Generation of Constitutively Active p90 Ribosomal S6 Kinase in Vivo

IMPLICATIONS FOR THE MITOGEN-ACTIVATED PROTEIN KINASE-ACTIVATED PROTEIN KINASE FAMILY

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p90 ribosomal S6 kinases (RSKs), containing two distinct kinase catalytic domains, are phosphorylated and activated by extracellular signal-regulated kinase (ERK). The amino-terminal kinase domain (NTD) of RSK phosphorylates exogenous substrates, whereas the carboxyl-terminal kinase domain (CTD) autophosphorylates Ser-386. A conserved putative autoinhibitory alpha helix is present in the carboxyl-terminal tail of the RSK isoforms (697HLVKGAMAATYSALNR712 of RSK2). Here, we demonstrate that truncation (Δα) or mutation (Y707A) of this helix in RSK2 resulted in constitutive activation of the CTD. In vivo, both mutants enhanced basal Ser-386 autophosphorylation by the CTD above that of wild type (WT). The enhanced Ser-386 autophosphorylation was attributed to disinhibition of the CTD because a CTD dead mutation (K451A) eliminated Ser-386 autophosphorylation even in conjunction with Δα and Y707A. Constitutive activity of the CTD appears to enhance NTD activity even in the absence of ERK phosphorylation because basal phosphorylation of S6 peptide by Δα and Y707A was ~4-fold above that of WT. A RSK phosphorylation motif antibody detected a 140-kDa protein (pp140) that was phosphorylated upon epidermal growth factor or insulin treatment. Ectopic expression of Δα or Y707A resulted in increased basal phosphorylation of pp140 compared with that of WT, presenting the possibility that pp140 is a novel RSK substrate. Thus, it is clear that the CTD regulates NTD activity in vivo as well as in vitro.

p90 ribosomal S6 kinase (RSK) is a member of a growing subfamily of mitogen-activated protein kinase-activated protein kinases (MAPAPKs) that contain two distinct kinase catalytic domains in a single polypeptide chain (see Fig. 1A). The three mammalian isozymes of RSK (RSK1, RSK2, RSK3), encoded by separate genes (1), are phosphorylated and activated in vivo by extracellular signal-regulated kinase (ERK).

The amino-terminal kinase domain (NTD) of RSK, residues 68–327 of human RSK2 (see Fig. 1A), is most closely related to p70 S6 kinase with regard to primary structure. To date, only the NTD has been shown to phosphorylate exogenous substrates for RSK, including the cAMP response element binding protein (2, 3), c-Fos (4, 5) and the estrogen receptor (6). The list of substrates suggests that RSK plays a role in transcriptional regulation. The carboxyl-terminal kinase domain (CTD) of RSK, residues 422–679 in RSK2 (see Fig. 1A), is related to calmodulin-dependent protein kinases (CaMKs) and autophosphorylates Ser-386 in the linker region between the two kinase domains (7).

Activation of RSK in vivo requires interaction between ERK and the ERK-docking site located in the extreme carboxyl terminus of RSK (8, 9). RSK activation also requires ERK phosphorylation of Thr-577 in the CTD activation loop and Ser-369 in the linker, as well as autophosphorylation of Ser-386 by the CTD (see Fig. 1A) (7). Attenuation of CTD activity by mutation of Thr-577 or the ATP binding pocket generates an enzyme that cannot be fully activated (7, 10, 11). Thus, it is postulated that the CTD is involved in autoregulation of NTD catalytic activity. The crystal structure of CaMK revealed an alpha helix near the carboxyl-terminal tail that interacts with the substrate-binding groove of the catalytic domain (12). This interaction inhibits substrate binding although not in the classical pseudosubstrate mode of autoinhibition. Carboxyl-terminal to the autoinhibitory alpha helix is a conserved phenylalanine (Phe-298) that is buried in the hydrophobic pocket of the substrate-binding groove. For proper orientation of the substrate to occur, this residue must be removed from the hydrophobic pocket. Calmodulin binding is likely to disrupt the interaction between the autoinhibitory alpha helix and the substrate-binding groove, reducing the ability of the alpha helix to compete for substrate binding. Truncation of the autoinhibitory alpha helix to remove Phe-298 resulted in constitutively active CaMK1 (13).

