90-kDa ribosomal S6 kinase-2 (RSK2) belongs to a family of growth factor-activated serine/threonine kinases composed of two kinase domains connected by a regulatory linker region. The N-terminal kinase of RSK2 is involved in substrate phosphorylation. Its activation requires phosphorylation of the linker region at Ser369, catalyzed by extracellular signal-regulated kinase (ERK), and at Ser386, catalyzed by the C-terminal kinase, after its activation by ERK. In addition, the N-terminal kinase must be phosphorylated at Ser257 in the activation loop by an as yet unidentified kinase. Here, we show that the isolated N-terminal kinase of RSK2 (amino acids 1–360) is phosphorylated at Ser257 by PDK1, a constitutively active kinase, leading to 100-fold stimulation of kinase activity. In COS7 cells, ectopic PDK1 induced the phosphorylation of full-length RSK2 at Ser257 and Ser386, without involvement of ERK, leading to partial activation of RSK2. Similarly, two other members of the RSK family, RSK1 and RSK3, were partially activated by PDK1 in COS7 cells. Finally, our data indicate that full activation of RSK2 by growth factor requires the cooperation of ERK and PDK1 through phosphorylation of Ser227, Ser369, and Ser386. Our study extend recent findings which implicate PDK1 in the activation of protein kinases B and C and p70S6K, suggesting that PDK1 controls several major growth factor-activated signal transduction pathways.

The 90-kDa ribosomal S6 kinases (RSK1–3)1 are a family of broadly expressed serine/threonine kinases that are activated by extracellular signal-regulated protein kinases (ERK1 and -2) in response to many growth factors, polypeptide hormones, and neurotransmitters (Refs. 1–3; reviewed in Refs. 4 and 5). Inactivating mutations in the RSK2 gene are responsible for the human Coffin-Lowry syndrome, which is characterized by severe mental retardation and progressive skeletal deformations (6, 7). At the cellular level, RSK2 has been proposed to regulate the activity of the transcription factor cAMP response element-binding protein (CREB) (8, 9) and the transcriptional co-activators p300 and CREB-binding protein (10). RSK1 can phosphorylate the estrogen receptor and enhance its transcriptional activity (11) and may also be an activator of the transcription factor NFκB through phosphorylation of IκB (12, 13). Besides a role in transcriptional control, findings in Xenopus laevis oocytes implicate RSK in stimulation of meiosis via inactivation of the p34cdc2-inhibitory kinase Myt1 (14). Finally, RSK can phosphorylate the Ras GTP-exchange molecule SOS and may thereby exert negative feedback of the Ras-ERK pathway (15). Recently, a family of two mitogen- and stress-activated protein kinases (MSK) has been discovered, which resembles RSK in having two kinase domains and other structural hallmarks (16, 17). MSK is activated by ERK as well as by p38 mitogen-activated protein kinase in response to growth factors and various cellular stress stimuli.

The two kinase domains of RSK are connected by a ~100-amino acid sequence, referred to here as the linker. The substrates of RSK identified so far are phosphorylated by the N-terminal kinase (NTK) (18–20), whereas the C-terminal kinase (CTK) and the linker participate in the regulation of the NTK (18, 20, 21). The mechanism of activation of RSK is complex and involves phosphorylation of at least four sites (Fig. 1), as demonstrated with RSK1 in cells treated with phorbol ester, a potent activator of ERK (21). As a probable sequence of events, ERK phosphorylates two sites, one in the linker and one in the activation loop of the CTK, leading to its activation (20–22). The CTK then phosphorylates an additional site in the linker (21, 23). Dual phosphorylation of the linker leads to increased phosphorylation of a serine residue in the activation loop of the NTK and full kinase activity (21). The critical role of this serine is indicated by the finding that its mutation to alanine abolishes the ability of all three RSK isotypes to phosphorylate exogenous substrates in vitro (6, 18, 21) and that this mutation in RSK2 can cause the Coffin-Lowry syndrome (6). The phosphorylation sites in the activation loop of the NTK and linker of RSK are situated in analogous positions to regulatory phosphorylation sites in p70S6K (24), protein kinase B (PKB) (25), and protein kinase C (PKC) (26, 27) (Fig. 1), suggesting a common structural basis of activation for these protein kinases.

