The 90-kDa ribosomal S6 kinases (RSK1–3) are important mediators of growth factor stimulation of cellular proliferation, survival, and differentiation and are activated via coordinated phosphorylation by ERK and 3-phosphoinositide-dependent protein kinase-1 (PDK1). Here we performed the functional characterization of a predicted new human RSK homologue, RSK4. We showed that RSK4 is a predominantly cytosolic protein with very low expression and several characteristics of the RSK family kinases, including the presence of two functional kinase domains and a C-terminal docking site for ERK. Surprisingly, however, in all cell types analyzed, endogenous RSK4 was maximally (constitutively) activated under serum-starved conditions where other RSKs are inactive due to their requirement for growth factor stimulation. Constitutive activation appeared to result from constitutive phosphorylation of Ser232, Ser372, and Ser389, and the low basal ERK activity in serum-starved cells appeared to be sufficient for induction of ~50% of the constitutive RSK4 activity. Finally experiments in mouse embryonic stem cells with targeted deletion of the PDK1 gene suggested that PDK1 was not required for phosphorylation of Ser232, a key regulatory site in the activation loop of the N-terminal kinase domain, that in other RSKs is phosphorylated by PDK1. The unusual regulation and growth factor-independent kinase activity indicate that RSK4 is functionally distinct from other RSKs and may help explain recent findings suggesting that RSK4 can participate in non-growth factor signaling as for instance p53-induced growth arrest.

The 90-kDa ribosomal S6 kinases (RSKs) are serine kinases that are activated by growth factors and many polypeptide hormones via the Ras-dependent mitogen-activated protein (MAP) kinase cascade composed of Raf, MEK, and extracellular signal-regulated kinase (ERK) (Refs. 1 and 2; for a review, see Ref. 3). RSKs contain two functional protein kinase domains, and in mammals three widely expressed homologues (RSK1, RSK2, and RSK3) have been identified. Many studies suggest that RSKs are central mediators of ERK in regulation of cellular division, survival, and differentiation via phosphorylation of numerous intracellular proteins. For instance, RSK mediates ERK-induced G1/M phase progression in meiosis I (4) and metaphase arrest in meiosis II (5, 6) of Xenopus laevis oocytes, which may in part occur via phosphorylation of the p34<sup>cdc2</sup>, inhibitory kinase Myt1 (7) and the protein kinase Bub1 (8), respectively. In somatic cell types, RSK may stimulate cell division through phosphorylation of substrates like p27<sup>Kip1</sup> (9) and glycogen synthase kinase-3 (10–12); protein synthesis and cell growth through substrates like elongation factor 2 kinase (13), glycogen synthase kinase-3 (10–12); transcription initiation factor IA (14), and tuberous sclerosis complex-2 protein (15); cell survival through Bad (16); and transcription through substrates like c-Fos (17) and estrogen receptor (18). Experiments with PC12 cells suggest that RSK is a key mediator of ERK in neurotrophin-induced neuronal differentiation (19). Finally genetic evidence from human and mouse has identified an important role for RSK2 in osteoblast differentiation and function through phosphorylation of activating transcription factor-4 (20) and in stimulation of white adipose tissue mass via an unknown mechanism (21). RSK is related to the mitogen- and stress-activated protein kinases (MSK1 and MSK2), which also contain two kinase domains. MSK, however, is activated by the ERK family as well as by p38 family MAP kinases and thereby functions in signal transduction of growth factors as well as of cellular stress stimuli like UV/radioactive irradiation and proinflammatory cytokines (22, 23).

The substrates of RSK are phosphorylated by the N-terminal kinase (NTK) domain (24–27) whose activity is regulated by MSK, mitogen- and stress-activated kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; MAP, mitogen-activated protein; NTK, N-terminal kinase; PDK1, 3-phosphoinositide-dependent protein kinase-1; HA, hemagglutinin; HEK, human embryonic kidney; CHO, Chinese hamster ovary; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ES, embryonic stem; IR, insulin receptor; Ab, antibody; GST, glutathione S-transferase; PBS, phosphate-buffered saline; EST, expressed sequence tag.
the C-terminal kinase domain and a linker region between the two kinase domains (Fig. 1). The activation mechanism of RSK is complex and involves sequential phosphorylation of four regulatory sites (28), which are Ser\(^{295}\), Ser\(^{297}\), Ser\(^{389}\), and Thr\(^{581}\), using RSK4 numbering. First, ERK, bound to a C-terminal MAP kinase docking site (29, 30), phosphorylates Ser\(^{295}\) in the linker and Thr\(^{581}\) in the activation loop of C-terminal kinase (28, 31). Phosphorylation of Thr\(^{581}\) activates C-terminal kinase, which thereafter autophosphorylates RSK at Ser\(^{389}\), located in a so-called hydrophobic motif in the linker (32). Phosphorylation of Ser\(^{389}\) in the hydrophobic motif generates a transient docking site that recruits as well as stimulates the activity of 3-phosphoinositide-dependent protein kinase-1 (PDK1) (25), which then phosphorylates Ser\(^{372}\) in the activation loop of the NTK of RSK (33, 34). After dissociation of PDK1 from RSK, the Ser\(^{389}\)-phosphorylated hydrophobic motif interacts with a binding pocket within the NTK domain and thereby activates the NTK in synergy with phospho-Ser\(^{372}\) (35). The phosphorylation of Ser\(^{372}\) enhances the activity of the NTK by a yet unknown mechanism (28, 33). Apart from these four activating sites, Thr\(^{581}\) is phosphorylated by ERK, but the site has not been found to regulate kinase activity (28). Moreover Ser\(^{742}\) appears to be phosphorylated by the activated NTK (28, 36), which results in decreased affinity of RSK for ERK, serving as an intramolecular feedback inhibitory mechanism that operates in RSK1 and RSK2 but not in RSK3 (36). The activation mechanism of MSK is thought to be very similar to that of RSK except for two features (22, 23, 37–39). First, the MAP kinase docking site can interact with both ERK and p88 MAP kinase, explaining why MSK is activated by two MAP kinase pathways. Second, the activation loop of the NTK is not phosphorylated by PDK1 but probably via autophosphorylation.

