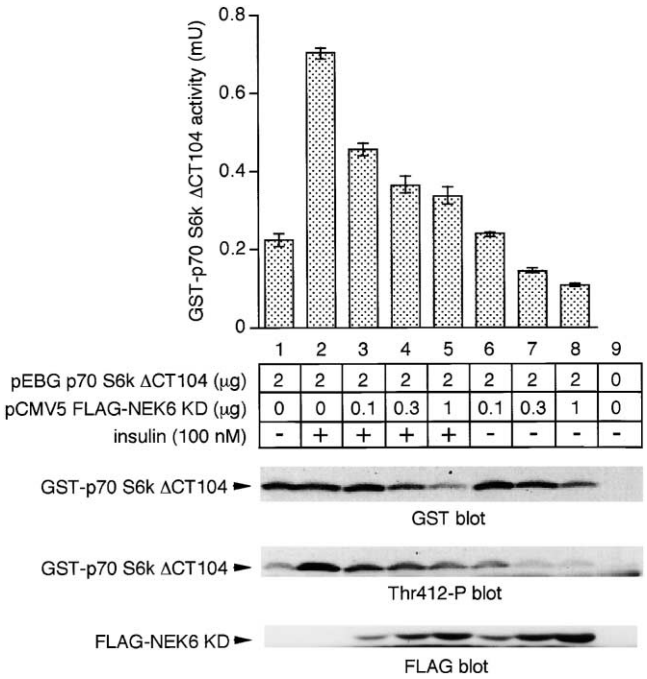
Aliquots of recombinant FLAG-NEK6 that was immunoprecipitated from transiently transfected HEK293 cells were treated with the serine/threonine protein phosphatase PP2A in the presence or absence of Calyculin A. The reaction was terminated by the addition of Calyculin A to the previously untreated samples, and NEK6-catalyzed GST-p70S6KΔCT104 phosphorylation was measured (Figure 7a). PP2A abolished the ability of NEK6 to phosphorylate GST-p70S6KΔCT104 and caused the FLAG-NEK6 polypeptide, which characteristically appears on immunoblots as a doublet, to collapse to a single species, corresponding to the faster migrating band (Figure 7a). The total amount of FLAG-NEK6 polypeptide is unaltered by PP2A, and the activity and electrophoretic mobil-

Figure 5



PDK1 and NEK6 do not regulate each other. **(a)** PDK1 does not phosphorylate NEK6 directly in vitro. PP2A-treated GST-NEK6 (lanes 2 and 3) or GST-PKB plus Ptd Ins 3,4,5P_3 (lanes 4 and 5) were incubated alone (lanes 2 and 4) or with baculovirus recombinant PDK1 (lanes 3 and 5) for 15 min at 30°C with Mg [γ-^{32}P]ATP (100 μM). The reaction mixtures were separated by SDS-PAGE and transferred to PVDF membrane (left panel); the autoradiograph (upper panel) Coomassie blue stain of GST PKB and immunoblots of PDK1 and GST-NEK6 are shown. In the bar graph on the right, the kinase activity of GST NEK6, incubated previously as indicated in the presence or absence of PDK1, toward GST-p70S6KΔCT104 (Thr252Ala) is shown. **(b)** NEK6 does not phosphorylate or activate PDK1 in vitro. PP2A-treated baculoviral PDK1 (lanes 2 and 3) or GST-p70S6KΔCT104 (lanes 4 and 5) were incubated as in (a) in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of FLAG-NEK6. ^{32}P incorporation into PDK1 (lanes 2 and 3) or p70S6KΔCT104 (lanes 4 and 5) is shown in the autoradiograph. The Coomassie blue stain of GST-p70S6K and immunoblots of PDK1 and FLAG-NEK6 are shown below. The bar graph on the right displays the ability of PDK1, incubated previously in the absence or presence of FLAG-NEK6 as described, to catalyze the phosphorylation of GST PKB in the presence of Ptd Ins 3,4,5P_3. **(c)** Coexpression of FLAG-NEK6 with PDK1 does not alter the activity of NEK6 assayed in vitro. A fixed amount of pCMV5 FLAG-NEK6 (0.3 μg) was transiently expressed in CHO-IR cells alone or with increasing amounts of pCMV5 Myc-PDK1 (0.1, 0.3 μg). The kinase activity of NEK6 toward MBP is shown in the bar graph. **(d)** Coexpression of Myc-PDK1 with NEK6 does not alter PDK1 activity assayed in vitro. A fixed amount of Myc-PDK1 (0.1 μg DNA) was transiently expressed alone or with increasing amounts of FLAG-NEK6. The kinase activity of Myc-PDK1 toward GST PKB, in the presence of Ptd Ins 3,4,5P_3, was assayed in vitro.