The identity of the kinase that phosphorylates the serine in the activation loop of the NTK is unclear. In RSK1, phosphorylation of this serine was greatly reduced when either the NTK or the CTK was inactivated by mutagenesis (21). These observations led to the suggestion that the NTK catalyzes the phosphorylation of the serine in its activation loop. The consensus substrate phosphorylation sequence of RSK is: Arg/Lys-
**FIG. 1. Domain structure and regulatory phosphorylation sites of RSK.** RSK is composed of two kinase domains connected by a regulatory linker region. The C-terminal tail contains a docking site responsible for complex formation with ERK (42, 43). The locations of the four known regulatory phosphorylation sites in RSK and the surrounding amino acid sequences (single-letter code) are shown. Amino acid numbering refers to murine RSK2. Ser<sup>369</sup> and Thr<sup>577</sup> are phosphorylated by ERK (21, 22), Ser<sup>386</sup> by the C-terminal kinase (21, 23), and Ser<sup>227</sup> by PDK1, as suggested by their regulatory phosphorylation sites in RSK and the surrounding amino acid sequences ([linker region](https://www.ncbi.nlm.nih.gov/pubmed/18360656)). The C-terminal tail contains a docking site responsible for complex formation with ERK (42, 43). The locations of the four known regulatory phosphorylation sites in RSK and the surrounding amino acid sequences (single-letter code) are shown. Amino acid numbering refers to murine RSK2. Ser<sup>369</sup> and Thr<sup>577</sup> are phosphorylated by ERK (21, 22), Ser<sup>386</sup> by the C-terminal kinase (21, 23), and Ser<sup>227</sup> by PDK1, as suggested by their regulatory phosphorylation sites in RSK and the surrounding amino acid sequences ([linker region](https://www.ncbi.nlm.nih.gov/pubmed/18360656)).

**Domain Structure:**
- **N-terminal kinase:** EKKA\[SGCGTVYE\]TPK\[PGPDPP\]PPG\[FRSTPV\]
- **ERK docking site:** LLI\[TMFY\]CTY
- **C-terminal kinase:** EKKA\[SGCGTVYE\]TPK\[PGPDPP\]PPG\[FRSTPV\]

**Regulatory Sites:**
- Ser<sup>369</sup> and Thr<sup>577</sup> are phosphorylated by ERK (21, 22)
- Ser<sup>386</sup> by the C-terminal kinase (21, 23)
- Ser<sup>227</sup> by PDK1

**Materials—**
- Human recombinant epidermal growth factor (EGF) was from PreproTech Inc. (Rocky Hill, NJ).
- S6 peptide (residues 231–239 of human 40 S ribosomal protein 6: RRLSSLRA) and phosphopeptide-specific antibodies to RSK (catalog nos. 66-824 and 66-826) were from Upstate Biotechnology (Lake Placid, NY).
- Anti-phospho(cAMP)-dependent protein kinase A (PKA) was from New England Biolabs (Beverly, MA). Anti-phospho(cAMP)-dependent protein kinase A (PKA) was from New England Biolabs (Beverly, MA).
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**EXPERIMENTAL PROCEDURES**

- **Materials—** Human recombinant epidermal growth factor (EGF) was from PreproTech Inc. (Rocky Hill, NJ). S6 peptide (residues 231–239 of human 40 S ribosomal protein 6: RRLSSLRA) and phosphopeptide-specific antibodies to RSK (catalog nos. 66-824 and 66-826) were from Upstate Biotechnology (Lake Placid, NY).
- Anti-phospho(cAMP)-dependent protein kinase A (PKA) was from New England Biolabs (Beverly, MA).

- **Plasmid Constructs—** N-terminally HA epitope-tagged rat RSK1, murine RSK2, or human RSK3 in pMT2 (35) were kindly provided by Christian Bjørbek (Beth Israel Hospital, Boston, MA). RSK1, originally cloned from rat hepatoma cell DNA [36], contained a glutamine insertion at position 157. A glutamine at this site has not been reported in any RSK sequence, nor have we found it in RSK1 cDNA from rat liver.

- **Purification—**
  - E. coli strains were grown in Luria-Bertani (LB) media supplemented with ampicillin at 100 μg/ml. Selection of plasmid-containing colonies was done using X-Gal and IPTG to measure β-galactosidase activity (37).
  - Bacterial extract-tryptone medium to an optical density of 0.8 was inoculated with overnight cultures and grown at 37 °C for 4 h.
  - Bacterial extracts were clarified by centrifugation at 12,000 × g, and GST-RSK was collected on glutathione-Sepharose beads (Amersham Pharmacia Biotech), washed, and eluted with 10 mM

- **Experimental Procedures—**
  - Purification of recombinant human RSK1 was performed by thrombin digestion of the pMT2-HA-RSK1 construct. The purified protein was resolved on 15% SDS-PAGE and transferred to nitrocellulose. Blots were incubated with the mouse anti-HA antibody, followed by detection with a horseradish peroxidase-conjugated secondary antibody and 4-chloro-1-naphthol as the substrate.
  - For the in vivo phosphorylation experiments, NIH 3T3 cells were grown to approximately 30% confluence in 10-cm tissue culture dishes. The cells were then incubated for 24 h in serum-free medium and stimulated with 100 ng/ml EGF for 5 min. The cells were then washed and lysed with a lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), and 1 mM iodoacetamide.
  - The samples were incubated at 30 °C for 30 min and then precipitated with 10% trichloroacetic acid. The precipitates were washed and resuspended in sample buffer and subjected to SDS-PAGE. Blots were probed with the mouse anti-HA antibody, followed by detection with a horseradish peroxidase-conjugated secondary antibody and 4-chloro-1-naphthol as the substrate.