Recently a search for the mental retardation gene in a complex disease locus at Xq21 resulted in the discovery of a gene encoding a new RSK/MSK homologue (40), which contains all the structural hallmarks of this kinase family (Fig. 1). Due to highest amino acid sequence identity to RSKs, the kinase was named RSK4 (gene symbol RPS6KA6), but no functional studies were performed to confirm the relationship. Although RSK4 was found to be deleted in several patients with alterations at Xq21, a definitive link to mental retardation could not be established. At present, the only characterization performed with RSK4 is the demonstration of RSK4 mRNA expression in various human (40) and fetal mouse (41) tissues. Very recently, a short interfering RNA screen targeting 7,914 human genes identified five genes, including RSK4, whose transcripts were required for growth arrest induced by the p53 tumor suppressor protein (42). This finding suggests that RSK4 has a function in growth inhibition and opens the possibility that RSK4 is a tumor suppressor protein. Moreover an expression screen with mouse mRNAs in X. laevis embryos showed that RSK4 expression can disrupt mesoderm formation induced by the fibroblast growth factor-Ras-ERK signaling pathway (43). RSK4 was therefore proposed to be an inhibitor rather than a mediator of growth factor signal transduction. However, the two recent studies provided no characterization of the RSK4 protein, its kinase activity, or regulation. Since RSK1–3 are activated by growth factors it is currently enigmatic how RSK4 can be an inhibitor of growth factor signal transduction and a mediator of p53, which is a transcription factor.

In this study, we characterized RSK4 and demonstrated that RSK4 is distinct from other RSKs by being constitutively activated in serum-starved cells in the absence of growth factor. Our data suggested that constitutive activity was due to constitutive phosphorylation of Ser\(^{295}\), Ser\(^{297}\), and Ser\(^{389}\) in part by very low basal levels of ERK activity and in part by less well defined mechanisms that may include enhanced autophosphorylation ability of RSK4 compared with other RSKs. The constitutive, growth factor-independent activity of RSK4 may help explain how RSK4 can participate in non-growth factor signaling, such as p53-induced growth arrest.

**EXPERIMENTAL PROCEDURES**

Antibodies to RSK4 (sc-17178) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Two different lots, C112 and H112, were used, and both were found to be specific as shown in the experiment depicted in Fig. 2B. Antibodies to RSK2 (sc-1430), the PDK1 phosphorylation site in RSK (sc-12445-R), MSK1 (sc-9392), lamin A/C (sc-7929), and the influenza hemagglutinin (HA) epitope tag (sc-805, used for immunochemistry and immunoblotting) were also from Santa Cruz Biotechnology, Inc. Antibodies to the phosphorylated hydrophobic motif (06-826) and the regulatory ERK phosphorylation site in the linker (06-824) of RSK were from Upstate Biotechnology (Lake Placid, NY). Antibody to phospho-Thr\(^{581}\), raised against RSK2, is described in Ref. 44. Anti-HA Ab used for immunoprecipitation was from the I2CA5 mouse hybridoma cell line. Antibody to p83/CLIMP-63 (43) was kindly provided by Dr. Hans-Peter Hauri (Basel, Switzerland). Antibody to α-tubulin was from the YL1/2 mouse hybridoma cell line. Epidermal growth factor (EGF) was from PreproTech Inc. (Rocky Hill, NJ). S6 peptide (RRLSSS-LRA) and cross-tide (GRPTTSSFAGG) were synthesized by K. J. Ross-Petersen AS (Copenhagen, Denmark). U0126 was from Promega (Madison, WI). SB203580 was from Calbiochem. (γ-32P)ATP was from Amersham Biosciences. Other chemicals were from Sigma.

