Cloning and Characterization of RLPK, a Novel RSK-related Protein Kinase*

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A novel protein kinase whose activity can be stimulated by mitogen in vivo was cloned and characterized. The cDNA of this gene encodes an 802-amino acid protein (termed RLPK) with the highest homology (37% identity) to the two protein kinase families, p90RSK and p70RSK. Like p90RSK and p70RSK, but not p70RSK, RLPK also contains two complete nonidentical protein kinase domains. RLPK mRNA is widely expressed in all human tissues examined and is enriched in the brain, heart, and placenta. In HeLa cells, transiently expressed epitope-tagged RLPK can be strongly induced by epidermal growth factor, serum, and phorbol 12-myristate 13-acetate, but only moderately up-regulated by tumor necrosis factor-α and other stress-related stimuli. The activity of RLPK stimulated by epidermal growth factor was not inhibited by several known protein kinase C inhibitors nor by rapamycin, a known specific inhibitor for p70RSK, but could be inhibited by herbimycin A, a tyrosine kinase inhibitor, and partially inhibited by PD089509 or SB203580, inhibitors for the mitogen-activated protein kinase pathways. Recombinant RLPK possesses high phosphorylation activity toward histone 2B and the S6 peptide, RRRRLSSLRA. Although purified recombinant RLPK can be phosphorylated by ERK2 and p38α in vitro, its activity is not affected by this phosphorylation. Moreover, the treatment of RLPK with acid phosphatase did not reduce its in vitro kinase activity. These data suggest that RLPK is structurally similar to previously isolated RSKs, but its regulatory mechanism may be distinct from either p70RSK or p90RSKs.

Two families of ribosomal S6 protein kinase, p90RSK and p70RSK, have been identified in mammals (1–4). Members of p90RSK family contain two nonidentical, but complete, kinase domains and have molecular masses ranging from 85 to 92 kDa (5–9). The two p70RSK isoforms have molecular masses of 70 and 85 kDa, but are believed to be transcribed from a single gene by a differential splicing (10, 11). Unlike p90RSK, p70RSK contains only one kinase domain, which is about 51% identical to N-terminal kinase domain of p90RSK. These two protein families were collectively called RSK because of their ability to phosphorylate ribosomal S6 protein of the 40 S ribosomal subunit (10, 12–17). However, the fact that specific inhibition by rapamycin of the p70RSK but not p90RSK inhibited growth factor-mediated phosphorylation of S6 protein in vivo demonstrated that p70RSK, rather than any isoforms of the p90RSK, was really responsible for growth-associated phosphorylation of S6 protein (18–20).

p90RSK appears to have a broad range of substrates in vitro that include glycosyn synthase kinase 3, Nur 77, c-Fox (21, 22), serum response factor (23), 40 S ribosomal subunit, and histones H1, H3, and H2B (12, 24). In addition, estrogen receptor α (25), actin-binding protein 280 (26), and CREB1 (27) were reported to be regulated by p90RSK in vivo. It has also been shown that p90RSK2 forms complexes with MAP kinase ERK1 (28, 29) and with a nuclear regulatory protein CBP (30). The scope of these interactions indicate the complexity of the regulation and function of p90RSKs. Although both p70RSK and p90RSK respond to growth factor-related stimuli, quite different mechanisms have been implicated in the regulation of these kinases. The p70RSK is rapamycin-sensitive and relies on multiple phosphorylation to be activated (31–33). The interactions with Rho family members such as RAC1 and Cdc2 may be crucial for p70RSK activation (34). As for p90RSKs, ERK1 and ERK2 were believed to be responsible, at least in part, for their activation (4, 35, 36). Despite the progress in this field, regulation mechanisms and biological functions of these two RSK protein kinase families are still largely unknown.

In this study, we reported cloning and characterization of RLPK, a novel protein kinase with two kinase domains. The overall sequence identity of this kinase to p90RSKs is about 37%, which is much lower than that within the p90RSK family (79–82%), suggesting that while it is homologous to p90RSKs, it is not an isoform of p90RSK family. Despite the distinct amino acid sequence of RLPK, we have found similarities in its substrate specificity and activation profile to that of both p90RSK and p70RSK. We demonstrated that the intrinsic activity of recombinant RLPK is not dependent on phosphorylation and cannot be regulated by MAP kinases in vitro. Therefore, RLPK may represent another class of kinase with two kinase domains, and its regulation mechanism and function may differ from that of p90RSK and p70RSK.