Figure 6



A kinase-deficient NEK6 inhibits the basal and insulin-stimulated activity of p70S6KΔCT104. CHO-IR cells were transfected with pEBG-encoded p70S6KΔCT104 alone (lanes 1 and 2) or together with pCMV5 FLAG-NEK6 (K74M, K75M; KD; lanes 3–8) in the amounts indicated. Following serum-deprivation for 16 hr, cells were treated with the vehicle (lanes 1,6-8) or insulin (100 nM; lanes 2–5) for 10 min prior to extraction. The activity of GST-p70S6KΔCT104, affinity purified on GSH-Sepharose from aliquots of cell lysates matched for protein, was assayed by the use of a synthetic peptide substrate (see Materials and methods). Aliquots of GSH isolates or total cell lysate were resolved by 7.5% SDS-PAGE and immunoblotted with GST-specific (top panel), phospho-Thr412-specific (middle panel), or FLAG-specific (bottom panel) antibodies. Similar results were observed in three experiments.

ity of FLAG-NEK6 is not altered if PP2A treatment occurs in the presence of Calyculin A. Thus, NEK6 activity requires NEK6 serine/threonine phosphorylation. It should be noted that the autophosphorylation of recombinant NEK6 in vitro is confined to the upper band of the doublet, and this observation suggests that only this upper, phosphorylated NEK6 polypeptide possesses kinase activity. Moreover, the slowed mobility of wild-type NEK6 on SDS-PAGE after expression in vivo appears to require NEK6 catalytic activity in light of the fact that NEK6 (K74M, K75M) migrates as a single band whose mobility corresponds to the faster, inactive band observed with wild-type NEK6.