- **Glutathione S-Transferase (GST) RSK Fusion Protein Synthesis and Purification—**
  - E. coli cells (BL21) transformed with various plasmids encoding GST-RSK fusions were grown to an OD<sub>600</sub> of 0.8. The cells were then induced with isopropyl β-D-thiogalactoside (IPTG) at 30 °C for 3 h. The cells were then harvested, resuspended in PBS, and lysed by sonication. The lysates were centrifuged at 12,000 × g, and GST-RSK was collected on glutathione-Sepharose beads (Amersham Pharmacia Biotech), washed, and eluted with 10 mM

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2. C. J. Jensen, S. Gammeltoft, and M. Frodin, unpublished observation.
3. M. Frodin, S. H. Hansen, and S. Gammeltoft, manuscript in preparation.
glutathione in 50 mM Tris-HCl (pH 8). Aliquots of the fusion proteins were fractionated by SDS-PAGE, and protein concentration and purity were assessed as described under "Protein Quantitation." Approximately 90% of the GST-RSK2, GST-RSK2–360, or GST-RSK2–360S227E, and GST-
RSK2–373K100A were in a non-degraded form, whereas only ~10% of full-length GST-RSK2 was non-degraded.

Protein Quantitation—Samples were solubilized in SDS-PAGE sample buffer (2% sodium dodecyl sulfate, 62 mM Tris (pH 6.8), 10% glycerol, 5% 2-β-mercaptoethanol, 0.1% (v/v) phenol red) and fractionated by 10% PAGE. Proteins were stained by incubation of the gel for 20 min in 5000-fold dilution of Sypro Orange™ (Molecular Probes, Eugene™) in 7.5% (v/v) acetic acid. After a brief wash in 7.5% (v/v) acetic acid, the gel was scanned on a STORM™ FluorImager (Molecular Dynamics) and quantified by the ImageQuant™ software using a dilution series of the broad range molecular mass marker from Molecular Probes as a standard.

Transfection and Immunoprecipitation—COS7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C in atmospheric air containing 5% CO2. Monolayers of ~3.2 × 10⁶ cells in 9.6-cm² dishes were incubated for 4–5 h in serum-free medium with a total of 1.5 μg of DNA complexed with 12 μl of LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. In double transfections, 0.75 μg of each DNA construct were used. After transfection, cells were cultured for 48 h and then plated in serum-free medium. After incubation for 3 h in the absence of serum, the cells were exposed (or not) to either growth factor, washed with phosphate-buffered saline, and solubilized for 15 min in 500 μl of lysis buffer (1% Nonidet P-40, 0.5% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 10% glycerol, 1 mM NaVO₄, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin, and 200 kallikrein inhibitor units/ml aprotinin) on ice. Subsequent manipulations were performed at 0–4 °C. Cell extracts were clarified by centrifugation at 15 min at 14,000 × g, and the supernatant was incubated for 3 h with antibody with the addition of 20 μl of Protein G- or Protein A-agarose beads (Amersham Pharmacia Biotech) and autoluminography. Scanning or by enhanced chemiluminescence development (Amersham Pharmacia Biotech) and autoradiography.

RESULTS

PKD1 Phosphorylates and Activates the N-Terminal Kinase of RSK2 in Vitro—We first investigated whether the isolated NTK of RSK2 can autoprophosphorylate or be phosphorylated by RSK2 or PKD1 in vitro. The deletion mutant RSK21–360, which lacks all known phosphorylation sites in RSK, except for Ser²²⁷ in the activation loop, was expressed as a GST fusion protein in E. coli and purified. PKD1 and RSK2 were transiently expressed in COS7 cells and immunopurified. Prior to lysis, RSK2 was activated by exposure of the cells to EGF. RSK2–360 was incubated for 1.5 h with Mg(γ-³²P)ATP alone or with PKD1 or active RSK2 followed by SDS-PAGE and autoradiography. As shown in Fig. 2A (lanes 1 and 2), RSK2–360 did not autoprophosphorylate and was poorly phosphorylated by RSK2. In contrast, RSK21–360 was heavily phosphorylated after incubation with PKD1 (Fig. 2A, lane 4). This phosphorylation was catalyzed by PKD1, rather than by a co-immunopurified kinase, since RSK21–360 showed no phosphorylation by incubation with a kinase-deficient mutant of PKD1 (Fig. 2A, lane 3). PKD1 appeared to phosphorylate RSK21–360 at Ser²²⁷, since the mutant RSK21–360S227E was not phosphorylated by incubation with PKD1 (Fig. 2A, compare lanes 5 and 7). No change in phosphorylation was observed by incubation of RSK2–360S227E with active RSK2.

