Characterization of the p90 Ribosomal S6 Kinase 2 Carboxyl-terminal Domain as a Protein Kinase*

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The carboxyl-terminal domain (CTD) of the p90 ribosomal S6 kinases (RSKs) is an important regulatory domain in RSK and a model for kinase regulation of FXXFXF(Y) motifs in AGC kinases. Its properties had not been studied. We reconstituted activation of the CTD in Escherichia coli by co-expression with active ERK2 mitogen-activated protein kinase (MAPK). GST-RSK2-(aa373–740) was phosphorylated in the P-loop (Thr277) by MAPK, accompanied by increased phosphorylation on the hydrophobic motif site, Ser386. Activated GST-RSK2-(aa373–740) phosphorylates synthetic peptides based on Ser386. The peptide RRQLFRGFSFVAK, which was termed CTDtide, was phosphorylated with \( K_m \) and \( V_{max} \) values of \(-140 \mu M \) and \(-1 \mu mol/min/mg \), respectively. Residues Leu at p5 and Arg at p-3 are important for substrate recognition, but a hydrophobic residue at p+4 is not. RSK2 CTD is a much more selective peptide kinase than MAPK-activated protein kinase 2. CTDtide was used to probe regulation of hemagglutinin-tagged RSK proteins immunopurified from epidermal growth factor-stimulated BHk-21 cells. K100A but not K451A RSK2 phosphorylates CTDtide, indicating a requirement for the CTD. RSK2-(aa1–389) phosphorylates the S6 peptide, and this activity is inactivated by S386A mutation, but RSK2-(aa1–389) does not phosphorylate CTDtide. In contrast, RSK2-(aa373–740) containing only the CTD phosphorylates CTDtide robustly. Thus, CTDtide is phosphorylated by the CTD but not the NH2-terminal domain (NTD). Epidermal growth factor activates the CTD and NTD in parallel. Activity of the CTD for peptide phosphorylation correlates with Thr277 phosphorylation. CTDtide activity is constrained in full-length RSK2. Interestingly, mutation of the conserved lysine in the ATP-binding site of the NTD completely eliminates S6 kinase activity, but a similar mutation of the CTD does not completely ablate kinase activity for intramolecular phosphorylation of Ser386 even though it greatly reduces CTDtide activity. The standard lysine mutation used routinely to study kinase functions in vivo may be unsatisfactory when the substrate is intramolecular or in a tight complex.

The RSKs1 (reviewed in Ref. 1) and the closely related nuclear enzymes MSK1 (2) and MSK2 (also known as Rsk-b (3)) are unusual among protein serine/threonine kinases because they possess two kinase domains. This feature was predicted from analysis of the first RSK cDNA after its isolation from Xenopus laevis (4). There are four human RSK genes encoding RSK1, RSK2, RSK3, and RSK4,2 and all have the same conserved domain structure (1). The NH2-terminal catalytic domain (NTD) of the RSKs is most similar to p70 ribosomal S6 kinases followed by members of the protein kinase B and protein kinase C families. All of these enzymes, including the RSK NTDs, require PDK1 phosphorylation of a conserved serine (Ser277 in RSK2) in the activation loop for activity (5, 6). The NTD once activated is functional for phosphorylation of several physiologic substrates (1), perhaps best demonstrated by the ability of a constitutively active form of RSK, containing only the NTD, to induce metaphase arrest in cleaving Xenopus embryos (7). The RSK CTD evaluated together with the adjacent COOH terminus is most similar in a standard BLAST search to CaM-activated protein kinases I and II. However, features in the activation loop and the COOH terminus reveal its functional relationship to the single domain MAPKAP kinases3 such as the MNKs (8) and MAPKAP kinase 2 (9). The RSK and MSK CTDs and the single domain MAPKAP kinases have a threonine residue followed by proline in the kinase activation loop, nine and eight residues from the APE motif. None of the calmodulin-activated protein kinases have threonine followed by proline at this position, a necessary feature for MAPK phosphorylation. Similarly, all of the MAPKAP kinases have a MAPK docking motif in the COOH terminus (10) that we believe to be similar in structural disposition to the calmodulin-binding domain encompassing the aR2 helix in CaM kinase I (11). Similar to other MAPKAP kinases, the CTD is activated

1 The abbreviations used are: RSK, p90 ribosomal S6 kinase; aa, amino acid(s); NTD, NH2-terminal catalytic domain; CTD, carboxyl-terminal catalytic domain; MNK, MAPK-interacting kinase; MSK, mitogen-and stress activated kinase; PDK1, 3-phosphoinositide-dependent protein kinase-1; MAPK, mitogen-activated protein kinase; MAPKAP, MAPK-activated protein; GST, glutathione S-transferase; CaM kinase, calmodulin-activated protein kinase; HA, hemagglutinin; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; ADRI-g, alcohol dehydrogenase repressor protein 1; WT, wild type.