EXPERIMENTAL PROCEDURES

Materials—Pfu DNA polymerase and PHAS-I were purchased from Stratagene (La Jolla CA). S6 peptide (RRRLSSLRA, amino acids 231–245) was synthesized by the Department of Immunology, The Scripps Research Institute. Antisera against p90RSK (5, 6) and p70RSK (8) were gifts from Dr. H. U. Schneggenburger (University of Freiburg, Germany). Anti-epitope-tag antibodies used in Western blot analysis were purchased from Pierce (Rockford, IL). Anti-human CREB antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-actin antibody was purchased from Sigma Chemical Co. (St. Louis, MO). Other reagents and chemicals were from standard suppliers.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF089009.

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‡ The abbreviations used are: CREB, cAMP response element-binding protein; MAP, mitogen-activated protein; EGF, epidermal growth factor; HA, hemagglutinin; GST, glutathione S-transferase; Ni-NTA, nickel-nitritotriacetic acid; PAGE, polyacrylamide gel electrophoresis; CBP, CREB-binding protein; EST, expressed sequence tags.
239), activated and partially purified p70^{S6K} and p90^{RSK} were purchased from Upstate Biotechnology (Lake Placid, NY). Epidermal growth factor (EGF), tumor necrosis factor-a (TNF-a), insulin-like growth factor-1, and phorbol 12-myristate 13-acetate were purchased from Genzyme (Cambridge, MA). Calcium ionophore A23187, histone H1, H2, H3, H4, and phospho-Y-218 H3 were purchased from New England Biolabs. Goat anti-HA (IgG) and mouse anti-actin (IgG) were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibody against HA was purchased from Santa Cruz Biotechnology. Rabbit anti-p90RSK antibody was purchased from Transduction Laboratories. Rabbit anti-p70^{S6K} antibody was purchased from Cell Signaling. Rabbit anti-p42 MAPK antibody was purchased from Cell Signaling. Affinity-purified rabbit anti-p90RSK, or rabbit anti-p42 MAPK from Transduction Laboratories was used as the control. The reactions were stopped by adding an equal amount of 2 mM SDS solution by boiling for 1 min and re-hybridized to a [a-32P]dCTP-labeled human beta-actin cDNA probe.

cDNAs Cloning of RLPK—As reported previously (37), two peptide sequences, LTPCTFYTPYAP and LTPCTANFVAP from MAPKAP-K2/3 and p90^{RSK2}, respectively, were used to search for new homologues of human MAP kinase-regulated protein kinase in the GenBankTM data base of expressed sequence tags. Two clones containing LTPCTFYTPYAP, but no other known signal peptide, were isolated. The longer one was completely sequenced and found to contain the full-length coding region of the protein.

Northern Blot Analysis—A multiple human tissue mRNA blot containing 2 mg of poly(A)+ RNA from eight different tissues was purchased from CLONTECH. The blot was hybridized to a probe prepared by random priming of the cDNA of RLPK in clone N57096 with [a-32P]dCTP as described before (38). After exposure to an x-ray film, the blot was stripped in 1% SDS solution by boiling for 1 min and re-hybridized to a [a-32P]dCTP-labeled human beta-actin cDNA probe.

cDNA Constructs and Reconstituted Proteins—The bacteria expression plasmids for His-tagged proteins or their mutants were constructed by inserting the full coding region of the cDNAs, generated by polymerase chain reaction using Pfu polymerase, into the pET-11 vector (Novagen, Madison, WI). The proteins were expressed and purified as described previously (38). The mammalian expression plasmid for RLPK was constructed by subcloning hemagglutinin-epitope tagged full-length RLPK cDNA into pcDNA3 vector (Invitrogen, San Diego, CA). All point mutations and truncations including human CREB N-terminal 2-158, RLPK (581A), RLPK (581D), RLPK (700A), and RLPK (700D) were created by a polymerase chain reaction-based method using the PCR mutagenesis kit (Novagen). The sequence of the oligonucleotides used for creating those constructs is available upon request. Each mutation was subjected to DNA sequencing to confirm the correct amino acid replacement. Expression and purification of MKK6(E), MKK7(D), MEK1, p38alpha, ERK2, JNK2, GST fusion protein of ATF-2(1–109) and GST-p38 were performed as described previously (37, 40).