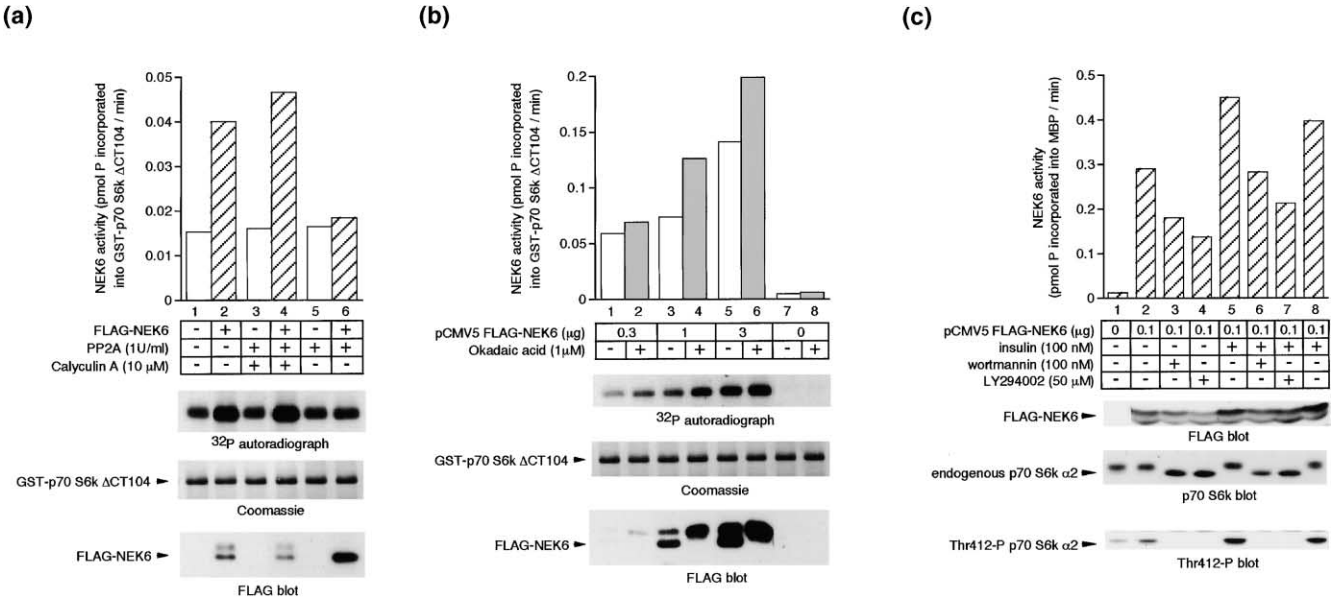
The effect of okadaic acid on the activity of recombinant NEK6 expressed in HEK293 cells was examined. The activity of FLAG-NEK6, expressed at several levels, was enhanced modestly (between 10%-60%) by the pretreatment of cells with okadaic acid prior to extraction (Figure 7b). Concomitantly, okadaic acid caused an increase in the fraction of NEK6 polypeptide migrating at the slower electrophoretic mobility. We interpret these results as an indication that NEK6 is activated by Ser/Thr phosphorylation; moreover, during transient expression the recombinant NEK6 undergoes partial activation that can be modestly augmented by phosphatase inhibitors and that requires NEK6 catalytic activity. These findings presumably reflect a need for autophosphorylation in vivo.

The phosphorylation of p70S6KThr412 in vivo in response to insulin occurs in a PI 3-kinase-dependent manner [10, 11]. We therefore investigated whether NEK6 activity is modified by insulin or the PI 3-kinase inhibitors, Wortmannin and LY294002. CHO-IR cells transiently expressing NEK6 were deprived of serum overnight; the carrier, Wortmannin (100 nM), or LY294002 (50 μM) was added 30 min prior to the addition of buffer or insulin (100 nM), and the cells were harvested 15 min later. Insulin caused a modest increase (1.3- to 1.6-fold) in the activity of recombinant NEK6, which increase was comparable to that seen with okadaic acid (Figure 7c, lanes 5,8). Insulin treatment also caused an increase in the proportion of NEK6 polypeptide in the more slowly migrating band on the immunoblot, and this increase was consistent with increased NEK6 phosphorylation. Wortmannin and LY24002 each caused a 30%–50% decrease in the basal and insulin-stimulated activity of NEK6. Insulin increased the activity of endogenous p70S6K approximately 1.5-fold in these experiments, and the basal and insulin-stimulated p70S6K activity was inhibited by Wortmannin and LY294002 (not shown). Recombinant NEK6 expression gave a clear-cut increase in the Thr412 phosphorylation of endogenous p70S6Kα2 (immunopurified from aliquots of the same extracts; Figure 7, lane 2 versus 1) and Thr412 phosphorylation was strongly suppressed by both Wortmannin and LY294002. In contrast to the modest inhibitory effects of Wortmannin and LY294002 pretreatment on recombinant NEK6, Rapamycin did not alter the mobility of recombinant NEK6 on SDS-PAGE or the NEK6 kinase activity assayed after extraction.