**Plasmid Constructs—** HA-RSK2 (mouse) and HA-RSK3 (human) in the mammalian expression vector pMT2 (2) were kindly provided by Dr. Christian Bjørkebæk (Beth Israel Deaconess Medical Center, Boston, MA). To generate pMT2-HA-RSK4, the entire human RSK4 coding region was PCR-amplified using pTL10Flag-RSK4 (see below) as template and primers introducing a 5′ Xhol site just prior to the start codon and a 3′ Kpn I site after the stop codon. PCR- product digested with the same enzymes. HA-RSK1 (rat) in pMT2 (46) was kindly provided by Dr. Joseph Avruch. HA-MSK1 in pMT2 is a plasmid vector. Other plasmids were described in Ref. 25. To generate pMT2-FLAG-RSK4, Marathon Ready Human Fetal Brain cDNA (Clontech) was used as template to PCR-amplify the human RSK4 coding region (amino acids 1–745) with primers introducing a 5′ BamHI site just prior to the start codon and a 3′...
EcoRI site after the stop codon. The PCR product was cloned into the pcR2.1 vector (Topo TA cloning kit, Invitrogen). RSK4 was then excised from the pcR2.1 vector using BamHI and cloned into the BamHI site of the mammalian expression vector pMT2-HA-RSK3* generated by PCR amplification of the HA tag and residues 1–40 of RSK2 with a 3′ primer introducing a SalI site and a 5′ primer annealing upstream of the PsI site in pMT2. The PCR product was digested with PsI and SalI and used to replace the corresponding 31 residues of RSK3 in pMT2 digested with PsI and XhoI. Point mutations were introduced in RSK4 using the QuikChange™ (Stratagene) mutagenesis procedure, and the entire coding region was sequenced to confirm that no other mutations were introduced. HA-RSK4-(1–730) and HA-RSK2-(1–725) in pMT2 were generated by introducing a stop codon after residues 730 and 725, respectively, in the wild-type constructs using QuikChange. To generate wild-type and T581A HA-RSK4-(1–730) and HA-RSK3-(731–745) in pMT2, the sequence encoding residues 371–745 of RSK4 was PCR-amplified with primers introducing a 5′ XhoI site just prior to residue 371 and a 3′ KpnI site after the stop codon using wild-type RSK4 and RSK4-T581A, respectively, as a template. RSK2 was excised from pMT2-HA-RSK2 using XhoI and KpnI and replaced with either of the two PCR products digested with the same enzymes. To generate glutathione S-transferase (GST)-JNK2α, the JNK2α(48 residues) coding sequence was amplified by PCR with primers introducing a 5′ NdeI and a 3′ HindIII site and cloned into the NdeI and HindIII sites of the mammalian expression vector pEBG in-frame with GST. The expression construct for HA-c-Jun is described in Ref. 49.

The mammalian expression vector pEBG was modified from pEBG7ram, modified from pEBG, resulting in N-terminal fusion of the FLAG epitope tag. pmT2-HA-RSK3* was generated by PCR amplification of the HA tag and residues 1–40 of RSK2 with a 3′ primer introducing a SalI site and a 5′ primer annealing upstream of the PsI site in pMT2. The PCR product was digested with PsI and SalI and used to replace the corresponding 31 residues of RSK3 in pMT2 digested with PsI and XhoI. Point mutations were introduced in RSK4 using the QuikChange™ (Stratagene) mutagenesis procedure, and the entire coding region was sequenced to confirm that no other mutations were introduced. HA-RSK4-(1–730) and HA-RSK2-(1–725) in pMT2 were generated by introducing a stop codon after residues 730 and 725, respectively, in the wild-type constructs using QuikChange. To generate wild-type and T581A HA-RSK4-(1–730) and HA-RSK3-(731–745) in pMT2, the sequence encoding residues 371–745 of RSK4 was PCR-amplified with primers introducing a 5′ XhoI site just prior to residue 371 and a 3′ KpnI site after the stop codon using wild-type RSK4 and RSK4-T581A, respectively, as a template. RSK2 was excised from pMT2-HA-RSK2 using XhoI and KpnI and replaced with either of the two PCR products digested with the same enzymes. To generate glutathione S-transferase (GST)-JNK2α, the JNK2α(48 residues) coding sequence was amplified by PCR with primers introducing a 5′ NdeI and a 3′ HindIII site and cloned into the NdeI and HindIII sites of the mammalian expression vector pEBG in-frame with GST. The expression construct for HA-c-Jun is described in Ref. 49.

**RESULTS**

We first cloned the predicted coding sequence of the human RSK4 gene into the mammalian expression vector pMT2, which resulted in fusion of the 9-amino acid HA epitope tag to the N terminus of RSK4. In transiently transfected human HEK293 cells, the RSK4 cDNA expressed a single protein product that was of the same size as a putative endogenous RSK4 protein immunoprecipitated from non-transfected HEK293 cells with an antibody directed to the C-terminal sequence of human RSK4 (Fig. 2A). To investigate the specificity of the anti-RSK4 Ab, we tested its ability to precipitate each of the four RSK homologues. To perform this experiment, we first had to generate a soluble mutant of RSK3 because >95% of wild-type RSK3 is lost to the insoluble fraction during clearing of crude cell lysates before immunoprecipitation (Ref. 2 and our data not shown). By constructing and analyzing RSK3-RSK2 chimeras, an RSK3 mutant (RSK3g), in which the N-terminal 40 amino acids derive from RSK2, was found to be soluble and was used for the experiment. N-terminally HA-tagged forms of the four RSKs were transiently expressed in COS7 cells, and cell lysate from each transfection was split and subjected to immunoprecipitation with anti-RSK4 Ab and anti-HA Ab, respectively. Anti-HA immunoblotting on the precipitates showed that the anti-RSK4 Ab precipitated RSK4 only (Fig. 2B). Similar experiments showed that the Ab also does not interact with MSK1 or MSK2 (data not shown). Using the anti-RSK4 Ab, we detected expression of RSK4 protein in lysates from human U2OS osteosarcoma cells, BJ fibroblasts, Ln148 glioblastoma cells, and monkey COS7 kidney fibroblasts (Fig. 2C), whereas no RSK4 was detected in HeLa and Chinese hamster ovary (CHO) cells (data not shown). Immunoblotting on lysates of adult mouse tissues showed RSK4 expression in whole brain, heart, cerebellum, kidney, and skeletal muscle, whereas the remaining tissues analyzed showed no detectable RSK4 (Fig. 2C).