Secondary structure prediction and alignment of the carboxyl-terminal tails of CaMK1 and RSK2 revealed a conserved putative autoinhibitory alpha helix in the RSKs (697HLVKGAMAATYSALNR712 of RSK2) (see Fig. 1). Recombinant RSK2 in which the putative alpha helix was truncated or mutated was examined to determine whether this region is an autoinhibitory domain for the RSK CTD. The results presented here indicate that autoregulation of RSK CTD and CaMK is remarkably comparable and suggest an autoinhibitory alpha helix as the mode of regulation for each of the MAPAPKs. Furthermore, the data clearly demonstrate that the CTD influences the activity of the NTD in vivo.

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Fig. 1. Schematic of RSK2 and secondary structure prediction of MAPKAPK carboxyl-terminal tails. A, schematic of RSK2 depicting the two kinase domains, the phosphorylation sites identified by Dalby, et al. (7), the putative autoinhibitory alpha helix, and the ERK docking site (8). B, secondary structure prediction was performed on sequences carboxyl-terminal to the conserved catalytic cores of RSK1/2, RSK-B (RMSK32), MAPKAPK2/3, MAPKAPK5 (PRAK), and MNK1/2. The amino acid sequences of these proteins can be accessed through the National Center for Biotechnology Information (NCBI) Protein Data base under the following accession numbers: human RSK1 (AAC324857); human RSK2 (P58123); human RSK3 (A57459); human MNK1 (AAC31171); human RSK-B (CAA09009); mouse MNK1 (C4A71966); mouse MNK2 (C4A71966); human MAPKAPK2 (P49137); human MAPKAPK3 (AAC50428); human MAPKAPK5 (NP_003659) and human CaMKI (Q14012). Hierarchical Neural Network Secondary Structure Prediction from Poˆle Bio-Informatique Lyonnais (which can be accessed on the World Wide Web) was used for the predictions. Putative autoinhibitory alpha helices in the MAPKAPKs are highlighted in the grey-shaded boxes. The autoinhibitory alpha helix in the carboxyl-terminal tail of CaMKI is outlined, and the critical Phe-298 residue is denoted with an asterisk. Δα-RSK2, created by truncation amino-terminal to the predicted autoinhibitory alpha helix, and location of the RSK2 point mutant Y707A are indicated.

EXPERIMENTAL PROCEDURES

Materials—Reagents and antibodies were obtained from the following sources: insulin (Humulin-R™), Eli Lilly and Co; epidermal growth factor (40001), Collaborative Biomedical Products; microcystin LR (475815) and PD 98059 (513000), Calbiochem; ImmunoPure™ protein A/G-agarose beads (20421), Pierce; BHK-21 (C-13) cells (hamster kidney cells; ATCC CCL-1), American Type Culture Collection; ribosomal S6 peptide (RRLLSSLRA, residues 231–239), University of Virginia Biomolecular Research Facility; hemagglutinin (HA) peptide (YPYDVPDYA), Howard Hughes Medical Institute Peptide Synthesis Facility, Duke University; polyclonal phospho-Ser-380 RSK1 antibody (06–826) and polyclonal anti-mouse IgG antibody (06–371), Upstate Biotechnology; monoclonal 12CA5 anti-HA antibody, University of Virginia Lymphocyte Culture Facility; polyclonal anti-HA antibody (PRB-101P), Berkeley Antibody Co.; polyclonal RSK phosphorylation motif (RPM) antibody;2 anti-rabbit IgG horseradish peroxidase-linked antibody (NA934), Amersham Pharmacia Biotech; anti-sheep IgG horseradish peroxidase-linked antibody (A34155), Sigma.

Plasmid Construction—The pK3H.RSK2-Y707A point mutant was generated from the parent vector pK3H.RSK2 (mouse) (8) by polymerase chain reaction. Oligonucleotide sequences are available upon request. pK3H.RSK2-Δα encodes a truncation mutant in which the

2 Characterization of the phospho-specific antibody developed against a known RSK phosphorylation motif will be presented in a later publication by Dr. Deborah A. Lannigan.