Discussion

This report demonstrates that, in rat liver, the major protein kinase activity capable of phosphorylating in vitro Thr412 on the p70S6K comprises a mixture of two closely related 35 kDa protein kinases in the NIMA family; these protein kinases are designated NEK6 and NEK7. Consistent with this conclusion, recombinant NEK6, isolated after transient expression in mammalian cells, phosphorylates the p70S6K in vitro on Thr412. This phosphorylation is accompanied by activation of the p70S6K catalytic function. In addition, NEK6 collaborates with PDK1 to produce a synergistic increase in the catalytic activity of p70S6K, in vitro and especially in vivo. The mechanisms

Figure 7



NEK6 activity requires serine/threonine phosphorylation. **(a)** The effect of PP2A on NEK6 activity in vitro. Aliquots of recombinant FLAG-NEK6 from transiently transfected HEK293 cells (lanes 2, 4, and 6) or control FLAG IP (lanes 1, 3, and 5) were treated in vitro with (lane 3–6) or without (lanes 1 and 2) protein phosphatase 2A (PP2A, 1 U/ml) in the presence (lanes 3 and 4) or absence (lanes 5 and 6) of Calyculin A (1 μM) for 1 hr. at 30°C. Calyculin A was then added to the samples that had not already received the drug (lanes 1, 2, 5, and 6), and NEK6 activity was assessed by the measurement of its ability to catalyze ^{32}P incorporation from [γ-^{32}]ATP into GST-p70S6KΔCT104 (top and middle panels). The effect of PP2A treatment on the electrophoretic mobility of NEK6 polypeptide is shown in the anti-FLAG immunoblot (lowest panel). **(b)** The effect of okadaic acid on NEK6 activity in intact cells. HEK293 cells transiently transfected with increasing quantities (0–3 μg) of pCMV5 FLAG-NEK6 were serum deprived for 16 hr prior to treatment with the cell-permeable PP2A inhibitor okadaic acid (1 μM) for 30 min. The phosphotransferase activity of immunoprecipitated FLAG-NEK6 is shown in the bar graph and autoradiograph. An anti-FLAG immunoblot is shown in the bottom panel. **(c)** NEK6 activity is enhanced by insulin and is sensitive to inhibitors of PI 3-kinase. CHO-IR cells transiently expressing the FLAG-NEK-6 expression vector were serum deprived for 16 hr prior to treatment with the vehicle (lane 2), Wortmannin (100 nM, lanes 3 and 6), or LY294002 (50 μM, lanes 4 and 7). Thirty minutes later, the vehicle (lanes 1–4) or insulin (100 nM, lanes 5–8) was added, and the cells were extracted 15 min later. The activity of FLAG-NEK6 toward MBP is shown in the bar graph, and immunoblots of recombinant FLAG-NEK6 and endogenous p70S6Kα2 polypeptide and Thr412PO_4^- are shown below. This is representative of four replicates.

by which NEK6 and PDK1 cooperate to generate a synergistic activation of p70S6K are not entirely known, but they do not appear to involve any crossactivation of NEK6 by PDK1 or vice versa. Based on in vitro experiments, it appears that prior phosphorylation of p70S6K by NEK6 increases both the extent of phosphorylation achieved by a subsequent incubation with PDK1 as well as the size of the increase in p70S6K catalytic activity engendered by any level of PDK1-catalyzed p70S6K phosphorylation. These responses are consistent with the previous demonstrations that the substitution of an acidic residue for Thr412 enhances the binding of PDK1 to p70S6K and that the simultaneous modification of Thr252, the primary site of PDK1 phosphorylation, and Thr412 generates a strong, positively cooperative activation of p70 catalytic activity [15]. Nevertheless, NEK6 clearly phosphorylates sites on p70S6K other than Thr412; the identification of those sites and their contribution to the regulation of p70S6K is yet to be determined. Moreover, the fact that the activation of p70S6K elicited by NEK6 and PDK1 coexpression in vivo is much more robust than that observed for purified PDK1 and NEK6 in vitro suggests that additional factors necessary for the full activation of p70S6K remain to be identified.