**In Vitro Activation of RSK**—Endogenous RSK4 or RSK2, immunopurified from ~3 × 10^6 serum-starved embryonic kidney (HEK) 293 cells per assay point, were incubated for 30 min at 30°C with 50 μM MgATP in the absence or presence of 50 ng of active ERK2 (Upstate Biotechnology) and 50 ng of active PKD1 (35) in 20 μL of 1.5X kinase assay buffer. Thereafter the kinase activity of RSK4 and RSK2 was determined as described under “Kinase Assays” except that in the present assay the blank reaction contained ERK2 and PKD1.

**Generation of RSK muteins**—The RSK4-specific immunoprecipitation mutant forms of RSK4, modified from pCSG (47), were incubated for 90 min with antibody with the addition of 20 μM MgATP, 10 mM MgCl₂, 1 μg/ml rabbit anti-HA antibody (Amersham Pharmacia Biotech), and 50 ng of active PDK1 (35) in 20 μL of 1.5X kinase assay buffer. Thereafter the kinase activity of RSK4 and RSK2 was determined as described under “Kinase Assays” except that in the present assay the blank reaction contained ERK2 and PKD1.
RSK4 immunoblotting on the nuclear, cytosolic, or membrane fraction of HEK293 cells suggested that RSK4 is localized to the cytoplasm (Fig. 2D). Control blots showed the expected distribution of the marker proteins lamin A/C, α-tubulin, and p63 in the various cell fractions. A predominantly cytosolic localization of RSK4 was confirmed by immunochemistry and confocal microscopy to HA-RSK4 transiently expressed in various cell types (Fig. 2E). However, a weak nuclear RSK4 signal was also observed, suggesting that a minor fraction of cellular RSK4 may be localized to the nucleus. This fraction might have leaked to the cytoplasmic fraction during the cell fractionation experiment depicted in Fig. 2D. The subcellular distribution of transiently expressed RSK4 was not affected by exposure of the cells to a variety of stimuli that activate MAP kinase pathways. These stimuli included 20 nM EGF or 150 nM PMA for 10, 20, 30, or 60 min; 120 J of UV irradiation and analysis after 20, 40, or 60 min or 2, 4, 7, or 10 h; and 10 μg/ml anisomycin for 30 or 60 min (data not shown). We noted an optimal bipartite nuclear localization signal in a loop within the N-terminal kinase domain of RSK4: 325KRHLFFANIDWDKLYKR341. Although this nuclear localization signal is conserved in all RSKs, it does not appear to be functional since the low nuclear RSK4 signal in transfected cells was not affected by its mutation as in the triple mutant RSK4-K325Q/R326A/K337A (data not shown).

To investigate how the kinase activity of RSK4 is regulated, endogenous RSK4 was immunoprecipitated and assayed from serum-starved HEK293 cells after exposure, or not, to EGF and/or U0126, a specific inhibitor of MEK1 (50). The anti-RSK4 Ab precipitated RSK4 to the same extent under all the conditions (Fig. 3A, lower left panel). Surprisingly the kinase activity of RSK4 was only slightly stimulated by EGF (Fig. 3A, lower left panel). In contrast, the activity of RSK2 precipitated from the same cell lysates was stimulated 10-fold by EGF as expected (Fig. 3A, lower right panel) and as previously demonstrated with RSK1, RSK2, and RSK3 in HEK293 cells (36). Moreover ERK, assayed on the same cell lysates, was robustly activated by EGF (Fig. 3A, upper panel). Incubation with U0126 for 2 h reduced the basal and EGF-stimulated kinase activity of RSK4 by 40–50%. This demonstrates the involvement of the MEK-ERK pathway in activation of RSK4 and also suggests that the very low level of ERK activity in unstimulated cells is sufficient to induce significant activation of RSK4 but not of RSK2. Serum starvation for 18 h, instead of 4 h as in Fig. 3, did not increase the responsiveness of RSK4 to growth factor stimulation nor did incubation for 18 h with U0126 or PD98059, another specific MEK1 inhibitor (51), decrease the activity of RSK4 by more than 50% (data not shown). In agreement with the activity measurements, EGF induced a profound electrophoretic mobility shift in RSK2 (Fig. 3A, lower right panel) most likely due to profound EGF-induced phosphorylation of RSK2. In contrast, EGF induced no significant mobility shift in RSK4 (Fig. 3A, lower left panel), suggesting that no significant phosphorylation was induced. Results identical to those shown in Fig. 3A were obtained by exposure of HEK293 cells to PMA, which is another strong activator of the ERK pathway in this and other cell types (data not shown). Intriguingly also in other cell types analyzed under serum-starved conditions, the activity of endogenous RSK4 was not significantly increased by EGF or PMA but was 30–50% inhibited by U0126. In contrast, RSK2 precipitated from the same cell lysates was robustly stimulated by EGF or PMA. Fig. 3, B–D, shows the results of such experiments with U2OS osteosarcoma cells, BJ fibroblasts, and COS7 cells. These experiments indicate that RSK4 is constitutively activated in cells.

Control experiments showed that one single protein that was reactive with the anti-RSK4 Ab was precipitated in the above experiments, and this protein was competed out by incubation of the anti-RSK4 Ab with the peptide used to raise the Ab (blocking peptide) (Fig. 3E). Note that the band indicated by an asterisk derives from the blocking peptide reagent and not from the cell lysate since it is present in a precipitation performed...
with omission of cell lysate (Fig. 3E, lane 1). Moreover the S6 peptide kinase activity present in HEK293 immunoprecipitates was reduced by 80–90% when the precipitations were performed with anti-RSK4 Ab incubated with blocking peptide or by omission of the anti-RSK4 Ab (Fig. 3F), confirming that kinases other than RSK4 account for only a minor fraction of the S6 peptide kinase activity. In fact, all the data with endogenous RSK4 kinase activity presented in this study have been adjusted by subtraction of a reaction blank, which was determined by performing parallel immune complex kinase assays with omission of the anti-RSK4 Ab in each experiment. We therefore conclude that the kinase activity measured in the experiments derives from RSK4.