In Vitro Protein Kinase Assay—In vitro kinase assays were performed using standard experimental conditions as described (38). Briefly, 2.5 mM of bacterially expressed and Ni-NTA column-purified wild type or mutant proteins of RLPK were used in a total reaction of 40 ml containing 10 mM of substrate protein, 100 mM cold ATP, and 10 mM of [γ-32P]ATP. In the coupling kinase assay, the reactions included 1 mg of purified p38, JNK2, or ERK2 and 0.2 mg of the corresponding activator MKK6(E), MKK7(D), or MEK1(E), respectively. The reactions were stopped by adding an equal amount of 2× SDS sample buffer. The proteins were then separated on SDS-PAGE. The intensities of 32P-labeled proteins were determined by phosphoimage analysis. For the S6 peptide phosphorylation assay, 50 ng of S6 peptide and an appropriate amount of protein kinase, which had been determined by normalizing equal activity of each kinase toward histone H2B, were incubated in a kinase reaction as described above. The same kinase reaction without S6 peptide was used as the control. The reactions were stopped by adding 8 volumes of 75 mM phosphoric acid and loaded onto phosphocellulose spin filters (Pierce). After three washes with 75 mM phosphoric acid, the incorporated 32P in the peptide substrates absorbed on the filter was determined by liquid scintillation counting.

Cell Transfection and Extracellular Stimulation—HeLa cells were plated in six-well plates at 60% confluence in Dulbecco's modified Eagle's medium supplemented with 10% FBS. After 12 h, the cells were transfected with expression vectors containing HA-tagged RLPK or empty pcDNA3 vector using LipofectAMINE (Life Technologies, Inc.). Forty-eight hours after transfection, the cells were incubated with or without presence or absence of different stimuli. For the stimulation by EGF, insulin-like growth factor, and serum, the transfected cells were washed twice by phosphate-saline buffer and kept in nonserum Dulbecco's modified Eagle's medium for 5 h before the treatment. Various inhibitors, as indicated in Fig. 3E, were added 30 min before EGF stimulation to the cells. HA-tagged RLPK was immunoprecipitated using anti-HA monoclonal antibody-conjugated agarose beads and quantitated by Western blot with monoclonal anti-HA antibody. HA-RLPK from each treatment was used in in vitro kinase assay using histone 2B as substrate. HSP27 was used as substrate for PRAK. The relative RLPK kinase activity by various stimuli and inhibitors was normalized to the amount of each HA-tagged protein detected on Western blot and then compared with the control activity.

Phosphatase Treatment of Recombinant RLPK—About 20 mg of recombinant RLPK bound to the Ni-NTA beads was washed three times with the binding buffer (40 mM Tris of pH 6.5, 0.3 M NaCl, and 10 mM imidazole) and split equally into two parts (namely 1 and 2). Part 2 was further treated with 5 units of potato acid phosphatase in phosphatase buffer for 15 min with shaking. Part 1 was incubated under the same conditions, but without potato acid phosphatase. After three washes with ice-cold binding buffer at pH of 7.9, RLPK from both parts was eluted out from Ni-NTA beads and used in kinase assay. The same procedure was used for PRAK except that kinase was pre-activated in the beads by incubating with 5 mg of GST-p88a and 1 mg of MKK6(E) in the presence of 900 mM ATP at 30 °C in kinase assay buffer with shaking for 15 min. GST-p38 and MKK6 were removed by washing the beads with the phosphatase buffer.