The mammalian family of NIMA-related kinases or NEKs currently numbers seven. NIMA, the founding member, is an *Aspergillus nidulans* gene product required, in collaboration with cdk1, for the initiation and progression of mitosis [23]. The functions of the mammalian NIMA-related kinases are in general poorly understood. The similarity between NIMA and the mammalian NIMA homologs is largely confined to their (usually) amino-terminally located catalytic domains, which share 40%–50% amino acid sequence identity. NEKs 1–3 contain completely distinct, nonhomologous carboxy-terminal noncatalytic tails, implying distinct functions and/or regulation. For example, NEK2 is centrosome-associated and, like NIMA, shows peak expression at the G2M boundary [24]. NEK2 is implicated in centrosome separation through the

phosphorylation of the centrosome-associated polypeptide NAP2 [25]. In contrast, NEK3 is a cytoplasmic enzyme that is somewhat more abundant in G0 and shows little variation in activity across the cell cycle [26].

NEK6 is the third protein kinase reported to phosphorylate p70S6KThr412, the others being mTOR [18, 19] and PDK1 [21] itself. The most significant question is whether any of these function physiologically as p70S6KThr412 kinases, and under what circumstances. The mTOR kinase phosphorylates p70S6K at Thr412 and several other sites in vitro, and it collaborates in vitro with PDK1 in a manner quite similar to that described herein for NEK6; phosphorylation of p70S6K by mTOR increases p70S6K catalytic activity per se and substantially amplifies the activation of p70S6K induced by a subsequent phosphorylation by PDK1 [19]. The phosphorylation of p70S6KThr412 is strongly inhibited in vivo by the highly specific mTOR inhibitor, Rapamycin [20]. Moreover, the activity of mTOR (at least as assayed in vitro with eIF-4E BP as a substrate) is reported to be modestly increased by insulin [27]. Nevertheless, several observations suggest that although an active mTOR is critical for the insulin-stimulated phosphorylation of p70Thr412 in vivo, this requirement is not attributable to mTOR's ability to act directly as an insulin–stimulated p70S6KThr412 kinase. Thus, the properties and specificity of the eIF-4E BP- and p70S6K-directed kinase activities of mTOR are quite distinct, and we have been unable to detect any insulin-induced increase in the mTOR kinase activity assayed with a p70S6K substrate [19]. In addition, the p70S6K mutant Δ2-46/ΔCT104, which is resistant to inhibition by Rapamycin in vivo, nevertheless undergoes substantial insulin-stimulated phosphorylation of Thr412 and activation in vivo in the presence of Rapamycin concentrations far in excess of those required for complete dephosphorylation of Thr412 on wild-type p70S6K and maximal inhibition of mTOR [20]. In view of these observations, we and others have suggested that mTOR regulates p70S6KThr412 phosphorylation in vivo primarily by the inhibition of a Thr412 phosphatase, probably PP2A [20, 28].

As regards PDK1, although its ability to phosphorylate p70Thr412 in vitro is less than 5% that of p70S6KThr252, coexpression of PDK1 with p70S6K engenders considerable p70S6KThr412 phosphorylation [15, 21]. In addition, PDK1 activity is necessary for p70S6KThr412 phosphorylation in vivo; ES cells lacking PDK1 exhibit no IGF-1 stimulated phosphorylation of p70S6KThr412 [17]. Thus, PDK1 may participate directly as a Thr412 kinase in vivo or may be necessary for the activation in vivo of a p70S6KThr412 kinase. Alternatively, the requirement for PDK1 in Thr412 phosphorylation may arise at a substrate level; we have observed that although the conversion of p70S6KThr252, the primary site of PDK1-directed phosphorylation, to Ala has no effect on PDK1-catalyzed Thr412 phosphorylation in vitro, it greatly diminishes insulin-stimulated p70S6KThr412 phosphorylation in vivo [10].