Kinase measurements showed that RSK2–360 incubated alone or with active RSK2 possessed little ability to phosphophorylate S6 peptide (Fig. 2B). Incubation with PKD1, however, increased the activity of RSK2–360 approximately 100-fold, whereas kinase-deficient PKD1 had no effect (Fig. 2B). Interestingly, the activity of RSK2–360S227E was reduced more than that of RSK2–360 (Fig. 2B), indicating that a negative charge, mimicking a phosphate group, at residue 227 is kinase-activating. More importantly, RSK2–360S227E was not activated by PKD1 (Fig. 2B). Finally, we used the catalytically inactive mutant RSK2–360K100A as a substrate for PKD1. This mutant was heavily labeled by incubation with Mg(γ-³²P)ATP and PKD1, confirming that the NTK of RSK2 is
PKD1 Activates RSK in Vitro and in Vivo

RSK2 activity

RSK2

RSK2

PDK1

PDK1-KD

A

B

C

FIG. 2. PKD1 phosphorylates the isolated NTK of RSK2 at Ser\(^{227}\) and stimulates its kinase activity in vitro. GST-RSK2\(^{1-360}\) or GST-RSK2\(^{1-360}S227E\) were incubated for 90 min at 25 °C with MgATP in the absence or presence of myc-RSK2 (from EGF-treated cells), myc-PDK1-KD (kinase-deficient) immobilized on agarose beads. A, incubations were performed in the presence of \(^{[\gamma-\text{32P}]}\)ATP. After the incubation period, the agarose beads were removed by centrifugation, GST was cleaved from the NTK by thrombin digestion, whereafter the samples were subjected to SDS-PAGE and autoradiography. The experiment was repeated twice with similar results. B, after the incubation period, the agarose beads were removed by centrifugation, and the kinase activity of GST-RSK2\(^{1-360}\) or GST-RSK2\(^{1-360}S227E\) was determined. Data are expressed as percent of the PKD1\(^{1-155}\)stimulated value (ranging from 0.5 to 1 \(\times 10^6\) cpm) and are means ± S.E. of three experiments performed in duplicate. The data of the following bars were compared by non-paired \(t\) test and were different (\(p < 0.01\)): 1 versus 4. In contrast, the following data were not statistically different (\(p > 0.05\)): 1 versus 2, 1 versus 3, 5 versus 6, 5 versus 7. C, GST-RSK2\(^{1-373}K100A\) (kinase-deficient) was incubated alone or together with myc-PDK1\(^{51-556}\) as described in A and thereafter subjected to SDS-PAGE and autoradiography. The experiment was repeated three times with similar results.

PKD1 Phosphorylates and Activates the N-terminal Kinase of RSK2 in Vivo—We next investigated whether HA epitope-tagged RSK2\(^{1-360}\) is phosphorylated by PKD1 or RSK2 when coexpressed in COS7 cells. Lysates prepared from transiently transfected cells were subjected to SDS-PAGE and immunoblotting with antibody to the HA epitope tag to visualize the amount of the kinases. C, GST-RSK2\(^{1-373}K100A\) (kinase-deficient) was incubated alone or together with myc-PDK1\(^{51-556}\) as described in A and thereafter subjected to SDS-PAGE and autoradiography. The experiment was repeated three times with similar results.

These findings indicate that activation loop phosphorylation is necessary and sufficient for activation of the NTK of RSK and that PKD1 can catalyze this event. In contrast, RSK2 is incapable of intra- or intermolecular autophosphorylation at this site and hence RSK2 is incapable of autoactivation.

PKD1 Phosphorylates and Activates the N-terminal Kinase of RSK2 in Vivo—We next investigated whether HA epitope-tagged RSK2\(^{1-360}\) is phosphorylated by PKD1 or RSK2 when coexpressed in COS7 cells. Lysates prepared from transiently transfected cells were subjected to SDS-PAGE and immunoblotting with anti-HA antibody in order to detect decreased electrophoretic mobility of RSK2\(^{1-360}\), indicative of its phosphorylation. Coexpression with RSK2, followed by exposure to EGF, did not affect the electrophoretic migration of RSK2\(^{1-360}\), whereas coexpression with PKD1 induced a profound mobility shift (Fig. 3A, lanes 1–3). Scanning of the gels revealed that more than 80% of RSK2\(^{1-360}\)S227E migrated with decreased mobility, indicating that PKD1 efficiently phosphorylates the NTK of RSK2 in vivo. RSK2\(^{1-360}S227E\) migrated at an intermediate position relative to phosphorylated/unphosphorylated RSK2\(^{1-360}\), and the band was not shifted by coexpression with PKD1 (Fig. 3A, lanes 4–6), indicating that PKD1 phospho-

FIG. 3. PKD1 phosphorylates the isolated NTK of RSK2 at Ser\(^{227}\) and stimulates its kinase activity in COS7 cells. Cells were transfected with plasmids expressing HA-RSK2\(^{1-360}\), HA-RSK2\(^{1-360}S227E\), myc-RSK2, or myc-PDK1 or with empty plasmid, indicated by −. Forty-eight hours after transfection and following a 3-h serum starvation period, the cells were lysed. Prior to lysis, cells expressing myc-RSK2 were exposed to 20 nm EGF for 15 min. A, an aliquot of the cleared lysates were subjected to SDS-PAGE and immunoblotting with antibody to the HA epitope tag on the RSK2\(^{1-360}\) constructs. The experiment was performed four times with similar results. B, HA-RSK2\(^{1-360}\) or HA-RSK2\(^{1-360}S227E\) were precipitated from the lysates remaining from A, using antibody to the HA epitope tag, and subjected to kinase assay. Data are expressed as percent of the PKD1-stimulated value (ranging from 0.6 to 1 \(\times 10^6\) cpm) and are mean values ± S.E. of three experiments performed in duplicate. The data of the following bars were compared by non-paired \(t\) test and were different (\(p < 0.02\)): 1 versus 2, 1 versus 3. In contrast, the following data were not statistically different (\(p > 0.5\)): 4 versus 5, 4 versus 6. Inset to B, to control for equal expression of myc-RSK2 and myc-PDK1, an aliquot of cleared lysate from assays 2 and 3 in B was subjected to SDS-PAGE and immunoblotting for the myc epitope tag.