2 The nomenclature for RSKs has been confusing, with varying designations as p90rsk, pp90rsk, MAPKAP kinase 1, and others making comprehensive literature searches difficult. The acronym RSK is used herein and recommended for the vertebrate orthologs of the four human RSKs. The accession numbers for the human RSKs are: RSK1 (Q15418), RSK2 (P51812), RSK3 (Q15349), and RSK4 (Q9UK32).

3 MAPKAP kinase is used to refer collectively to the MAPK-activated protein kinases (RSKs, MSKs, MNKs, and MAPKAP kinase 2, 3, and 5), just as in current usage MAPK is used collectively for the ERKs, c-Jun NH2-terminal kinases, and p38 MAPK enzymes.

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by MAPK via phosphorylation of a conserved T-P site in its activation loop (12), facilitated by the MAPK docking motif (10). The CTD phosphorylates one known substrate, a conserved Ser (Ser369 in RSK2) (12) in the linker domain that joins the NTD and the CTD. The majority of this linker corresponds to the carboxyl terminus of p70 ribosomal S6 kinase by alignment and thus belongs to the NTD by inference. Within it are several regulatory sites of phosphorylation that correspond to phosphorylation sites in p70 ribosomal S6 kinase, protein kinase B, and protein kinase C enzymes.

Activation of the RSK NTD is dependent on phosphorylation of the regulatory sites in the linker domain (Ser369 and putative MAPK sites Thr465 and Ser469). Ser369 and Ser386 may be the most important of these because S369A- and S386A-type mutants of full-length RSK are virtually inactive and unresponsive to agonist stimulation (6, 12), whereas T365A-type mutants are nearly wild type (12). Ser386 lies within a docking motif for PDK1, and Ser386 phosphorylation is required for PDK1 binding and subsequent activation of the NTD (6). The role of Ser369 phosphorylation is unknown. Ser369 is not significantly phosphorylated in truncated RSK2 (amino acids I–389), which has significant constitutive activity, suggesting that Ser369 phosphorylation plays a role in the regulation of full-length RSK. Some conformational states of inactive full-length RSK may sterically inhibit NTD activity independent of Ser277 phosphorylation in the P-loop because a portion of RSK1 is phosphorylated basally at this site, yet is inactive (12, 13). Phosphorylation of Ser369, and possibly Ser386 as well, may contribute to relief of this inhibition in full-length RSK. The Ser369 kinase(s) are U0126-inhibitable (14), pointing to ERK1–2 or ERK5 as the upstream kinases for Ser369 phosphorylation in vivo. Also consistent with this conclusion, phosphorylation of the equivalent serine in avian RSK1 is blocked by deletion of the MAPK docking motif (14). Thus, ERKs are the likely physiologic Ser369 kinases.

Presently there are multiple models for RSK activation, possibly because of the existence of a multiplicity of activation mechanisms for this key signaling protein. One current model for RSK activation is vectorial (6). In this model, MAPK activates the CTD, which in turn phosphorylates Ser369 in cis, creating a binding site for PDK1, which in turn phosphorylates Ser277, activating the NTD. However, some evidence suggests that NTD activation may not always proceed vectorially from CTD activation. A portion of Ser277 in RSK1 is phosphorylated basally (12) as already mentioned, obviating the requirement for PDK1 phosphorylation. Furthermore, myristoylated avian RSK1 targeted to the plasma membrane is activated in serum-starved cells independent of evident ERK activation (15). RSK mutants rendered kinase-defective in the CTD (by mutation of the essential lysine) are still activated by growth factors but not as robustly as wild type (16, 17).

In comparison with the NTD, much less is known about the CTD. In the current view, the NTD is assumed to be the only domain of the two capable of substrate phosphorylation in trans. The properties of the activated CTD as a protein kinase have never been studied. Our results demonstrate that the isolated, MAPK-activated RSK2 CTD is functional as a protein kinase toward peptide substrates. Furthermore, the CTD but not the NTD portion of the full-length protein selectively phosphorylates the best of these peptides (RRQLFRGFSFVAR), which is referred to herein as CTDtide. This peptide substrate allowed us to probe CTD regulation independently in full-length RSK2.