As regards NEK6, its ability to phosphorylate and activate p70S6K both in vitro and in vivo and to act synergistically with PDK1 is supportive of a physiologic role. In addition, overexpression of a mutant, inactive NEK6 interferes significantly with the ability of insulin to activate p70S6K and promote Thr412 phosphorylation; this finding suggests that NEK6 can interact with and sequester one or more of the elements necessary for the insulin activation of p70S6K. In light of the fact that insulin-stimulated p70Thr412 phosphorylation is inhibited by Wortmannin and LY294002, we anticipated that the responsible protein kinase would also be activated by insulin and inhibited by Wortmannin and LY294002. It is evident that recombinant NEK6 spontaneously acquires significant catalytic activity during transient expression in mammalian cells; this activity can be abolished by treatment with PP2A in vitro. Coexpression with PDK1 does not increase NEK6 activity further, although PDK1 strongly activates p70S6K. Thus, NEK6 is not a PDK1-activated kinase. The activity of recombinant NEK6 is increased modestly (30%–60%) by treatment in vivo with okadaic acid; this increase is associated with slowed mobility on SDS-PAGE, and a comparable mild activation is elicited in response to insulin. The physiologic significance of this modest insulin stimulation is uncertain. The small activation may reflect an artefactually high basal activity associated with transient overexpression. The relatively high basal activity of recombinant NEK6 is modestly inhibited by the PI 3-kinase inhibitors, Wortmannin and LY294002, at concentrations that engender strong inhibition of p70S6K. This finding indicates that the dependence of recombinant NEK6 activity on PI 3-kinase activity is only partial. Thus, the regulatory inputs that control NEK6 activity under physiologic conditions, apart from its dependence on phosphorylation, are as yet unclear. Our efforts to evaluate the regulation of endogenous NEK6/7 have been hampered by an inability to generate antibodies capable of immunoprecipitating endogenous NEK6/7 polypeptides; cell lines expressing recombinant NEK6 are currently in preparation.

In conclusion, NEK6 is a NIMA-related protein kinase that is a novel candidate regulator of the p70S6K. It is capable of phosphorylating and activating this ribosomal protein kinase in vitro and in vivo.

Materials and methods

Assay of Thr412 kinase activity

Each kinase reaction (30 μl) contained 5 μl of extract (or buffer B as a control), 1 μg of Rapamycin-treated baculoviral p70S6KΔCT104, 50 mM MOPS, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 20 mM β-glycerophosphate, and 1 μM Calyculin A. The reactions were initiated by the addition of nonradioactive ATP to a final concentration of 100 μM.

Assays were carried out at 30°C for 10 minutes and terminated by the addition of 10 μl 4× Laemmli sample buffer. Samples were resolved by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-phospho-Thr412 specific antibodies as described in [10]. As a positive control for the phospho-Thr412 Western blot, active, recombinant p70S6KΔCT104, purified from Sf9 cells coinfected with baculoviral p70S6KΔCT104 and a constitutively active PI 3-kinase p110 catalytic subunit [12], was run in a separate lane.