RESULTS

Cloning of a Novel Human Protein Kinase with Two Kinase Domains—We reported previously cloning of a novel MAP kinase-regulated protein kinase by searching a conserved MAP kinase regulatory site in the GenBankTM data base (37). Another candidate for an unknown protein kinase containing the sequence LKTP located in the T-loop was found. It was encoded in part by two EST clones (GenBankTM accession numbers N57096 and H09985 containing overlapping cDNA sequence). The full-length sequence of this gene was cloned by using the insert of the N57096 EST clone as a probe to screen a human placental library. The longest cDNA isolated from the screening had a length of 2840-base pair with an in-frame stop codon preceding the first ATG predicted as the starting codon for the gene. The open reading frame contained 2406 base pairs, which encoded 802 amino acids. Alignment of the deduced protein to the GenBankTM data base indicated that it was most similar to p70^{S6K} and p90^{RSK}, representing two different groups of S6 protein kinases. Thus, we named this putative protein kinase RLPK, which stands for RSK-like protein kinase. Protein domain analysis (using the Expasy profile domain search program) revealed that, like p90^{RSK}s, RLPK contained two non-identical, but complete, protein kinase domains. The first kinase domain was located in amino acids 40–340, and the second one was within amino acids 420–720. Protein sequence analysis indicated that RLPK was 36.7, 36.8, 36.7 and 36.7% identical to human p70^{S6K}, p90^{RSK1}, p90^{RSK2} and p90^{RSK3}, respectively, and had less than 28% identity to any other known human protein kinase. Like p90^{RSK}, the terminal kinase domain of RLPK had homology to p70^{S6K}, while the N-terminal kinase domain was similar to MAP kinase-regulated protein kinase such as MAPKAPK2, MNK1, and PRAK. Although RLPK and p90^{RSK} shared the common structural feature of two kinase domains, the percentages of identity among three p90^{RSK} isoforms were much higher (79–82%) than that between p90^{RSK} and RLPK. As shown in a computer-generated phylogenetic tree (Fig. 1B), RLPK is
FIG. 1. A, amino acid sequence comparison of human RLPK (the second line), p70<sup>RSK</sup> (designated as S6K70), and three p90<sup>RSK</sup> isoforms (designated as RSK1, RSK2, and RSK3, respectively) by Lasergene Megaline program (DNAStar, Madison, WI). Residues conserved between two
rather dissimilar to p90RSK group as p70RSK. This suggested that RLPK was not a new isoform of p90RSK, but rather represented a new class of two-kinase domain kinase and that RLPK may have diverged earlier in its structure and physiological function from a two-kinase domain ancestral protein kinase than did isoforms of the RSK family under evolutionary selection pressure.

Northern blot analysis showed a single band of 4.4-kilobase transcript of RLPK in all eight tissues examined; however, the mRNA of this gene seemed much more abundant in heart, brain, and placenta than in lung, kidney, and liver (Fig. 1C).

Kinase Activity of RLPK In Vitro—Wild type RLPK was expressed as His6-tagged protein in bacteria and purified for biochemical analysis. The recombinant protein was tested in vitro on a panel of proteins, which were known substrates for various kinases, including MAP kinase, MAPK-activated protein kinases, and RSKs (Fig. 2A). We found that histone 2B was the most preferred substrate in this panel. Myelin basic protein, histone 1, and CREB were also phosphorylated by RLPK, but to a lesser extent and the rest of others were poor substrates. Since it was known that S6 protein, one of the components in small ribosomal 40 S, was a preferred substrate for both p90RSK and p70RSK, we sought to compare the activity of RLPK toward S6 protein with that of p90RSK and p70RSK. Because p90RSK and p70RSK used in our experiment were partially purified, the absolute quantities of the two enzymes were not determined. Therefore, we first normalized RLPK activity to the p90RSK and p70RSK using histone 2B as substrate. A peptide from S6 protein (231RRRLSSLRA239), widely used as standard in vitro substrate for both types of RSKs or related protein kinases, was used in the in vitro kinase assay. As shown in Fig. 2C, RLPK possessed an activity toward the S6 peptide similar to that of p90RSK and p70RSK.

RLPK Is Activated by Growth-related Stimuli in Culture Cells—To determine which extracellular stimuli can activate RLPK in vivo, a variety of agonists were tested for their ability to activate transient expressed HA-tagged RLPK in HeLa cells. The activity of RLPK was measured in an immunokinase assay with histone 2B as the substrate. As shown in Fig. 3A, RLPK activity was significantly stimulated by 12-myristate 13-acetate, EGF, and serum, while the proinflammatory cytokine tumor necrosis factor-α and cell stress stimuli calcium ionophore A23187, anisomycin, and arsenite also increased RLPK activity, but to a lesser extent. A Western blot demonstrated the amount of RLPK that was used in each reaction (lower panel of Fig. 3A).