**EXPERIMENTAL PROCEDURES**

*Materials—* The plasmids pET-MEK1 R4F/His$_{6}$ ERK2 (18), pMT2-RSK2-(aa1–389) and its S386A mutant (6), and pGEX-5X-MK2-EE (19) were generously provided by Melanie Cobb (University of Southwestern Medical Center, Dallas, TX), Steen Gammeltoft (Glostrup Hospital, Glostrup, Denmark), and Matthias Gaestel (Max Delbrück Centrum Molecular Medicine, Berlin, Germany), respectively. We obtained the murine RSK2 cDNA as pmT2 HA-RSK2 (20) from Christian Bjerbaek (Beth Israel Medical Center, Boston, MA). K451A and K451E mutants for pKH3-RSK2 (10) as GSK3 (1, 2) accession number AY083469. The monoclonal antibody to RSK2 Thr(P)$^{277}$ (21) was kindly given to us by Paolo Sassone-Corsi (CNRS, Strasbourg, France). The synthetic peptides related to the NH$_2$ terminus of glycogen synthase were a generous gift of Sir Philip Cohen (University of Dundee, Dundee, UK), and the alcohol dehydrogenase repressor protein 1 (ADR1-g), synapsin, and glycogen synthase peptides were generous gifts from Anthony Means (Duke University, Durham, NC). Goat polyclonal anti-RSK2 (C-19) and the horseradish peroxidase-linked anti-goat antibodies were purchased from Santa Cruz Biotechnology; GammaBind Sepharose plus, glutathione-Sepharose 4B, horse-radish peroxidase-linked anti-mouse, and anti-rabbit antibodies were from Amersham Biosciences; epidermal growth factor was from Collaborative Biomedical Products; microcin$\text{\textregistered}$ and PD98059 were from Calbiochem; BHK-21 cells were from American Type Culture Collection; Syntide-2 was from the American Peptide Company; S6 and all RSK peptides were from the University of Virginia Biomedical Research Facility; and the HA peptide was from the Howard Hughes Medical Institute Peptide Synthesis Facility (Duke University). All of the other reagents and proteinases were from Sigma (St. Louis, MA) or from Amersham Biosciences.

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**Construction of pAC-pET RSK2 CTD—** A PCR-based strategy was used to engineer murSK2-(aa373–740) (wild type and a K451A kinase-defective mutant) in frame into the BamHI and XhoI sites of pET41b (Novagen). The GST-RSK2 CTD and the kinase-defective mutant were transferred to pACYC184, a plasmid that has a p15A origin of replication, using a strategy suggested to us by Dr. Peter Sheffield (Center for Cell Signaling, University of Virginia Health Sciences Center, Charlottesville, VA). PCR was used to add Bell sites to a ~2.3-kb fragment amplified with Pfu polymerase (Promega) from pET41a-RSK2-(aa373–740). This fragment spans the T7 promoter for RNA polymerase, the GST tag, the pET41b multidicing site, RSK2-(aa373–740) with its stop codon, and the T7 terminator. The amplified fragment was cloned into the single BamHI site of pACYC184 (GenBank; accession number X86403). The construct was verified by sequencing. The plasmids pAC-pET RSK2-(aa373–740) and pAC-pET RSK2-(aa373–740)(K451A) have a chloramphenicol resistance marker. The expressed proteins (exclusive of GST) have a 72-amino acid leader polypeptide (derived from codons within pET41b) that contains His$_{6}$ and 8 tags (Novagen) in addition to thrombin and enterokinase cleavage sites.

**Phosphospecific Antibody to RSK2 Ser(P)$^{277}$—** The immunogen was [Cys]-Gly-Arg-Phε-Phε-Val-Ala conjugated to keyhole limpet hemocyanin via the cysteine, and the antisera were produced in rabbits by Research Genetics (Huntsville, AL). The IgG fraction was purified from the production bleed by affinity chromatography on immobilized protein G.

**Protein Production and Purification—** The vectors encoding GST- and His$_{6}$-tagged proteins were transformed into Escherichia coli BL21 cells with or without the bicistronic vector that encodes constitutively activated MEK1 and wild type ERK2 (18). These cells were grown at 37°C to an A$_{600}$ of ~0.3 and then induced with 0.5 mM isopropyl-$\beta$-D-thiogalactopyranoside for 6–8 h at room temperature. The proteins were purified from the cell lysates using either glutathione Sepharose or Ni$^{2+}$-nitritilotriacetic acid-agarose (Qiagen) essentially as directed by the manufacturer but with the addition of 2 M lithium chloride washes for the GST-tagged RSK proteins. The purified proteins were quantified using the Bio-Rad protein assay with bovine serum albumin as the standard. Active EE-MAPKAPK2 was made according to Engel et al. (19) using pGEX-5X-MK2-EE.

**Kinase Activity and K$_{d}$ Determination—** PS1 paper assays were used to monitor kinase activity of the purified GST or His$_{6}$-tagged proteins. The kinase assays were performed in 40-μl reactions containing (final concentrations) 25 mM Hapes, pH 7.4, 2 mM diethiothreitol, 0.25 mg/ml bovine serum albumin, 10 mM MgCl$_{2}$, a peptide substrate (as indicated in the figure legends), 50 μM [γ-$^{32}$P]ATP (~4000 cpm/pmml) at 30°C for 15 min or the indicated times in the figure legends. Phosphate incorporation into peptide substrate was determined using PS1 phosphocelluloseulose paper as described previously (10).