To investigate the activity and regulation of RSK4 expressed from the RSK4 cDNA clone, HEK293 cells were transiently transfected with plasmid expressing HA-RSK4 or HA-RSK2 for comparison. Exogenous RSK4 and RSK2 responded to EGF and U0126 in basically the same way as the endogenous kinases except that a 2-fold stimulation of exogenous RSK4 was observed in response to EGF (Fig. 4A). To determine the specific kinase activity of RSK4 compared with that of RSK2, immunoprecipitations for kinase assays were titrated to precipitate similar amounts of HA-RSK4 and HA-RSK2 from transiently transfected COS7 cells. As shown in Fig. 4B, RSK4 has in fact higher specific activity than RSK2 when measured against S6 peptide, the most frequently used in vitro RSK substrate, or against cross-tide, derived from glycogen synthase kinase-3β/H9252 and containing Ser9, which is a physiological RSK phosphorylation site (37). Using full-length glycogen synthase kinase-3β as a substrate, similar results were obtained (data not shown). When correlating for RSK protein levels, the specific activity of RSK4 was 10–20-fold higher than that of RSK2 in serum-starved COS7 cells and 2–3-fold higher than that of RSK2 in EGF-treated COS7 cells. As observed with HEK293 cells, exogenous RSK4 could be stimulated 2–3-fold by EGF in COS7 cells in contrast to the endogenous RSK4, which was not stimulated by EGF in these cell types, as shown in Fig. 3. It should be noted that the above in vitro results do not imply that S6 protein or glycogen synthase kinase-3β are physiological substrates of RSK4 but only reflect that the proteins contain...
The involvement of ERK in activation of RSK4 was supported by the finding that RSK4 can co-immunoprecipitate ERK1 from lysates of transiently transfected COS7 cells (Fig. 7A). In contrast, the mutant RSK4(1–730) with deletion of the C-terminal 15 residues, which contain the predicted MAPK docking site, did not precipitate ERK1, showing that the MAPK docking site is functional. Interestingly RSK4 co-precipitated from 2 to 3 times as much ERK1 as did similar amounts of RSK2 (Fig. 7A). Finally RSK4 showed no ability to co-immunoprecipitate with p38α or JNK2α2 (Fig. 7, B and C). As controls, p38α and JNK2α2 co-immunoprecipitated with MSK1 and c-Jun, respectively. Thus, these results provide further evidence that RSK4 is regulated by ERK-type MAP kinases but not by stress-activated MAP kinases.

The results presented in Figs. 3–7 support the intriguing conclusion that endogenous RSK4 is maximally (constitutively) activated in serum-starved cells and suggest that the low basal ERK activity is responsible for inducing at least 30–50% of the RSK4 activity under these conditions. RSK4 is thereby markedly distinct from other RSKs, which require growth factor stimulation to obtain significant kinase activity. To further investigate the basis of constitutive RSK4 activity, endogenous RSK4 and RSK2 were precipitated from HEK293 cells and analyzed with phosphospecific antibodies to the three phosphorylation sites known from analysis of RSK1 (28) and RSK2 (33, 35) to directly stimulate catalytic activity, i.e. Ser372, Ser387, and Ser397. This analysis revealed that RSK4 had a very similar level of phosphorylation in serum-starved and EGF-stimulated cells at all three sites (Fig. 8A). By contrast in RSK2, EGF-stimulated phosphorylation 2-fold at Ser372 and >10-fold at Ser377 and Ser395 in a U0126-sensitive manner. The 40–50% reduction of RSK4 kinase activity by U0126 (Fig. 3A) was not paralleled by a 40–50% reduction of phosphorylation of one particular site by U0126 in Fig. 8A. Conceivably U0126 induces a small decrease of phosphorylation at perhaps all of the sites, which is difficult to detect by immunoblotting but which together result in 50% reduction of kinase activity, which can readily be measured by the very accurate kinase assay.

We next subjected endogenous RSK4 or RSK2, immunopre-
Phosphorylation by active ERK2 and PDK1, whereas the activity of RSK4 was not affected (Fig. 8C). By contrast in RSK2, mutation of Ser389 profoundly decreased the kinase activity of RSK4 (Fig. 8C). By contrast, kinase assays on mutants of Thr581 and Thr582 suggested that these sites have no role in the basal activity of exogenous RSK4 in COS7 cells but contribute to the EGF-stimulated activity.

Analysis of RSK4 point mutants surprisingly revealed that phosphorylation of the predicted PDK1 site, Ser389, was not affected by mutation of Ser389 in the predicted PKD1 docking site (Fig. 8C, upper panel). By contrast in RSK2, mutation of the corresponding serine in the PKD1 docking site (Ser386) completely abolished phosphorylation of the PKD1 phosphorylation site (Fig. 8C, upper panel). Also unexpectedly, mutation of Thr581 only slightly decreased the ability of EGF to induce phosphorylation of Ser389. To investigate whether this is due to an inability of the C-terminal kinase of RSK4 to be activated via phosphorylation of Thr581, we analyzed EGF-induced activation of the isolated C-terminal kinase domain with the linker including the Ser389 site (RSK4-(371–745)). As shown in Fig. 8C (lower panel), EGF promoted wild-type RSK4-(371–745), but not RSK4-(371–745)-T581A, to autophosphorylate at Ser389. This shows that the C-terminal kinase of RSK4 is functional. Kinase assays showed that mutation of Ser389 profoundly decreased the kinase activity of RSK4, suggesting that this phosphorylation site has a major role in the basal as well as EGF-stimulated catalytic activity of RSK4 (Fig. 8C). By contrast, kinase assays on mutants of Thr581 and Thr582 suggested that these sites have no role in the basal activity of exogenous RSK4 in COS7 cells but contribute to the EGF-stimulated activity.