Cloning of NEK6 and NEK7 and construction of expression vectors

The entire open reading frame of human NEK6 and mouse NEK7 was derived by interrogation of the dbEST database at the National Center for Biological Information (http://www.ncbi.nlm.nih.gov/dbEST/index.html) for overlapping est sequences. In addition, a λZAP II F442A mouse 3T3 L1 adipocyte cDNA library was screened by the polymerase chain reaction (PCR) and the use of nested forward or reverse primers corresponding to complementary sequences within regions found to be invariable in overlapping ESTs (encoding subdomain II of mouse NEK7 kinase domain and subdomain VIb of mouse NEK6) and primers annealing to the T3 and T7 promoters in λZAP II. Confirmation of the sequence of the entire open reading frame of mouse NEK6 and NEK7 was achieved by automated DNA sequence analysis of PCR fragments. Amplification by PCR of the open reading frame of human NEK6 was obtained from a human skeletal-muscle cell library by the use of the forward primer 5′-CCGGAATTCTATGGCAGGACAGCCCGGCCACATGCCCCATGGAGGGAGTTCC-3′ and the reverse primer 5′-GGCGAATTCTCAGGTGCTGGACATCCAGATGTGCAT-3′. The product was subcloned as an EcoRI fragment into the mammalian expression vector pCMV5-FLAG, screened for correct orientation by digestion with BamHI, and verified by DNA sequencing. An identical approach was used for the generation of a pCMV5-FLAG-NEK7 expression construct, for which the forward primer 5′-CCGGAATTCTATGGATGAACAATCACAAGGAATGCAAG-3′ and the reverse primer 5′-CCGGAATTCCTATTAGGTGCTTGCGGTACATGCATGC-3′ were used, with both mouse embryonic or T-cell cDNA libraries as a template.

A kinase-deficient version of NEK6 was constructed by PCR mutation of both K74 and K75, lysine residues presumably critical for ATP phosphate binding, to M. Subcloning both wild-type and K74M/K75M NEK6 variants into the pEBG2T plasmid enabled the expression of NEK6 variants as GST-fusion proteins in mammalian cells. The construction of pEBG p70S6Kα1ΔCT104 variants and Myc-tagged PDK1 has been described previously [15].

Northern analysis

A blot of murine mRNA purchased from Clontech was sequentially hybridized with a ^{32}P-labeled PCR fragment of NEK6 (281 nucleotides), NEK7 (270 nucleotides), and beta-actin under standard conditions.

Cell culture and transient transfections

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Sigma) at 37°C in an atmosphere containing 5% CO_2. CHO-IR cells were maintained under 0.66 μg/ml Geneticin (Gibco BRL) selection in Ham F-12 nutrient mixture (Gibco BRL) supplemented with 10% FCS.

For the examination of NEK6 regulation in CHO-IR cells, 10 cm plates at 80% confluency were transfected with pCMV5 FLAG-NEK6 plasmid DNA (0.03–1 μg) in 5 ml OPTI-MEM1 media by the use of Lipofectamine reagent (Gibco BRL) according to the manufacturers instructions. After 5 hr, cells were supplemented with 5 ml Ham F-12 (20% FCS) media containing 0.66 μg/ml Geneticin. After 16 hr, the medium was replaced with Ham F-12 (10% FCS) media. After a further 10 hr, the cells were serum-deprived overnight (16 hr) before treatment with Wortmannin, LY294002, and/or insulin. Cells were extracted in ice-cold buffer A supplemented with Complete protease inhibitor cocktail (1 tablet/50 mls), 50 nM Calyculin A, 1 mM Na_3VO_4, and 0.2% Triton X-100 and centrifuged for 20 minutes at 13,000 × g. Anti-FLAG antibody (2 μg/tube) precoupled to Protein G-Sepharose was added to aliquots of supernatant containing equal amounts of total protein and incubated for 3 hr at 4°C. Immunoprecipitates were washed three times in lysis buffer, three times in lysis buffer containing 0.5 M LiCl, and three times in kinase buffer (50 mM MOPS, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 20 mM β-glycerophosphate, and 1 μM Calyculin A). An assay of immunoprecipitated recombinant NEK6 was carried out under conditions identical to those described for the assay of rat liver kinase activity toward p70S6KThr412 (see above) except that nonradioactive ATP was replaced by 10 μM [γ-^{32}P]ATP (5000–6000 cpm/pmol), and occasionally myelin basic protein (0.5 mg/ml) was used in place of p70S6KΔCT104. Phosphate transfer into NEK6 substrate was quantified by liquid scintillation counting.