The kinase activity of immunoprecipitated RSK2\(^{1-360}\) was very low when RSK2\(^{1-360}\) was expressed alone in COS7 cells (Fig. 3B). Coexpression with RSK2, followed by exposure to EGF, induced a 5-fold increase in RSK2\(^{1-360}\) activity (Fig. 3B, lane 2). The activation of RSK2\(^{1-360}\) was mediated by coexpression of RSK2, since it was not observed in control cells treated only with EGF (data not shown). Coexpression with PKD1 increased 120-fold the RSK2\(^{1-360}\) activity in the precipitates (Fig. 3B, lane 3). Depending on the experiment, however, coexpression with PKD1 also increased the protein level of RSK2\(^{1-360}\) by 2–3-fold (see Fig. 3A), so the actual degree of stimulation of RSK2\(^{1-360}\) by PKD1 was 40–60-fold. The specific activity of RSK2\(^{1-360}\) activated by PKD1 corresponded to 30–60% of the activity of full-length RSK2 activated by EGF (data not shown). PKD1 and RSK2 were equally abundant in the cells (Fig. 3B, inset), showing that their differential ability to activate RSK2\(^{1-360}\) was not due to a difference in protein levels. PKD1 and RSK2 did not increase the activity of RSK2\(^{1-360}S227E\) above the basal level (Fig. 3B, lanes 4–6), indicating that both kinases stimulate RSK2\(^{1-360}\) by phosphorylation of Ser\(^{227}\).

Finally, the ability of PKD1 to phosphorylate the NTK of RSK2 in vivo was analyzed in COS7 cells metabolically labeled with \(^{32P}\)orthophosphate. RSK2\(^{1-360}\) was weakly phosphorylated in cells in the basal state or in cells treated with EGF (Fig. 4A, lanes 1 and 2). In contrast, coexpression with PKD1 resulted in strong labeling of RSK2\(^{1-360}\) but not of RSK2\(^{1-360}S227E\) (Fig. 4, lanes 3 and 5).

These data confirm our findings in vitro that autophosphorylation and autoactivation of the NTK of RSK2 occurs inefficiently and show that PKD1 is an efficient activator of the NTK of RSK2 in vivo by phosphorylation of Ser\(^{227}\).
FIG. 4. [32P]Orthophosphate labeling of the isolated NTK of RSK2 in COS7 cells. Cells were transfected with plasmids expressing HA-RSK2, HA-RSK2(S227E), or myc-PDK1-51–556 or with empty plasmid, indicated by −. Forty-eight hours after transfection, cells were incubated with [32P]Orthophosphate for 3.5 h in phosphate- and serum-free medium and exposed, or not, to 20 nM EGF for 15 min followed by lysis. The NTK of RSK2 was precipitated with antibody to the HA epitope tag and subjected to SDS-PAGE and autoradiography. The position and size (in kDa) of molecular mass markers are indicated to the left of the gel.

Full-length RSK1, RSK2, and RSK3 Are Activated when Coexpressed with PDK1 in COS7 Cells—The ability of PDK1 to activate full-length RSK was investigated in COS7 cells transfected with HA epitope-tagged versions of RSK1, RSK2, or RSK3, alone or together with PDK1. For comparison, activation of RSK after treatment of cells with EGF was measured. The activity of RSK was measured using S6 peptide as a substrate, which is phosphorylated by the NTK, but not by the CTK of RSK (20). All three RSKs had relatively high basal activity in COS7 cells that was stimulated 3–10-fold by EGF, depending on isoform (Fig. 5A), in agreement with a previous study (35). Coexpression with PDK1, increased the activity of RSK1 to approximately 40% of the EGF-stimulated value. In contrast, PDK1 increased the activity of RSK2 and RSK3 to 70–80% of that observed in cells exposed to EGF (Fig. 5A). Measurements of the protein amounts of RSK in the immunoprecipitates showed that PDK1 induced a ~1.2-fold increase of RSK2 and a 3–4-fold increase of RSK3. Accordingly, the activity data shown in Fig. 5A are normalized to RSK protein. Immunoblotting for PDK1 showed that its protein level was ~50% reduced by coexpression with RSK1 compared with coexpression with RSK2 or RSK3 (Fig. 5B). However, variable expression levels of PDK1 resulted in the same activation of RSK (Fig. 6), suggesting that PDK1 is not limiting in these cotransfection experiments and that the lower level of activation of RSK1 by PDK1 is not due to less PDK1 expression.