Only in one of eight cell lines tested did RSK4 show a low basal activity compared with stimulated kinase activity. Thus, in CHO cells stably transfected with the insulin receptor (IR) (53), the activity of exogenous RSK4 was stimulated about 5-fold by insulin. No endogenous RSK4 protein could be detected in CHO-IR cells (data not shown). Since endogenous RSK4 was not stimulated in any cell type tested, the stimulation in CHO-IR cells may not be physiological. However, this cell line was considered suitable for further analysis of the role of the individual phosphorylation sites in regulation of the catalytic activity of RSK4 due to the low basal versus stimulated activity. Wild-type RSK4 and mutants of the six phosphorylation sites identified in RSKs were expressed and assayed in CHO-IR cells. Stimulation of RSK4 activity by insulin was mediated by ERK since it was completely abolished by U0126.
Functional Characterization of RSK4

RESULTS

Kinase Activity

Individual mutation of Ser372 and Thr581 decreased the activity of RSK4 by 50%, whereas the double mutant S372A/T581A showed a 2-fold reduction in activity compared with wild-type RSK4 (Fig. 9A). Phosphoimmunoblot analysis of RSK4 from CHO-IR cells showed that, as in COS7 cells, mutation of Ser232 did not abolish phosphorylation of Ser232, although a 2-fold reduction was observed (Fig. 9B). Also in COS7 cells, mutation of Thr581 had only a small effect on insulin-stimulated phosphorylation of Ser232, explaining why the T581A mutation had only a small effect on kinase activity.

FIG. 8. Constitutive activation of endogenous RSK4 in serum-starved cells is due to constitutive phosphorylation. A, endogenous RSK4 or RSK2 was immunoprecipitated from serum-starved HEK293 cells treated as described in the legend to Fig. 3A and subjected to immuno blotting with phosphospecific Abs to Ser232, Ser372, or Ser389. The experiment was performed three times with similar results. B, HEK293 cells were serum-starved for 4 h and then lysed. Endogenous RSK4 or RSK2 was immunoprecipitated from the cell lysates and incubated for 30 min with MgATP in the absence or presence of active ERK2 and active PDK1. Thereafter the kinase activity of RSK4 and RSK2 was determined using S6 peptide as a substrate and expressed as percent of the activity after incubation with ERK2 and PDK1. C, COS7 cells were transfected with plasmid expressing wild-type or mutant HA-RSK4 or HA-RSK2. After 20 h and a final 4-h serum starvation period, cells were exposed, or not, for 20 min to 20 nM EGF and then lysed. The lysates were subjected to immunoprecipitation with Ab to the HA tag. Aliquots of the precipitates were subjected to immunoblotting with the Abs indicated in the panel or to kinase assay using S6 peptide as a substrate. Kinase activity is expressed as percent of maximal RSK4 activity, and data are means ± S.D. of three independent experiments. *p, phosphothreonine; pS, phosphoserine.

The finding that the kinase activity of PDK1-deficient ES cells treated with PMA in these experiments, but RSK4 had similar activity in PMA-treated and untreated cells (data not shown). To investigate whether RSK4 may autophosphorylate at Ser232, PDK1-deficient ES cells were transfected with RSK4 mutants (RSK4-K105A or RSK4-P242A/E243A) in which the NTK had been inactivated by mutation of critical residues conserved in virtually all kinases. These mutants had minimal kinase activity and showed profoundly decreased phosphorylation at Ser232 compared with wild-type RSK4 (Fig 10B), suggesting that RSK4 can autophosphorylate at Ser232. Finally, like exogenous RSK4, endogenous RSK4 showed equal phosphorylation of Ser232 in wild-type and PDK1-deficient ES cells (Fig. 10A). To obtain maximal RSK2 activity, the ES cells were treated with PMA in these experiments, but RSK4 had similar activity in PMA-treated and untreated cells (data not shown). The constitutive activity of RSK4 appears to result from constitutive phosphorylation of key activating sites. Thus, RSK4 has different signaling properties and is functionally distinct from other RSKs in that it does not require PDK1 for phosphorylation of Ser232 and activation.

DISCUSSION

We performed the first functional characterization of the putative new RSK family member RSK4. Our data showed that RSK4 is a functional protein kinase that belongs to the RSK, rather than MSK, family since RSK4 interacts with and is regulated by ERK, whereas no evidence for regulation by stress-activated MAP kinases was obtained. However, our data also showed that RSK4 has important features that clearly distinguish it from other RSK proteins. Thus, RSK4 has growth factor-independent, constitutive activity in many cell types and does not require PDK1 for activation. These features imply that RSK4 has different signaling properties and is functionally distinct from other RSKs.