**Cell Culture and Transfection—** BHK-21 cells were grown in a humidified incubator at 37°C with 10% CO$_{2}$ in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum. During transfections, the cells were also supplemented with 100 units/ml of penicillin and 100 μg/ml of streptomycin. The cells were transfected...
with 20 μg of DNA using a calcium phosphate precipitation system (Pre-
mea) on 150-mm dishes as described previously (22). Post-transfection
(45 h) the cells were serum-starved for 3 h with or without 50 μM PD
98059 and then treated with epidermal growth factor (100 ng/ml) for
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**RESULTS AND DISCUSSION**

**Design of a Plasmid for Reconstitution of Active RSK2 CTD in Bacteria**—To address whether the RSK2 CTD is functional for phosphorylation of an exogenous substrate, we felt it necessary to test an active, recombinant protein from *E. coli* for several reasons. Kinase activity detectable from a CTD recovered from Sf9 or mammalian cells could be ascribed to a contaminant in the preparation. *E. coli* do not express protein serine/threonine kinases, and protein kinase cascades can be reconstituted in bacteria. In particular, a bicistronic plasmid for inducible expression of untagged active MEK1 together with His$_6$-tagged ERK2 was created to produce active ERK2 for crystallization (18). The active MEK1 phosphorylates ERK2 in bacteria, producing doubly phosphorylated, fully active ERK2. Active ERK2 activates RSK2 (reviewed in Ref. 1). We hypothesized that this strategy could be used to produce active recombinant MAPKAP kinases, including the RSK2 CTD, provided the MAPKAP kinase would fold correctly in bacteria. That the RSK2 CTD might fold correctly was suggested by previous work (17), wherein GST-RSK1 (aa386–752) was expressed in *E. coli* as a soluble protein and was active as revealed by its ability to autophosphorylate via an intramolecular (concentration-independent) mechanism.

For the above reasons, we engineered muRSK2-(aa373–740) to be expressed as a GST fusion protein (see “Experimental Procedures”). The plasmid pAC-pET RSK2 CTD (Fig. 1) derived from pACYC184 has a p15A origin of replication and is compatible with the ColE1 replicator in pET-MEK1 R4F/His6 ERK2.

**GST-RSK2 CTD Is Activated by ERK2 in *E. coli*—**We used phosphospecific antibodies in Western blots to compare the levels of phosphorylation of Ser$^{386}$ and Thr$^{577}$ in wild type GST-RSK2 CTD and K451A RSK2 CTD obtained from two conditions: expression alone or expression together with active ERK2 (Fig. 2A). RSK2-(aa373–740) encompasses the Ser$^{386}$ site, which is known to be an intramolecular substrate in RSK2 for the CTD. The blots were first probed with anti-RSK as a loading control (Fig. 2A, top panel) and then stripped and reprobed with anti-Ser$^{386}$ (Fig. 2A, middle panel). No signal was detected for the K451A mutant expressed by itself. Ser$^{386}$ was phosphorylated in wild type protein expressed in the absence of active ERK2. Expression with active ERK2 greatly increased Ser$^{386}$ phosphorylation for both the mutant and wild type proteins. The specificity of the phosphospecific Ser$^{386}$ antibody was verified using RSK2 CTD treated with and without calf intestine alkaline phosphatase (Fig. 2B). Because Ser$^{386}$ is phosphorylated by the CTD (12, 23) and is not a MEK or ERK2 substrate, RSK2 CTD enzymatic activity is up-regulated by ERK2 in bacteria.

The blot was stripped and reprobed a third time with a monoclonal antibody (21) to Thr$^{577}$, and the regulatory site was phosphorylated by MAPK in the activation loop of the RSK2 CTD (Fig. 2A, bottom panel). Neither GST-RSK2 CTD nor the kinase-defective K451A mutant expressed alone was immunoreactive with this antibody. Co-expression with active ERK2 induced a large and easily detectable signal from both active and kinase-defective RSK2 CTD, establishing that active ERK2 produced from the bicistronic plasmid phosphorylates RSK2 CTD on Thr$^{577}$ in bacteria.

It was somewhat surprising that the K451A protein was detectably phosphorylated on Ser$^{386}$ because this serine is not a substrate for ERK2 or MEK1 R4F. Although it is remotely possible that some portion of the Ser$^{386}$ phosphorylation occurring in situ is due to the activating kinases, it is more likely that the K451A mutant retains kinase activity. In kinases that contain the conserved lysine in subdomain II (not all do), mutation of that residue decreases but does not completely eliminate kinase activity. The residual retained activity of these mutated kinases is variable and dependent upon the kinase and the amino acid substitution. For example, ERK2 K52R retains ~5% of the activity of wild type ERK2 (24) and still weakly autophosphorylates (25). Our results indicate that the K451A mutant of RSK2 CTD retains reduced activity that is also up-regulated by ERK2. This reaction should be facilitated because it can occur intramolecularly (17), if, as seems almost certain, the unidentified site in that study was Ser$^{386}$.