Mechanism of Activation of RSK2 by PDK1—In the present study, PDK1-51–556 (31) and full-length PDK1 were used interchangeably. Although the two forms were expressed at somewhat different levels in COS7 cells (Fig. 6A), they stimulated RSK2 activity to the same degree (Fig. 6B).

We investigated whether the PH domain of PDK1 is required for activation of RSK2. However, a mutant of PDK1, in which the PH domain had been deleted, stimulated RSK2 activity to the same extent as wild-type PDK1 (Fig. 6B), suggesting that targeting to the plasma membrane, directed by the PH domain, is not involved in the activation of RSK2 by PDK1. Furthermore, wortmannin, an inhibitor of the phosphoinositide 3-OH-kinase, did not inhibit the stimulation of RSK2 by PDK1 (data not shown).

In order to investigate whether PDK1 activates RSK indirectly, via its upstream activator ERK, we measured whether PDK1 was able to stimulate the activity of ERK2 in cells coexpressing the two kinases. PDK1, however, had no effect on ERK2 activity in the cells, in contrast to EGF, which induced a 10-fold increase (Fig. 7).

Phorbol ester-induced activation of RSK1 involves phosphorylation of four sites in the linker and kinase regions (21). We analyzed whether the corresponding phosphorylation sites in RSK2 are involved in its activation by PDK1 by using RSK2 in which the phosphorylation sites had been mutated and phosphopeptide-specific antibodies directed against the two regulatory phosphorylation sites in the linker of rat RSK1 (21). These antibodies were found to cross-react with phosphorylated murine RSK2.

The mutant RSK2(S227E) had low basal activity compared with wild-type RSK2 and was not activated by coexpression with PDK1 (Fig. 8A). RSK2/S227E, however, was greatly stimulated by EGF, indicating that the S227E mutation was not obstructive to RSK2 function but specifically eliminated the serine through which PDK1 exerts its stimulatory effect. Immunoblotting of the precipitated RSK2 with phosphopeptide-specific antibodies showed that EGF induced strong phosphorylation of Ser386 and Ser369 in both wild-type RSK2 and RSK2/S227E (Fig. 8, B and C, lanes 2 and 5). PDK1 induced no phosphorylation of Ser369 in RSK2 (Fig. 8C), but, surprisingly,
were present in the precipitates (Fig. 8). RSK2 constructs confirmed that roughly equal amounts of RSK
0.8).

Cells were transfected with plasmid expressing HA-ERK2 together with myc-PDK1,51–556 or with empty plasmid, indicated by COS7 cells. Cells were transfected with plasmids expressing HA-RSK2 together with myc-PDK1 1–556 (wild type), or myc-PDK1 1–458 (lacking the PH domain) or with empty plasmid, indicated by.

Furthermore, RSK2(S369A) showed high basal phosphorylation of Ser369 and Ser386 in the linker and Ser227, catalyzed by PDK1 compared with wild-type RSK2 (Fig. 8 A). Thus, induction of Ser386 phosphorylation is important for activation of RSK2 by EGF as well as by PDK1.

We next investigated the effect of mutating the serine or threonine in the two regulatory sites phosphorylated by ERK. Activation by EGF of RSK2(S369A) (Fig. 9A) and RSK2(T577A) (data not shown) was reduced by 50% compared with wild-type RSK2, whereas mutation of both sites, abolished activation of RSK2 by EGF (Fig. 9A). In contrast, these mutations had no effect on the ability of PDK1 to activate RSK2, nor did the mutations decrease the basal level of RSK2 activity (Fig. 9A).

EGF induced strong phosphorylation of Ser386 in wild-type RSK2 and in RSK2(S369A), but induced no Ser386 phosphorylation in RSK2(S369A/T577A) (Fig. 9B). In contrast, these mutations had no effect on the ability of PDK1 to activate RSK2, nor did the mutations decrease the basal level of RSK2 activity (Fig. 9A).

Since the double mutant should contain an ERK-unrespon- sive CTK, this finding suggests that EGF induces the phosphorylation of Ser386 in RSK2 by activation of the CTK. PDK1 induced robust phosphorylation of Ser386 in wild-type RSK2 and in RSK2(S369A), whereas in RSK2(S369A/T577A) the effect was sometimes less pronounced (Fig. 9B, lanes 3, 5, and 8). Compared with wild-type RSK2, however, the double mutant is hypophosphorylated and will migrate less as a smear and therefore appear slightly less abundant.