Fig. 2. In vivo Ser-386 autophosphorylation. BHK-21 cells were transiently transfected with DNA plasmids encoding HA-tagged wild type (WT), Y707A, Δα, K451A, and K451A/Y707A RSK2. Cells were serum-deprived and incubated in the presence or absence of PD 98059 (PD) (50 μM) for 3 h followed by incubation in the presence or absence of EGFR (100 ng/ml) for 30 min. Supernatants from cells lysates were electrophoresed on a 10% SDS-polyacrylamide gel and immunoblotted for phospho-Ser-386 using the RSK1 phospho-Ser-380 polyclonal antibody and for HA-RSK2 with the polyclonal anti-HA antibody.

Codons for the carboxyl-terminal 54 amino acids of RSK2 were deleted. Two carboxyl-terminal kinase-dead mutants, pK3H.RSK2-K451A and -K451A/Y707A, were generated by shuffling the BstBI-MunI fragment from pMT2.RSK2-C (kindly provided by Christian Bjørbæk, Harvard Medical School, Boston, MA) into pK3H.RSK2 and pK3H.RSK2-Y707A. pMT2.RSK2-C-Lys was generated by mutating the critical ATP-binding site Lys (Lys-451) to Ala in the RSK2 CTD. The mutations, cloning sites, and all DNA subjected to polymerase chain reaction were verified using a Perkin-Elmer Applied Biosystems automated sequencer.

Cell Culture and Transfection—BHK-21 (C-13) cells were grown as described previously (8). Cells were plated at 1.8 × 10⁶ cells/150-mm dish 24 h prior to transfection. The cells were transfected with 25 μg of DNA/150-mm dish (pK3H.RSK2, Y707A, Δα, K451A, K451A/Y707A, or pK3H) as described in the Calcium Phosphate Precipitation™ System (Promega) manual protocol for 100-mm dishes (scaled up by ×1.35). 45 h post-transfection, cells were serum-deprived in the presence or absence of 50 μM PD 98059 for 3 h prior to treatment with 94 nM insulin (12 min), 100 ng/ml EGFR (30 min), or vehicle. It was determined that addition of the MAPK/ERK kinase (MEK) inhibitor, PD 98059, to serum-starved cells decreased RSK activity compared with that observed with serum starvation alone. Therefore, basal activity of WT and mutant RSKs was measured in cells pre-incubated with PD 98059. Two 150-mm dishes per condition were scraped into 750 ml of lysis buffer (50 mM Hepes, pH 7.4, 450 mM NaCl, 1% Nonidet P-40, 0.5% Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 10 μg/ml each of leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride, 200 μM Na₃VO₄, and 1 mM microcystin LR). Cells were lysed by incubation on ice for 30 min. Supernatant was clarified by centrifugation at 12,000 × g for 10 min at 4 °C and pre-cleaned by incubation for 30 min with immobilized protein A/G-agarose beads (20-μl bed volume).