The residual Ser$^{386}$ kinase activity of K451A RSK helps to rationalize the ability of full-length K451A-type RSK mutants to be partially activated by ERK (Refs. 17 and 22 and this study). The Ser$^{386}$ site must be phosphorylated or else mutated to a substrate for ERK2 or MEK1 R4F. Although it is remotely possible that some portion of the Ser$^{386}$ phosphorylation occurring in situ is due to the activating kinases, it is more likely that the K451A mutant retains kinase activity. In kinases that contain the conserved lysine in subdomain II (not all do), mutation of that residue decreases but does not completely eliminate kinase activity. The residual retained activity of these mutated kinases is variable and dependent upon the kinase and the amino acid substitution. For example, ERK2 K52R retains ~5% of the activity of wild type ERK2 (24) and still weakly autophosphorylates (25). Our results indicate that the K451A mutant of RSK2 CTD retains reduced activity that is also up-regulated by ERK2. This reaction should be facilitated because it can occur intramolecularly (17), if, as seems almost certain, the unidentified site in that study was Ser$^{386}$.

**Bound ERK2 Is Removed by Lithium Chloride Washes—**
ERK2 Activates RSK2 CTD in E. coli. A, GST tagged-RSK2 CTD and K451A mutant proteins were purified (see “Experimental Procedures”) from cells co-expressing active ERK2 (right two lanes) or not (left two lanes). Equal amounts of these purified proteins were subjected to Western blot analyses. Top panel, anti-RSK2, loading control. Middle panel, anti-Ser(P)386-RSK. RSK CTD phosphorylates Ser386 in the absence of ERK activation, but the K451A mutant cannot. ERK2 enhances the ability of RSK CTD to phosphorylate Ser386 in both the WT and K451A enzymes. Bottom panel, anti-Thr(P)577-RSK. Thr577 phosphorylation requires co-expression with active ERK2. B, GST-RSK2 CTD (4 μg) purified from bacteria expressing active ERK2 was treated with (lane 2) and without (lane 1) 13 units of calf intestinal alkaline phosphatase for 30 min at 37 °C, and equal amounts of protein (0.64 μg) were analyzed by Western blotting. Top panel, anti-RSK2, loading control. Bottom panel, anti-Ser(P)386-RSK2. The antibody is phosphospecific.

After purification, K451A GST-RSK CTD reproducibly retained more ERK2 than GST-RSK2 CTD (Fig. 3B, bottom panel). The relevant difference between these proteins is the amount of Ser386 phosphorylation (Fig. 3B, middle panel). This serine is contained within an FXF motif (in RSKs, FSF) that is an ERK docking site in proteins such as Elk1, Lin-1, KSR, and phosphodiesterase (28–30). This motif mediates interactions with ERK2 that are independent of the D domain MAPK docking site. Binding of ERK2 to FXF occurs at physiologically relevant affinities that are decreased up to 10-fold by disruption of the motif (30). Our data suggest that ERK2 is interacting with the Ser386 site in GST-RSK2 CTD and that phosphorylation of Ser386 decreases the affinity of the interaction. The carboxyl-terminal docking domain is the predominant mechanism of ERK-RSK interaction. However, it is plausible that the FSF site in RSKs is contributing to interaction with ERK2. Supporting this, ERK2 binds avidly to phenyl-Superose (31). In other proteins, this motif alone can direct phosphorylation of neighboring ERK sites (28).

RSK2 CTD Phosphorylates Ser386 Peptide—Having established that WT-RSK2 CTD was able to phosphorylate Ser386 in bacteria (Fig. 2A), we tested RSK2 CTD for enzymatic activity toward an exogenous Ser386 synthetic peptide (RRQLFRGFS-FVAI) (Fig. 4). WT-RSK2 CTD activated by ERK2 phosphorylated the peptide (solid squares), but WT-RSK2 CTD did not. The residual ERK2 in the preparation should not phosphorylate the peptide because the serine is not followed by a proline. This was confirmed by finding (Fig. 4, crosses) that ERK2, with an activity toward MBP that is 20 times greater than the activity of the wild type RSK toward the Ser386 peptide, did not
phosphorylate the Ser\textsubscript{386} peptide. The residual ERK2 in the CTD preparations can be detected using MBP as the phosphoacceptor (data not shown), but Ser\textsubscript{386} is not an ERK2 substrate. To our knowledge, this is the first proof that the isolated Ser\textsubscript{386} synthetic peptide (Fig. 4, open squares) significantly phosphorylates the peptide. K451A CTD co-expressed with ERK2 (\(\bullet\)) shows minimal activity (inset). The results are expressed as picomoles of phosphate incorporated into the peptide at each time point. Error bars indicate the range of duplicates. (Where error bars or symbols are not visible, they are contained within the symbol or obscured.) These results are representative of three independent experiments.