For evaluation of the regulation by NEK6 and/or PDK1 of p70S6KΔCT104 in intact cells and the ability of kinase-deficient NEK6 to interfere with insulin activation of p70S6KΔCT104 in intact cells, CHO-IR cells were cotransfected with the appropriate pCMV5 expression constructs (or empty vectors) together with pEBG2T p70S6KΔCT104 as the reporter plasmid; a constant total amount of DNA was used. GST-p70S6KΔCT104 was affinity purified from cell lysates on GSH-Sepharose, and its activity was measured by a peptide kinase assay, described below.

Activation of p70S6KΔCT104 by NEK6 and/or PDK1 in vitro

GST-p70S6KΔCT104 purified from Rapamycin-treated HEK293 cells was further dephosphorylated with PP2A in a buffer containing 50 mM Tris (pH 8.0), 0.1 mM EGTA, 1 mM DTT and 1U/ml PP2A. After incubation at 30°C for 30 min, Calyculin A was added to a final concentration of 10 μM. The activation of GST-p70S6KΔCT104 by NEK6 and/or PDK1 in vitro was carried out in three stages. In the first stage, a 5 μl aliquot of either GST-NEK6 (1.2 μg), baculoviral PDK1 (2.2 μg), or the vehicle was added to 2 μg PP2A-treated GST-p70S6KΔCT104 or GST. The phosphorylation reaction (25 μl), containing 50 mM MOPS (pH 7.4), 10 mM $MgCl_2$, 2 mM EGTA, 20 mM β-glycerophosphate, and 1 μM Calyculin A, was initiated by the addition of unlabeled ATP (100 μM) and incubated for 30 min at 30°C. After stage 1, reactions were placed on ice. In the second stage of the assay, 15 μl of either GST-NEK6 (1.2 μg), baculoviral PDK1 (2.2 μg), or the vehicle diluted in the same kinase buffer constituents (including 100 μM unlabeled ATP) was added to the appropriate tubes, which were incubated for a further 30 min at 30°C. In stage 3, the activity of GST-p70S6KΔCT104 was measured by the use of a synthetic peptide as a substrate; two equal aliquots were taken from each tube and mixed with the vehicle or the p70S6K substrate peptide KKRNRTLTV. The p70S6K reaction (40 μl containing 100 μM KKRNRTLTV peptide and 100 μM [γ-^{32}P]ATP (200–400 cpm/pmol) was initiated by the addition of [γ-^{32}P]ATP and terminated after 10 min at 30°C by spotting onto P81 phosphocellulose paper. The filters were washed in 75 mM H_3PO_4 and quantified by liquid scintillation counting. The amount of peptide-incorporated ^{32}P that was attributed specifically to p70S6K activity was calculated by subtraction of the ^{32}P-cpm from identical control reactions containing GST rather than GST-p70S6KΔCT104. There was no difference in the filter-bound ^{32}P-cpm in reactions with GST- p70S6KΔCT104 or GST when the peptide was absent. One unit (U) of p70S6K activity is defined as the incorporation of 1 nmol phosphate into KKRNRTLTV peptide per minute.

Phosphorylation of GST-p70S6KΔCT104 by NEK6 and/or PDK1

For quantification of the amount of phosphate incorporated into GST-p70S6KΔCT104 by NEK6 and/or PDK1 in each stage of the assay described above, identical reactions were carried out in parallel except that [γ-^{32}P]ATP (2000–3000 cpm/pmol) was used instead of unlabeled ATP during the first and second stages. These reactions were terminated by the addition of Laemmli sample buffer (1× final concentration). Samples were resolved by SDS-PAGE and transferred to PVDF membrane. After Coomassie blue staining and visualization by autoradiography, the membranes were immunoblotted with anti-phospho-Thr412 specific antibodies. Coomassie-stained bands corresponding to GST-

p70S6KΔCT104 were excised thereafter, and the ^{32}P content of each band was quantified.

Supplementary material

Additional methodological details, including information about the purification of kinase activity toward p70S6KThr412 from rat liver and the expression and purification of GST fusion proteins, is available as supplementary material at http://images.cellpress.com/supmat/supmatin.htm.

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