The constitutive activity of RSK4 appears to result from constitutive phosphorylation of key activating sites. Thus, endogenous RSK4 showed the same level of phosphorylation of Ser232, Ser372, and Ser389 in starved and growth factor-stimulated cells, and mutational analysis demonstrated that these sites are important in stimulation of kinase activity. In a simple explanation for the constitutive activation, the expression level (and/or the degree of dephosphorylation) of RSK4 is so low that the basal activity of a highly expressed activating kinase is sufficient to induce maximal phosphorylation of RSK4. This is

likely to be partly the case since the basal activity of ERK contributed to at least 30–50% of the activity of endogenous RSK4 as evidenced by U0126 sensitivity, although basal ERK activity was very low, amounting to less than 5% of the EGF-stimulated ERK activity (Fig. 3A and data not shown). In addition, ERK is a very abundant kinase with an estimated concentration of 1–3 μM in mammalian cells (54), whereas RSK4 has very low expression as discussed below. The above explanation is also indirectly supported by the finding that, in the case of overexpressed RSK4 in COS7 cells, the basal activity of endogenous ERK was not sufficient to induce maximal phosphorylation of the ERK sites in RSK4 conceivably due to high amounts of RSK4 relative to ERK.

The experiments with exogenous RSK4 in COS7 cells also

FIG. 9. Role of individual phosphorylation sites in regulation of RSK4 kinase activity. A, CHO-IR cells were transfected with plasmid expressing wild-type or mutant HA-RSK4 or HA-RSK1. After 36 h and a final 4-h serum starvation period, cells were exposed, or not, for 15 min to 150 nM insulin and then lysed. The lysates were subjected to immunoprecipitation with Ab to the HA tag. Aliquots of the precipitates were subjected to kinase assay using S6 peptide as a substrate or to immunoblotting with Ab to the HA tag (only precipitates from starved cells are shown). Kinase activity is expressed as percent, and data are means ± S.D. of at least three independent experiments. B, wild-type and mutant HA-RSK4 were expressed and purified from CHO-IR cells as described in A and subjected to immunoblotting with the Abs indicated in the panel. The experiment was performed three times with similar results. I.P., immunoprecipitate; pT, phosphothreonine; pS, phosphoserine.

FIG. 10. RSK4 does not require PDK1 for phosphorylation of Ser232 or activation. A, for RSK4, wild-type (+/+) or PDK1-deficient (−/−) mouse ES cells were transfected with plasmid expressing FLAG-tagged RSK4. After 18 h, the cells were exposed to 150 nM PMA for 20 min and then lysed. The lysates were subjected to immunoprecipitation with Ab to the FLAG tag. The precipitates were subjected to kinase assay using S6 peptide as a substrate followed by the addition of SDS-PAGE sample buffer to the precipitates and immunoblotting with Ab to Ser(P)232 or the FLAG tag. To obtain equal amounts of precipitated RSK4 from the two ES cell lines, more lysate from −/− cells than from +/+ cells was used for immunoprecipitation since the −/− cells were transfected with low efficiency. Kinase activity is expressed as percent, and data are means ± S.D. of triplicate determinations. For RSK2, endogenous RSK2 was precipitated with anti-RSK2 Ab from ~107 non-transfected PDK1(+/+) or PDK1(−/−) ES cells exposed for 20 min to 150 nM PMA and then analyzed as described for RSK4. The experiments were performed four times with similar results. B, PDK1-deficient (−/−) ES cells were transfected with plasmid expressing wild-type or mutant HA-RSK4. HA-RSK4 was precipitated using anti-HA Ab and analyzed as described in A. To obtain similar amounts of precipitated RSK4, 5-fold less cell lysate was used for immunoprecipitation of wild-type compared with mutant RSK4 as the mutants showed decreased expression. Kinase activity is expressed as percent, and data are means ± range of two independent experiments. C, approximately 107 wild-type (+/+) or PDK1-deficient (−/−) ES cells were exposed to 150 nM PMA for 20 min and then lysed. Endogenous RSK4 was precipitated with anti-RSK4 Ab and subjected to immunoblotting with Ab to Ser(P)232 or to RSK4. The experiment was performed twice with similar results. pS, phosphoserine; WT, wild type.
suggested that the U0126-insensitive (i.e. apparently ERK-independent) RSK4 activity derives from phosphorylation of Ser\textsuperscript{232} and Ser\textsuperscript{389} since the RSK4-S372A/T581A mutant with all regulatory ERK sites mutated showed considerable basal phosphorylation of Ser\textsuperscript{232} and Ser\textsuperscript{389} as well as considerable basal kinase activity. Mutational analysis furthermore confirmed that Ser\textsuperscript{232} and Ser\textsuperscript{389} are the most important phosphorylation sites in stimulation of RSK4 catalytic activity. This is not surprising since the two sites are conserved in many kinases of the so-called AGC kinase superfamily, which includes RSK, MSK, protein kinase B (PKB), p70 S6 kinase, and serum- and glucocorticoid-inducible kinase, where the two sites have an essential and synergistic effect on kinase activation (35). An absolute requirement for PDK1 in phosphorylation of the activation loop in the NTK of RSK1–3 has been demonstrated biochemically (33, 34) and genetically (37, 55). It is therefore surprising that PDK1 is not required for Ser\textsuperscript{232} phosphorylation and activation of RSK4 as demonstrated here genetically in PDK1-deficient ES cells. Instead RSK4 may autophosphorylate at Ser\textsuperscript{232} as indicated by the reduced Ser\textsuperscript{232} phosphorylation observed in the catalytically inactive RSK4 mutants in PDK1-deficient ES cells. Our results, however, do not exclude that PDK1 may contribute to phosphorylation of Ser\textsuperscript{232} in PDK1 wild-type cells.