**FIG. 4.** Kinase activity of purified RSK2 CTD toward peptide substrate. The RSK (380–390) peptide, which encompasses the Ser\textsubscript{386} site, was used as a substrate in P81 paper assays as described (see “Experimental Procedures”). Preparations of WT RSK CTD (squares) and K451 RSK CTD (circles) obtained from co-expression with (solid symbols) and without (open symbols) active ERK2 were assayed (0.25 \(\mu\)g) for peptide kinase activity. Active ERK2 alone (crosses) was tested at 70 ng or 15 ng (inset). Only purified WT RSK CTD co-expressed with ERK2 (\(\bullet\)) significantly phosphorylates the peptide. K451A CTD co-expressed with ERK2 (\(\bullet\)) shows minimal activity (inset). The results are expressed as picomoles of phosphate incorporated into the peptide substrate at each time point. Error bars indicate the range of duplicates. (Where error bars or symbols are not visible, they are contained within the symbol or obscured.) These results are representative of three independent experiments.

We tested the RSK2 CTD for stringency in substrate selection by comparing the phosphorylation of several mutant peptides to the parent Ser\textsubscript{386} peptide, each at 0.1 mM (Fig. 5, bottom panel). The sequence similarity of RSK CTD to CaM kinase I and MAPKAP kinase 2 dictated inclusion of the peptide sequence surrounding Ser\textsubscript{386} from p −5 to p +4. The Ser\textsubscript{386} motif (Fig. 5, top panel) is an exact match to the current consensus for CaM kinase I (BXXXXX(S/T)XXXB) (32) and for MAPKAP kinase 2 (XXBXXXXXXX) (33), where B is a subset of hydrophobic residues in each case. For MAPKAPK2, the optimal p −5 hydrophobic residue is bulky (Phe > Leu > Val > Ala) and is leucine in the physiologic MAPKAPK2 substrate Hsp27 (LNRLQG\_SS). For CaM kinase I, bulky hydrophobic residues are required at both the p −5 and p +4 positions (32, 34). The Ser\textsubscript{386} site of RSK includes bulky hydrophobic residues at p −5 and p +4, which could be important. In addition, hydrophobic residues are important at other positions in the consensus sequence of other members of the CaM kinase superfamily to which the CTD is related (35, 36). CaM kinase II and phosphorylase kinase both select Phe or another bulky hydrophobic residue strongly at p +1 (36). The “hydrophobic motif” FXYFXF(Y) (37) for PDK1 docking contains phenylalanines that could be important for Ser\textsubscript{386} phosphorylation.

The L381K peptide was not a substrate, suggesting that the p −5 position is important for recognition by the CTD. Arg\textsubscript{398} at p −3 was required because the R383K and R383G peptides were compromised or not phosphorylated at all, respectively. These data show that p −5 and p −3 are critical for peptide phosphorylation by the CTD and suggest that like MAPKAPK2 the CTD prefers a hydrophobic residue at Arg at these positions. We made leucine substitutions for the phenylalanines. All of these mutants (F382L, F385L, and F387L) were phosphorylated by the RSK2 CTD nearly as well as the peptide substrate. Some members of the CaM kinase superfamily (AMP-activated protein kinase (34) and CaM kinase I (32)) strongly prefer a hydrophobic residue at p +4. The RSK CTD resembles CaM kinase II (36) in tolerating Lys replacement at p +4.
In the case of full-length RSK, intramolecular phosphorylation of Ser386 a priori could be due to the combined effects of structural presentation of the site in the linker and to preference for a primary sequence. This will require detailed structure-function studies of the linker. However, the fact that residues in the linker (p = 5 and p = 3) are both critical for peptide phosphorylation and are conserved across RSKs suggests that recognition of Ser386 for intramolecular phosphorylation will be dependent on the primary sequence in the linker.

As a final caveat, we do not know whether the peptide sequence is an optimal sequence. ERKs autophosphorylate intramolecularly on regulatory tyrosine and threonine sites (38, 39) that do not conform to the (S/T)P consensus that is absolutely required for phosphorylation of exogenous substrates. We also do not imply existence of additional physiologic substrates for RSK2 CTD from these data, although our findings make the possibility more plausible.

RSK2 CTD Is a Selective Kinase—We tested ~20 peptides that have been used to characterize CaM kinase-related enzymes (Table I and data not shown). For comparison, the peptides in Table I were assayed with an equal amount of the active EE substrates for RSK2 CTD from these data, although our findings make the possibility more plausible.