To assess which signal pathways were involved in regulating RLPK activity in vivo, a panel of inhibitors with either specific or broad inhibitory effects on PKC, tyrosine kinase, p70RSK, MEK1, and p38α/β were used. HeLa cells that transiently expressed HA-tagged RLPK were pretreated with individual inhibitors before EGF stimulation. As shown in Fig. 3B, tyrosine kinase inhibitor herbimycin A had the most profoundly inhibitory effect with more than 60% inhibition being observed. Inhibitions by two other tyrosine inhibitors, genistein and erbstatin analog, were also observed, suggesting that EGF stimulation of RLPK was mediated through a tyrosine kinase. There was a partial inhibition of RLPK activity when the MEK1 inhibitor, PD98059, or the p38 inhibitor, SB203580, was used, suggesting a potential direct or indirect involvement of both ERK and p38 MAP kinases in RLPK signal pathway. In contrast, rapamycin, a p70RSK specific inhibitor in vivo, did not show any inhibitory effect on RLPK activation nor did the PKC inhibitors, GF109230X, H7, or Ro318220. When Ro318220 was used in an in vitro kinase assay, the IC50 for RLPK was determined to be ~500 nM (data not shown) that was much higher than that of p80RSK (5 nM) and p70RSK (100 nM) (41). Therefore, it appeared that the regulation of RLPK was apparently differ-

Fig. 2. In vitro kinase activity of RLPK. A, approximately 2 μg of recombinant RLPK purified from bacteria was used in each kinase reaction with 10 μg of His6-HSP27, His6-CREB(2/158), His6-eIF4e, Phas-1, myelin basic protein, histone 2B, histone 1, or GST-ATF2(1–109) as substrates. The kinase reactions were stopped by adding SDS sample buffer, and the reaction products were analyzed by SDS-PAGE. Substrate phosphorylation was detected by autoradiography as shown. Comparable results were obtained in two independent experiments. B, confirmation of an equal amount of each protein used in A by a parallel loading in a separate SDS-PAGE stained by the Coomassie Blue. C, comparison of the relative activities of RLPK, p90RSK, and p70RSK to phosphorylate the S6 peptide (RRRLSSLRA). The amounts of protein kinases used in this assay were normalized to histone 2B phosphorylation activity (data not shown). The phosphorylation activity for each kinase was defined as the total counts of counts/min from a kinase reaction containing both kinase and the S6 peptide subtracting the counts from the autophosphorylation of the corresponding kinase. The results shown here for S6 peptide phosphorylation represent three independent experiments.
ent from that of p70 RSK and may also be distinct from that of p90 RSK. Furthermore, it suggested that EGF-mediated activation of RLPK did not necessarily go through PKC.

RLPK Can Be Phosphorylated, but Not Activated, by MAP Kinases in Vitro—As shown in Fig. 1A, RLPK contained a putative MAP kinase phosphorylation site, LTP, in the T-loop of C-terminal kinase domain. Therefore, RLPK could be a MAP kinase-regulated protein kinase. To test this hypothesis, we performed a coupled kinase assay in which different MAP kinases activated by their corresponding MKKs were included in each kinase reaction to measure kinase activity of RLPK using histone 2B as substrate. As shown in Fig. 4A, RLPK was apparently phosphorylated by activated p38α and ERK2 (lanes 2 and 6), but not by activated JNK2 (lane 4). However, the activity of RLPK toward histone 2B was not influenced by the phosphorylation as compared with the control (lane 1). These results suggested that either RLPK was not regulated by MAP kinase phosphorylation or that the recombinant RLPK had been already phosphorylated at regulatory sites during its biosynthesis or during the process of purification. To examine the second possibility, we took two approaches. First we employed dephosphorylation, in which the bacterially expressed wild type RLPK was treated with potato acid phosphatase before being used in a kinase assay. As shown in Fig. 4B, phosphatase