Our findings indicate that EGF activates RSK2 by a mecha- nism similar to the one described for phorbol ester-induced activation of RSK1 (21), involving phosphorylation of Ser369 and Ser377 by ERK, leading to activation of the CTK and phosphorylation of Ser386. The combined phosphorylation of Ser369 and Ser377 in the linker and Ser277 catalyzed by PDK1, leads to full activation of the NTK of RSK2. The partial activation of RSK2 achieved by overexpression of PDK1, results from phosphorylation of Ser277 and Ser386 in an ERK-independ-
ERK and PDK1 Cooperate in Activation of Full-length RSK2 in Vitro—Taken together, our findings suggest that growth factor-induced activation of RSK2 involves the cooperative action of PDK1 and ERK. To determine whether the two kinases cooperate in activation of RSK, GST-RSK2 was incubated in vitro with PDK1 and active ERK2, either alone or together. As shown in Fig. 10A, basal RSK2 activity was low and was slightly increased by incubation with active ERK2, whereas incubation with PDK1 resulted in strong activation of RSK2. Addition of ERK2 and PDK1 together, increased RSK2 activity 2-fold compared with incubation with PDK1 alone. In control reactions without RSK2, essentially no S6 peptide phosphorylation was observed (Fig. 10A). Analysis of RSK2 phosphorylation by immunoblotting with anti-phospho-Ser221 antibody, showed that only RSK2 incubated with ERK2 was positive (Fig. 10B). Thus ERK2 had phosphorylated and activated the CTK of RSK2 with subsequent autophosphorylation of Ser386.

These findings indicate that PDK1 is sufficient to induce substantial activation of RSK2, whereas ERK2 is not. However, ERK2 can cooperate with PDK1 in stimulation of RSK2 activity.

DISCUSSION

In the present study, we have shown that PDK1 phosphorylates Ser221 in the activation loop of the NTK of RSK2, leading to substantial activation of the kinase in vitro and in vivo. Furthermore, our findings suggest that constitutively active PDK1 cooperates with ERK in activation of RSK following exposure of cells to growth factor. The role of PDK1 in activation of RSK is analogous to that described for p70S6K (30, 31), PKB (28, 29), and PKC (32), in that activation loop phosphorylation by PDK1 cooperates with phosphorylation of a conserved region C-terminally to the kinase domain in full activation of the kinases.

The mechanism of activation of the NTK of RSK has been enigmatic. Recently, it was suggested that the NTK autophosphorylates as part of the activation mechanism based on the observation that the Asp205 → Ala mutant of RSK1 has an inactive NTK and shows decreased phosphorylation of Ser221, equivalent to Ser386 in RSK2 (21). We have tested this hypothesis experimentally, and find that the isolated NTK of RSK2 does not autophosphorylate at Ser221 in vivo, nor during prolonged incubation at a high concentration in vitro. Additionally, in full-length RSK2, the NTK did not autoactivate in vitro, even when ERK had activated the CTK with subsequent phosphorylation of the linker. Our findings are consistent with two previous studies that failed to detect autophosphorylation in vitro of the NTK of avian RSK synthesized in E. coli (20, 23) and consistent with the fact that Ser221 is situated in a motif that lacks important features of a RSK consensus phosphorylation site (19). All together, our results strongly suggest that the NTK requires a heterologous kinase to catalyze the phosphorylation in its activation loop and that PDK1 may be this kinase. PDK1 acted as an efficient Ser221 kinase, capable of phosphorylating nearly all NTK molecules in cotransfected cells and increasing NTK activity 100-fold in vitro. Some previous observations are consistent with the idea that PDK1, or a kinase with similar characteristics, phosphorylates the activation loop serine in the NTK of RSK. First, RSK1 shows high basal phosphorylation of Ser221 in COS1 cells under conditions where no RSK1 activity was detectable (21), suggesting that the serine is phosphorylated by a constitutively active kinase, like PDK1. A kinase activity with properties very similar to PDK1 has been partially purified from COS1 cells (38). Second, the putative consensus phosphorylation site of PDK1 (33) is
conserved in all RSK isotypes and across species, including *Drosophila melanogaster*, which also has a homologue of PDK1 (39). Furthermore, in one Coffin-Lowry patient, a threonine to isoleucine mutation in the putative PDK1 consensus motif was reported (7). In RSK1, we have mutated this threonine to a glutamic acid and observed a complete loss of kinase activity. Moreover, we have recently been able to co-immunoprecipitate RSK2 and PDK1 from transiently transfected cells. So far, however, we have not been able to inhibit RSK activity by overexpression of the kinase-deficient mutant PDK11-558K111Q/D205A/D223A. Perhaps the mutant with three mutations in the active site is structurally altered and unable to compete with endogenous PDK1.

Our findings with the isolated NTK of RSK demonstrate that it is a functional catalytic entity in the absence of most of the linker and the CTK and provide positive evidence that Ser227 phosphorylation stimulates the activity of the NTK, switching it from very low to very high. The previous notion that the isolated NTK of RSK is inactive (18, 20, 23) is probably due to the lack of phosphorylation of the activation loop serine under the conditions used.