S6 Peptide Activity of HA-RSK2 Proteins—HA-RSK2 proteins were immunoprecipitated at 4 °C from pre-cleared supernatant with 25 μg of 12CA5 antibody for 45 min followed by a 30-min incubation with 25 μg of anti-mouse IgG. Immobilized protein A/G-agarose beads (30-μl bed volume) were incubated with the supernatant for 1 h on a Nutator rocker. The beads were pelleted by brief centrifugation and washed once each with 500 μl of lysis buffer, kinase wash buffer (50 mM Hepes, pH 7.4, 7 mM β-glycerophosphate, pH 7.4, 7.5 mM EGTA, 150 μM Na₃VO₄, 1.5 mM DTT, 2 μg/ml protein kinase A inhibitor peptide, 30 μM MgCl₂), and elution buffer (35 mM Hepes, pH 7.4, 5 mM β-glycerophosphate, pH 7.4, 4 mM EGTA, 1.5 mM DTT, 50 μM MgCl₂, 150 μM Na₃VO₄, 1 μM microcystin LR). The HA peptide (686 S6 peptide (RRRLSSLRA), 150 μM MgCl₂, 150 μM EDTA, 150 μM MgCl₂, 150 μM Na₃VO₄, 1 μM microcystin LR) was incubated with pelleted beads for 16 h in an Eppendorf Thermomixer at 4 °C with shaking at 1300 rpm. The eluted HA-RSK2 (5 μl) was incubated in 56 kinase mix (45 μl; 25 mM Hepes, pH 7.4, 5 mM β-glycerophosphate, pH 7.4, 7.35 mM EGTA, 1.5 mM DTT, 30 μM MgCl₂, 6 μg/ml protein kinase A inhibitor peptide, 6 μg/ml protein kinase C inhibitor peptide, 150 μM Na₃VO₄, 1 μM microcystin LR, 300 μg/ml S6 peptide (RRRLSSLRA), 150 μM ATP and γ-[³²P]ATP (~2000 cpm/μmol) at 30 °C for 13.5 min. Each assay was performed in triplicate. Phosphate incorporation into peptide substrate was determined using P81 phosphocellulose paper as described previously (8). Duplicates of eluted HA-RSK2 proteins were processed for Western analysis with anti-HA polyclonal antibody. The intensity of each immunoblot band from numerous exposures of film was quantitated in the linear range of detection, and the relative amount of HA-RSK2 protein was determined using NIH Image, Version 1.61. Specific activity was normalized for the amount of immunoprecipitated
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**RESULTS AND DISCUSSION**

MAPKAPKs are divided into two sub-groups: those containing two distinct kinase domains, RSK 1/2/3, MSK1, and RSK-B (MSK2); and those with a single kinase domain, MAPKAPK 2/3, MAPKAPK 5 (PRAK), and MNK1/2. The catalytic cores of the single domain MAPKAPKs share greater than 30% identity with the CTDs of the dual domain kinases and are closely related to calmodulin-dependent protein kinases. As seen in Fig. 1B, secondary structure prediction suggests that the similarities extend beyond the catalytic cores. These predictions indicate that each of these MAPKAPKs contains an alpha helix within fifty amino acids following the carboxyl-terminal end of the conserved catalytic core, a position similar to the autoinhibitory domain of CaMK1.

Truncation of CaMK1 to remove Phe-298 generated a constitutively active enzyme (13). Comparing the autoinhibitory domain of CaMK1 to the predicted alpha helix of RSK2 revealed a Tyr (Tyr-707) near the carboxyl-terminal end of the helix, similar to the position of Phe-298 in CaMK1. HA-tagged WT RSK2 and mutants of RSK2 in which the alpha helix was removed by truncation of the carboxyl-terminal fifty-four amino acids (Δα) or Tyr-707 was replaced with Ala (Y707A) (Fig. 1B) were examined to determine whether the predicted alpha helix was inhibitory to the CTD of RSK.

Ser-386 of RSK2, located in the linker region between the two kinase domains, is an autophosphorylation site for the CTD (7) and is, therefore, an indicator of CTD activity. BHK cells transfected with WT or mutant RSKs were serum-deprived and incubated either with the MEK inhibitor PD 98059 or EGF. PD 98059 specifically inhibits activation of MEK (14, 15), the *in vivo* activator of ERK, thereby reducing phosphorylation and activation of downstream components to basal levels. A phospho-specific antibody was used to examine Ser-386 autophosphorylation of the HA-tagged RSKs. As seen in Fig. 2, Ser-386 autophosphorylation of WT was stimulated by EGF but was undetectable when cells were pre-treated with PD 98059. However, mutation of Tyr-707 to Ala or truncation of the alpha helix resulted in significant autophosphorylation of Ser-386 in the presence of PD 98059. To determine whether the increased Ser-386 autophosphorylation in the presence of PD 98059 was the result of CTD activity, the critical Lys in the CTD ATP-binding domain was replaced with Ala (K451A). Ser-386 autophosphorylation was eliminated by this mutation and was not restored when K451A was combined with the activating Y707A mutation (K451A/Y707A) (Fig. 2) or the Δα mutation (K451A/Δα) (not shown). Thus, under basal conditions, Ser-386 autophosphorylation by Y707A and Δα resulted from CTD activity elicited by mutation in or deletion of the autoinhibitory alpha helix.