Characterization of the I390K Peptide as CTDtide—The I390K peptide was the best substrate for purified, active RSK2 CTD and is referred to as CTDtide (Fig. 5 and Table I). The apparent $K_m$ for this peptide is ~140 $\mu$M. This $K_m$ is in the upper range for most protein serine/threonine kinases. The specific activity toward the peptide was 1 mol/min/mg (Fig. 6), indicative of an efficient and properly folded enzyme. For reference, the catalytic subunit of protein kinase A, produced in E. coli, has a $K_m$ of ~40 $\mu$M for Leu-Arg-Ary-Ala-Ser-Leu-Gly (Kemptide) and a specific activity of ~20 mol/min/mg for phosphate transfer (41). For CTDtide to be useful as a probe for CTD activity, the CTD should phosphorylate the peptide in the full-length protein. The NTD should not phosphorylate the peptide or at least should do so poorly in comparison with CTD.

Specificity of the CTD was tested using constructs created to inactivate the two kinase domains in full-length RSK2 (Fig. 7, A and B). K100A RSK2 is kinase-defective for the NTD; K451A RSK2 is kinase-defective for the CTD. The HA-tagged proteins were immunopurified from EGF-treated cells for assay. The K100A mutant is completely kinase-dead for phosphorylation of S6 peptide but still phosphorylates CTDtide. The K451A mutant is kinase-dead for phosphorylation of CTDtide but not for phosphorylation of S6 peptide (25% of wild type). Note the difference in scales. S6 peptide is a much better substrate for the NTD in full-length RSK than CTDtide is for the CTD. To eliminate the possibility that the NTD could account for a significant proportion of CTDtide activity, we compared intact and a kinase-defective RSK2-(aa1–389) (Fig. 7C). A S386A mutant is as kinase-dead as a S227A mutant of the P-loop because it prevents PDK1 from phosphorylating the P-loop (6). Mutation of S386A caused nearly complete loss of S6 kinase activity but did not significantly affect the small amount of apparent CTDtide activity co-purified in this experiment. This residual incorporation is most likely due to contaminating kinases in the immunopurified RSK. Corrections to CTDtide activity were made from mock immunoprecipitates from empty vector controls, usually around 1–5% of CTDtide activity of wild type or K100A RSK2.

We suspect that the CTDtide activity is constrained in the full-length protein. This would be the expected result if the CTD has evolved to phosphorylate only the Ser386 site in the linker. Evidence for constraint of CTDtide activity in full-length RSK is

|Peptide name| Peptide sequence| CTD activity$^a$| MAPKAP kinase 2$^b$| Reference|
|---|---|---|---|---|
|RSK (S386)| R R Q L F R G S F V A I| 100| 30| |
|I390K| R R Q L F R G S F V A K| 306| 91| |
|Peptide 1$^a$| K R E R T S V A| 15| 566| 33|
|Peptide 2$^c$| K R E R T S V A| 3| 100| 33|
|Peptide 3$^c$| K R E R T S V A| 0| 6| 33|
|Peptide 4$^c$| K R E R T S V A| 0| 2| 33|
|Syntide 2| P L A R T L S V A G L P F G K K| 2| 27| 35|
|ADR1-g| L K K L E R A S F S G Q| 1| 23| 40|
|Syntide (4–13)| L E R A R L S D A N F| 0| 6| 40|
|GS (1–10/13)| F L R B T L S V A A| 1| 116| 40|
|AMARA| A R A R A A A S A A L A R R R| 0| 1| 34|

$^a$ Activity was measured using the p81 paper assay (see “Experimental Procedures”). The concentration of enzyme and substrate were 6.25 $\mu$g/ml and 100 $\mu$g/ml, respectively. The reactions were run in duplicate for 13 min; 100% incorporation was 6.7 or 7 pmol of phosphate for RSK2 CTD and EE-MAPKAP 2, respectively.

$^b$ Peptides 1–4 are related to the GS (1–10) peptide and were used previously to characterize MAPKAP2 specificity (33).

$^c$ GS, glycogen synthase.
Activity here is selective for S6. (Note difference in scale for apparent CTD kinase activity; the N-terminal constitutively active wild type RSK2-(aa1–373) kinase activity of wild type, K451A, and K100A HA-RSK2.

C is required for phosphorylation of CTD.

A activity, same protein preparations as in epidermal growth factor for 5 min (see Table II). Specific activity than CTD in full-length RSK2. HA-tagged RSK2 proteins (Table II) and CTD kinase activities are compared with the full-length wild type kinases. The data presented in Fig. 7 are normalized for total RSK protein from the Western signal for HA because we observed differences in level of expression of the separated domains. The CTD alone (RSK2-(aa373–740)) is consistently underexpressed, and the NTD alone (RSK2-(aa1–389)) is overexpressed compared with the full-length proteins (data not shown). Normalization was done as carefully as possible, making preliminary runs to find dilutions that were similar and would be in the linear range of the film after nonextended exposures.