Mutational analysis also suggested that phosphorylation of Ser\textsuperscript{372} and Thr\textsuperscript{581} plays a more modest role in stimulation of RSK4 kinase activity than does phosphorylation of Ser\textsuperscript{232} and Ser\textsuperscript{389}. In insulin-stimulated CHO-IR cells, double mutation of Ser\textsuperscript{372} and Thr\textsuperscript{581} had a much greater effect on RSK4 kinase activity than did the single mutations, suggesting that the sites can compensate for each other. It is possible that the unexpected phospho-Thr\textsuperscript{581}-independent phosphorylation of Ser\textsuperscript{389} induced by insulin in the T581A mutant (Fig. 9B) could contribute to the apparent compensating function of the two sites, but the mechanism behind this observation is unknown.

Based on this and previous studies on the RSK activation mechanism, a model for constitutive activation of endogenous RSK4 in serum-starved cells is proposed. Constitutive activation is due to maximal phosphorylation of Ser\textsuperscript{232}, Ser\textsuperscript{372}, and Ser\textsuperscript{389} of which Ser\textsuperscript{232} and Ser\textsuperscript{389} synergize to stabilize the NTK in the active conformation thereby inducing about 80% of the RSK4 activity. The low basal ERK activity is responsible for induction of at least 50% of the RSK4 activity via maximal phosphorylation of Ser\textsuperscript{372} and Thr\textsuperscript{581}. Phosphorylation of Ser\textsuperscript{372} directly stimulates the activity of the NTK, whereas phosphorylation of Thr\textsuperscript{581} stimulates NTK by promoting partial phosphorylation of Ser\textsuperscript{389}, and this event may possibly enhance autophosphorylation of Ser\textsuperscript{232}. The remaining 50%, apparently ERK-independent, RSK4 activity derives from phosphorylation of Ser\textsuperscript{232} and Ser\textsuperscript{386}, which may be catalyzed by autophosphorylation by the NTK and the C-terminal kinase, respectively, although a contribution from additional kinases cannot be excluded.

Two recent studies suggest that RSK4 may have unique cellular functions compared with RSK1–3. In the first study, a short interfering RNA screen was carried out to identify mediators of p53-induced growth arrest, and it was discovered that knock-down of RSK4 abolishes p53-dependent G\textsubscript{1} cell cycle arrest induced either by conditional activation of p53 or by DNA damage via ionizing irradiation (42). It was also demonstrated that RSK4 knock-down strongly suppressed expression of mRNA for the cyclin-dependent kinase inhibitor p21\textsuperscript{WAF1}, a major component of the antiproliferative response to p53. However, the regulation and mechanism of action of RSK4 in the p53 response was not addressed. In the present study, we found no regulation of RSK4 by UV irradiation, which also induces DNA damage and p53 activation, or by p53 transfection in U2OS or BJ cells, the two cell types analyzed in the study mentioned above. Thus, no change in RSK4 activity or subcellular distribution was detected within 8 h after UV irradiation nor did UV irradiation or transfection with p53 plasmid increase the RSK4 protein level within 24 h, although a robust growth arrest was observed (data not shown). It is therefore possible that RSK4 may function as an indirect mediator of p53 in induction of downstream effectors for instance by phosphorylating a protein in the p53 transcriptional activation complex on the p21\textsuperscript{WAF1} promoter or by stimulating p21\textsuperscript{WAF1} mRNA stability in the cytoplasm. The constitutive activity of RSK4 demonstrated here would enable RSK4 to function as such an indirect mediator of p53 signaling.

In the second study, a functional screen of mouse mRNAs showed that injection of RSK4, but not RSK1–3, transcribes into X. laevis one-cell embryos disrupted the subsequent formation of mesoderm, which is induced by the fibroblast growth factor-Ras-ERK pathway (43). The mechanism of inhibition was not resolved, but the inhibitory action appeared to take place at a level downstream of Ras, resulting in reduced phosphorylation of MEK and ERK at the activating sites. In early mouse development, RSK4 shows particularly high mRNA expression in extraembryonic tissue, and RSK4 expression is inversely correlated with the presence of active ERK as detected by anti-active ERK immunostaining. The authors proposed that RSK4 is an inhibitor, rather than a mediator, of growth factor signal transduction via the Ras-ERK pathway in selected cellular contexts. This hypothesis would require that activation of RSK4 can occur in a growth factor-independent manner. In the present study, we found evidence for growth factor-independent activation of RSK4 that may occur via low basal levels of ERK activity and/or autophosphorylation.

Although a quantitative analysis was not performed, the low signal in kinase assays and immunoblots of endogenous RSK4 suggests that RSK4 may be expressed at 10–20 times lower levels than other RSKs in most cell types. This conclusion is supported by the extraordinarily low number of human RSK4 expressed sequence tags (ESTs) in the public data bases. Thus, as of January 2005, Unigene listed 16 RSK4 ESTs, 289 RSK1 ESTs, 282 RSK2 ESTs, and 351 RSK3 ESTs (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene), indicating that low RSK4 protein expression is due to low RSK4 mRNA levels. In agreement with this conclusion, Kohn et al. (41) noted that RSK4 shows relatively broad but low expression compared with RSK2 in fetal mouse tissues. Since RSK4 appears to be constitutively activated in cells and may function to suppress Ras-ERK signal transduction and cell proliferation, the expression level of RSK4 may be low in most cell types to allow cell growth. Conversely up-regulation of RSK4 expression may be one mechanism to restrict cell growth. It can further be speculated that the biological activity of RSK4 is regulated primarily at the level of expression rather than at the level of catalytic activity, the major level of regulation of RSK1–3.

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