FIG. 3. Activation and inhibition of RLPK in cultured cells. A, the activity of various extracellular stimuli were tested for stimulating RLPK in HeLa cells under the conditions as described under “Experimental Procedures.” Samples of approximately 10⁶ HeLa cells, transiently transfected with HA-tagged RLPK, were treated with tumor necrosis factor-α (100 ng/ml), 12-myristate 13-acetate (100 nM), Ca²⁺ ionophore A13257 (5 μM), arsenite (200 μM), anisomycin (50 ng/ml), EGF (1 ng/ml), insulin-like growth factor (2 ng/ml), or 20% serum at 37 °C for 20 min. RLPK was immunoprecipitated from the cell lysates using HA-affinity beads. Kinase activity in each of immunoprecipitates was assayed using histone 2B as substrate (upper panel), and the amount of HA-RLPK used in each kinase reaction was determined by Western blotting (lower panel). The relative fold activation of RLPK by different stimuli was calculated using the intensity of phosphorylated H2B that has been normalized to the amount of HA-RLPK used in each reaction to compare with that of the control. B, various inhibitors were tested for their ability to inhibit RLPK activity in vivo. HeLa cells that transiently expressed HA-RLPK were serum-starved for 12 h and then pretreated with GF-10923X (10 μM), H7 (20 μM), HA1004 (20 μM), Ro318220 (5 μM), herbimycin A (10 μg/ml), genistein (20 μg/ml), rapamycin (5 ng/ml), erbstatin analog (1.5 μg/ml), FD98059 (20 μM), or SB203580 (20 μM) at 37 °C for 20 min before EGF (1 ng/ml) stimulation. After 30-min EGF stimulation, HA-tagged RLPK was immunoprecipitated from cell lysate. Kinase activity in each of the immunoprecipitates was assayed (upper panel) and HA-RLPK was quantitated (lower panel) as described for A. The relative inhibition of EGF-induced RLPK activation by each drug was defined by following formula: \((1 - A/C) \times 100\%\), in which \(A\) represents intensity of phosphorylated H2B from a given reaction after normalization to the amount of HA-RLPK used in that reaction, and \(C\) is the intensity of phosphorylated H2B from EGF-induced sample without any drug treatment.
treatment did not substantially reduce the RLPK activity toward histone 2B. In contrast, the activity of PRAK, a characterized p38-regulated protein kinase (37) as the control, was almost completely abolished by the same phosphatase treatment. Thus, phosphorylation may not be required for this RLPK activity. In the second approach, mutations on the putative MAP kinase phosphorylation sites were created. Since previous studies have suggested that p90RSKs were regulated by ERK \textit{in vivo} (36) and the Thr residue in LTP motif of p90RSK2 is the regulatory phosphorylation site (4), we reasoned that if the conserved threonine residue in the LTP sequence of RLPK was an operational regulatory site, mutation of this site to a nonphosphorylatable alanine residue would prevent activation of RLPK, otherwise, mutation to a negative charged aspartic acid would mimic a phosphate group and result in constitutively activated RLPK. We constructed two sets of mutants by converting Thr-581 and Thr-700 to alanines or aspartic acids. Thr-700 was selected as another site to be mutated because the surrounding sequence, PLMTPD (located immediately downstream of the XI kinase subdomain in the C-terminal kinase domain of RLPK), was very similar to that of the conserved regulatory motif. As shown in Fig. 4C, none of the mutants of RLPK exhibited substantial change in their kinase activity toward histone 2B as compared with that of the wild type RLPK, implying that neither Thr-581 nor Thr-700 functioned as a regulatory site in RLPK. Collectively, our data suggested that histone 2B phosphorylation activity by bacterially expressed RLPK was not subjected to direct regulation by ERK, JNK, and p38 MAP kinases nor necessarily dependent on phosphorylation.

**DISCUSSION**

We have identified and cloned a novel RSK-like protein kinase, RLPK. This protein shows 37% overall identity to p70RSK and p90RSK and has been demonstrated to share similar \textit{in vitro} substrates to these two families of kinases. Structurally like p90RSKs, RLPK contains two kinase domains. The N-terminal kinase domain of RLPK is homologous to p70RSK and the C-terminal kinase domain shows homology to a group of known MAP kinase-regulated protein kinases. In addition to this two-kinase domain structure, the activation profile of RLPK is also more similar to p90RSK than to p70RSK in that RLPK is insensitive to rapamycin inhibition. However, by contrast, several structural features of RLPK distinguish it from the p90RSK family. First, the overall similarity of RLPK to the p90RSK group as a whole is much lower than that within the p90RSK family (37% versus 79–82%). Second, RLPK contains an additional 49-amino acid C-terminal serine and threonine-rich tail, which is missing in all isoforms of p90RSKs. Third, although the