Coexpression of full-length RSK2 or RSK3 with PDK1 led to substantial activation of RSK, apparently by inducing the phosphorylation of Ser227 and Ser386. The phosphorylation of Ser227 is likely catalyzed by PDK1. In contrast, the identity of the kinase that phosphorylates RSK2 at Ser386 in PDK1-transfected cells is not clear, but the CTK is a likely candidate. PDK1 itself is also a candidate, since it was recently shown that PDK1 can phosphorylate the corresponding site in PKB under certain conditions in *vitro* that include the presence of a peptide homologous to the sequence surrounding Ser386 (40). In the present study, PDK1 did not phosphorylate Ser386 *in vitro* (Fig. 10). Interestingly, RSK2(S227E) showed high basal phosphorylation of Ser386 *in vivo* that was not enhanced by coexpression with PDK1. This strongly indicates that phosphorylation of Ser227 in the NTK promotes the phosphorylation of Ser386 in the linker, possibly by causing a structural change in RSK that disposes Ser386 to phosphorylation by the CTK, PDK1, or another kinase. Finally, PDK1 appeared to activate RSK2 without involvement of ERK, since PDK1 neither stimulated ERK2 activity in COS7 cells nor induced phosphorylation of Ser386 in RSK2 and since mutation of Ser386 and Thr377 did not affect activation of RSK2 by PDK1. Moreover, basal RSK2 activity was not affected by the S369A/T577A mutations, but abolished by the S227E mutation. This raises the possibility that basal RSK2 activity in resting cells may be attributed to PDK1 in an ERK-independent manner. In support of this model, basal RSK2 (and RSK3) activity in COS7 cells was not affected by PD98059, an inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, the kinase that activates ERK (35).

EGF was found to activate RSK2 by a mechanism similar to the one described for phosphorostel-ester-induced and ERK-mediated activation of RSK1 in COS7 cells (21). EGF stimulated the phosphorylation of Ser227 and Ser386 in the linker of RSK2, apparently via activation of ERK and the CTK, respectively, and both sites contributed to the activation of RSK2 by EGF, as evidenced by mutational analysis. RSK2, however, differs from RSK1, in that mutation of Ser227 reduced EGF-stimulated RSK2 activity by only 50%, whereas the same mutation in RSK1 abolished its activation by phosphorostel ester (21) or by EGF. The linker may therefore exert tighter inhibitory control of the NTK in RSK1 than in RSK2, in agreement with the fact that RSK1 has lower basal activity than RSK2. ERK alone was not sufficient to activate GST-RSK2 *in vitro*, in agreement with the previous finding that RSK1–3, immunopurified from EGF-treated cells and dephosphorylated by incubation with protein phosphatase 2A, cannot be reactivated by incubation with active ERK1 (35). Only in the presence of PDK1 was ERK able to stimulate the activity of RSK2, suggesting that the two ERK-induced phosphorylations in the linker enhance the activity of the NTK only in conjunction with its phosphorylation by PDK1. In p70S6K, phosphorylation of the threonine corresponding to Ser227 in RSK2 promotes phosphorylation of the activation loop during serum stimulation of cells and mutation of the threonine to a glutamic acid enables PDK1 to catalyze activation loop phosphorylation in a deletion mutant of p70S6K *in vitro* (30, 41). One role of Ser386 phosphorylation in RSK may therefore be to facilitate phosphorylation of the activation loop of the NTK by PDK1. This might partially explain how ERK and PDK1 cooperate in activation of RSK2 *in vitro* and why Ser227 phosphorylation is increased 2–3-fold during ERK-mediated activation of RSK1 in COS1 cells (21). However, our finding that RSK2(S227E) was stimulated 20-fold in response to EGF clearly shows that the EGF-induced phosphorylation(s) can stimulate the activity of the NTK even after it has been phosphorylated in the activation loop. It is possible that phosphorylation of the linker may stabilize the phosphorylated NTK in an active conformation. However, since the isolated NTK phosphorylated at Ser227 displayed high activity despite missing most of the linker (Fig. 2), we speculate that in full-length RSK phosphorylation of the linker serves to release an inhibition of the NTK exerted by the unphosphorylated linker.

In conclusion, we suggest that the level of RSK2 activity in cells is determined by the balanced input from PDK1 and ERK, which act hierarchically, in that ERK cannot activate RSK2 in the absence of PDK1 activity, whereas the opposite is possible. In resting cells, constitutively active PDK1 accounts for basal RSK2 activity, the magnitude of which is a function of the level of available PDK1. Extracellular stimuli that activate ERK increase RSK2 activity above the basal level, because the ERK-induced phosphorylations in the linker cooperate with PDK1 in stimulation of the NTK.

**RSK, p70S6K, PKB, and PKC are activated by growth factors and function in partly distinct signaling pathways that regulate proliferation, protein synthesis, cell survival, and other key processes. It will be important to elucidate the role of PDK1 as a common control mechanism for these pathways. In principle, the level of PDK1 activity may dictate the responsiveness of cells to growth factor action. Furthermore, overexpression of PDK1 results in activation of RSK in a growth factor- and ERK-independent manner. Similarly, ectopically expressed PDK1 has been found to activate PKB in some cell types in the absence of exogenous growth factor (28, 29). High expression of PDK1 may therefore act as an internal activator of some growth factor signaling pathways.**

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