The separated CTD domain consistently had higher specific activity when compared with the full-length proteins. In additional experiments, portions of the assayed RSK proteins were also analyzed for reactivity to anti-Thr(P)577, and specific activities relative to Thr577 phosphorylation were calculated (Table II). The enhanced specific activity of the isolated CTD is partially explained by increased stoichiometry of Thr577 phosphorylation. However, the specific activity of CTD alone, normalizing to Thr577 phosphorylation, is still 6-fold higher than that of either full-length protein (wild type or K100A). This suggests differences in conformation between the isolated CTD and the CTD in the full-length protein that alter the specific activity. Structures to determine the accessibility of the active site in the isolated CTD versus the CTD in full-length RSK would be of interest.

The I390K peptide can be considered CTDtide because the CTD, but not the NTD, portion of the RSK protein phosphorylates it. CTDtide can be used to assay CTD activity in relation to NTD activity in RSK provided that RSK is purified to remove other kinases, such as MAPKAPK2, that would also phosphorylate it and that proper controls are performed. The specific elution of bound RSK with HA peptide in our experiments may also have helped to reduce the amount of contaminating kinases that may stick to beads but are not elutable with HA peptide.

**CTD Activation Is Rapid and Parallels NTD Activation**—Previously it has not been possible to determine whether the NTD and CTD kinase activities are congruent (i.e. on together/ off together) or dissociated (i.e. CTD-activated and inactivated, toward S6 (hatched bars), in arbitrary units normalized for RSK protein. In C and D: left ordinate, S6; right ordinate, CTDtide. The data are the averages of duplicates (ranges indicated by error bars) but are representative of six experiments.

| Protein       | CTDtide activity/HA-RSK2 | pT577/HA-RSK2 | CTDtide activity/phospho-T577 |
|---------------|--------------------------|---------------|-------------------------------|
| Wild type     | 0.1                      | 11.1          | 0.5                           |
| K451A         | 0.0                      | 16.0          | 0.0                           |
| K100A         | 0.1                      | 12.0          | 0.4                           |
| RSK 373–741   | 1.4                      | 48.6          | 2.9                           |
| RSK 1–389     | 0.0                      |               |                               |

" Activity of HA-RSK proteins, eluted from immunoprecipitates of cells treated for 15 min with EGF (3 μl), was measured in duplicate using the p81 paper assay with CTDtide as substrate (see Experimental Procedures). The eluted proteins were then analyzed for protein content using western blot analysis (anti-HA), and the results are presented as specific CTDtide activity per the HA signal in arbitrary units.

" Eluted immunoprecipitated proteins from above were then analyzed for the amount of Thr577 phosphorylation (anti-Thr(P)577), and the results are presented as ratio of Thr(P)577 to HA signal, expressed as a percentage.

" The activity results from the p81 assays above presented as specific CTDtide activity per Thr577 phosphorylation in arbitrary units.
causes a weak but detectable increase in this phosphorylation (Fig. 8D, compare 0 min to 5 min). This is consistent with data discussed above that were obtained in bacteria. The kinetics of Ser^{386} phosphorylation in the wild type, included as a control, show EGF activation at the earliest time point, 2.5 min. These kinetics are compatible with the vectorial model for RSK activation because intramolecular phosphorylation of Ser^{386} would be extremely rapid. Furthermore, PDK1 binding and phosphorylation of Ser^{277} is potentially too fast to produce detectably slower NTD activation relative to the CTD. The PDK1 steps may even be preempted. There is evidence (13) for a pool of inactive RSK that is already phosphorylated on the NTD activation loop (12). Activation of this pool of RSK would only require phosphorylation of Ser^{386} and/or linker MAPK sites and a conformational change. Because this pool is inactive in unstimulated cells despite phosphorylation of the NTD activation loop, RSK activation must also include relief of intrasteric inhibition (43).

The Future for Other MAPKAP Kinases—Reconstitution of activation of other MAPKAP kinases (MNKs, etc.) along similar lines should be feasible. Although it is possible to generate active mutants that circumvent phosphorylation requirements by truncation or acidic replacements, structural studies of enzyme regulation are most informative with authentic phosphorylated enzyme. This will require generation of additional bicistronic vectors to produce the specific activated MAPKs. Reconstitution of protein cascades in bacteria may also prove to be surprisingly specific. MAPKAP kinase 2 was discovered biochemically as an enzyme that was activated in vitro by ERK (44) but was later shown to be activated by p38α,β MAPK specifically in mammalian cells. Consistent with this, we found that co-expression of MAPKAP kinase 2 with active ERK2 in bacteria caused only modest activation (data not shown), which we found surprising. EE-MAPKAP kinase 2 produced in bacteria, in contrast, was much more active (19) (data not shown).

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