Immunoprecipitated HA-tagged RSKs were assayed to determine the effect of the mutations on S6 peptide kinase activity of the NTD. Phosphorylation of S6 peptide by WT was stimulated 4-fold by insulin (Fig. 3A). Interestingly, the basal kinase activity of Y707A and Δα immunoprecipitated from PD 98059 pre-treated cells was 4-fold greater than that of WT, similar to that of insulin-stimulated WT. These data indicated that activation of the CTD, because of alteration of the autoinhibitory alpha helix, increased NTD activity toward exogenous
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substrate. EGF stimulated the activity of WT and Y707A by \( \sim 18 \)- and \( \sim 14 \)-fold, respectively, over that of WT basal activity but failed to increase kinase activity of \( \Delta \alpha \) (Fig. 3B). This is expected because truncation also removed the ERK-docking site of RSK which is required for in vivo activation. K451A and K451AY707A were activated by EGF but to a lesser extent than the activation of WT. We conclude that these mutants are functional enzymes and that CTD activity is required for full activation of RSK2 (Fig. 3C), consistent with previous reports (7, 10, 11).

EGF or insulin (not shown) treatment of cells transfected with WT resulted in phosphorylation of a 140-kDa protein (pp140) detected by a phospho-specific antibody developed against a known RSK phosphorylation motif (anti-RPM)\(^2\) (Fig. 4A). PD 98059 pre-treatment of cells transfected with WT reduced phosphorylation of pp140 to barely detectable levels (Fig. 4A). However, phosphorylation of pp140 was enhanced in PD 98059-treated cells expressing the active mutants Y707A or \( \Delta \alpha \) (Fig. 4B). A protein migrating at \( \sim 200 \) kDa was recognized nonspecifically by anti-RPM and was used to demonstrate equal sample loading and transfer to nitrocellulose. The results indicate that the kinase activity of these proteins led to phosphorylation of pp140, which functioned as an in vivo reporter of RSK activity.

Taken together, our data suggest that catalytic activity of the RSK CTD is autoregulated by an inhibitory domain located immediately carboxyl-terminal to the catalytic core, similar to that of CaMK1. The primary and predicted secondary structure similarities between the RSK CTD and the single kinase domain MAPKAPKs suggest that these MAPKAPKs are also autoregulated by a similar inhibitory domain. Therefore, alteration of the predicted alpha helix in the single domain MAPKAPKs would produce constitutively active enzymes. This is supported by the observation that truncation or mutation of the alpha helix in MAPKAPK2 did indeed generate a constitutively active enzyme (16, 17).

Full activation of RSK requires ERK phosphorylation of Thr-577 and Ser-369, as well as RSK autophosphorylation of Ser-386 (7, 10). However, relief of CTD autoinhibition (\( \Delta \alpha \) and Y707A) was sufficient to increase NTD activity in the absence of ERK phosphorylation. This is confirmed in two ways: (i) Y707A and \( \Delta \alpha \) were active in the presence of the MEK inhibitor PD 98059, and (ii) \( \Delta \alpha \) lacks the ERK-docking site and is not activated by ERK in vivo (Fig. 3A and B). The increased NTD activity elicited by disrupting autoinhibition of the CTD was either direct, through autophosphorylation by the CTD, or indirect, by a conformational change resulting from CTD activation. Increased phosphorylation of pp140 by expression of the constitutively active RSK mutants provides the first clear evidence that the CTD regulates NTD activity in vivo.

Identification of pp140 and determination of the placement and role of the protein in the mitogenic pathway are currently in progress. The data presented here suggest that pp140 may be a novel RSK substrate and clearly indicate that pp140 is phosphorylated as a consequence of RSK activation. Thus, physiological responses to RSK activation can be assessed using the constitutively active mutants. Creation of constitutively active MAPKAPKs will be a powerful new tool to examine the in vivo functions of various members of the MAPKAPK family isolated from the influences and cross-talk of the upstream pathways.

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