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**Fig. 4. Effects of phosphorylation and mutation on RLPK activity.** A, the effects of three different MAP kinases, p38α, JNK2, and ERK2 (1 μg of each), activated by their corresponding upstream activators, M KK6(E), M KK7(D), and MEK1(E) (0.2 μg of each), respectively, (not shown in the figure), on the activity of RLPK (3 μg) were assessed by \textit{in vitro} coupling kinase assays using histone 2B (10 μg) as substrate. The plus signs indicate the presence of the proteins in the reaction. The phosphorylation of the proteins was determined by phosphoimaging. The position of each protein on the SDS-PAGE are as marked. B, effect of dephosphorylation on recombinant RLPK activity. His-tagged wild type RLPK bound to the Ni-NTA beads was incubated with or without potato acid phosphatase under the conditions as described under “Experimental Procedures,” then subjected to kinase assay using histone 2B as substrate. As a control, His-tagged PRAK was pre-activated by GST-p38 and M KK6 on the Ni-NTA beads and then treated with phosphatase under the same conditions as RLPK. The substrate used in PRAK kinase assay was HSP27 (10 μg). C, kinase activity of mutant RLPK. An equal amount (3 μg) of recombinant RLPK, RLPK(T581A), RLPK(T581D), RLPK(T700A), or RLPK(T700D) was used in protein kinase assay with histone 2B (10 μg) as substrate.
Thr-581 residue of RLPK falls in the conserved LXXTP motif in the T-loop of the C-terminal kinase domain, the residue in the X position is a positively charged lysine. This is unique to all of known MAP kinase-regulated protein kinase, including all members of p90RSK, that have a neutral (methionine) residue instead (Fig. 1A). These features indicate that RLPK represents a new class of protein kinase rather than being an isoform of p90RSK.

RLPK activity was found to be tightly controlled in vivo (Fig. 3A). However, kinase activity associated with recombinant RLPK was not influenced by phosphorylation with MAP kinase or dephosphorylation with phosphatase. These data argue against our original prediction that RLPK is a MAP kinase-regulated protein kinase. However, if the following assumptions are true, RLPK may still be considered a MAP kinase-regulated protein kinase: 1) pre-existing post-translational modifications such as phosphorylation or protease cleavage is required for RLPK to be regulated by MAP kinases; 2) other co-factors are involved in RLPK regulation by MAP kinases. It has been proposed that the two kinase domains in p90RSK comprise different substrate specificities and that the N-terminal domain was believed to be responsible for the phosphorylation of c-Fos and S6 protein (9). We have found that the sequence identity between N-terminal kinase domain of RLPK and p90RSK is much higher (\textasciitilde 51\%) than the overall sequence identity (\textasciitilde 37\%). The sequence identity between N-terminal kinase domain of RLPK and that of p70RSK is also 51\%. Such high similarity may account for the similar substrate specificity observed among these different groups of kinases. When the C-terminal kinase domain sequence of RLPK and that of the p90RSKs are compared, only 39\% sequence identity is found, suggesting this domain in RLPK may differ more from its p90RSK counterpart in its function or regulation mechanism than the N-terminal kinase domain. As we mentioned in the results section, the C-terminal kinase domain of RLPK is similar to MAP kinase-regulated protein kinases. Although we showed that phosphorylation of RLPK by ERK or p38 in vitro had no influence on its activity toward histone 2B, we cannot exclude the possibility that phosphorylation did enhance the kinase activity of the C-terminal kinase domain, and the inability to detect such activation in our kinase assay would be the result of the inability of the C-terminal kinase domain to use histone 2B as substrate. Nevertheless, the role of phosphorylation of RLPK by MAP kinases still needs further investigation.

Although RLPK is activated by growth factors, as are p90RSK and p70RSK, the studies using chemical inhibitors indicated that the upstream signal pathways of RLPK differ from both p90RSK and p70RSK. However, the possibility that the different kinase domains phosphorylate specific substrates suggested that the data present in Fig. 3 may only partially represent the activation and regulation of RLPK, since stress stimuli may have increased RLPK activity to substrates other than histone 2B. The partial inhibition of RLPK activation by PD98059 and SB203580 may have also resulted from the use of unsuitable substrate for the kinase assay. A key question to be investigated is the existence and identification of specific substrates for each kinase domain.

Crystal structure studies have revealed that some kinases such as ERK2 functioned as dimers and that phosphorylation is required for the dimer formation, which is essential for the kinase normal ligand-dependent relocalization (42). The two kinase domain structure of RLPK and p90RSK may be functionally equivalent to a dimerized pair of kinase molecules. If this is true, a study of truncated RLPK derivatives containing two kinase domains independently would provide valuable insight into their separate regulation and substrate specificity. Although the independent activities of each kinase domain is important, a cooperative effect between the two kinase domains is also potentially of great interest. Furthermore, as discussed above, the role of post-translational modification of RLPK in kinase relocalization and interaction with other proteins needs to be defined.

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Addendum—During the preparation of this manuscript, Deak et al. (43) reported the sequence of a human kinase termed MSK1 that is identical to RLPK.