Identification of Regulatory Phosphorylation Sites in Mitogen-activated Protein Kinase (MAPK)-activated Protein Kinase-1a/p90\textsuperscript{RSK}
That Are Inducible by MAPK*

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Mitogen-activated protein kinase-activated protein kinase-1 (MAPKAP-K1; also known as p90\textsuperscript{RSK}) contains two protein kinase domains in a single polypeptide. The N-terminal kinase domain is necessary for the phosphorylation of peptide substrates, whereas the C-terminal kinase domain is required for full activation of the N-terminal domain. Here we identify six sites in MAPKAP-K1 that become phosphorylated in transfected COS-1 cells. The inactive form of MAPKAP-K1a in unstimulated cells is partially phosphorylated at Ser\textsuperscript{222} and Ser\textsuperscript{733}. Stimulation with phorbol 12-myristate 13-acetate induces the phosphorylation of Thr\textsuperscript{360}, Ser\textsuperscript{364}, Thr\textsuperscript{774}, and Ser\textsuperscript{381} and increases the phosphorylation of Ser\textsuperscript{222} and Ser\textsuperscript{733}. Our data indicate that mitogen-activated protein kinase activates the C-terminal kinase domain by phosphorylating Thr\textsuperscript{574} and contributes to the activation of the N-terminal kinase domain by phosphorylating Ser\textsuperscript{264}. The activated C-terminal domain phosphorylates Ser\textsuperscript{264}, which, together with phosphorylation of Ser\textsuperscript{264}, activates the N-terminal kinase domain. The phosphorylation of Ser\textsuperscript{222} and Ser\textsuperscript{733}, which can be catalyzed by the N-terminal domain, does not activate MAPKAP-K1a \textit{per se}, but Ser\textsuperscript{222} phosphorylation appears to be required for activation. Ser\textsuperscript{222}, Ser\textsuperscript{364}, and Ser\textsuperscript{381} are situated in analogous positions to phosphorylation sites in protein kinase B, protein kinase C, and p70\textsuperscript{S6K}, suggesting a common mechanism of activation for these growth factor-stimulated protein kinases.

As implied by their name, the isoforms of mitogen-activated protein kinase-activated protein kinase-1 (MAPKAP-K1; also known as p90\textsuperscript{RSK}) are only active \textit{in vitro} if they have been phosphorylated by the p42 or p44 mitogen-activated protein kinases (MAPKs) (1). Several lines of evidence indicate that the MAPKAP-K1 isoforms are also activated by MAPKs \textit{in vivo} via the Ras-dependent protein kinase cascade that is triggered by growth factors or tumor-promoting phorbol esters, such as phorbol 12-myristate 13-acetate (PMA). Thus, signals that activate MAPKs also activate MAPKAP-K1, and the phosphorylation (2) and activation (3) of MAPKAP-K1 induced by insulin or insulin-like growth factor-1 are prevented by PD 98059, a compound that specifically inhibits the activation of MAPK kinase-1 (3), the major upstream activator of MAPKs. Cyclic AMP-elevating agents that prevent the activation of MAPKs also prevent the activation of MAPKAP-K1 (4).

MAPKAP-K1 has a broad substrate specificity \textit{in vitro}, phosphorylating proteins and peptides at serine residues that lie in Arg-Xaa-Arg-Xaa-Xaa-Ser or Arg-Arg-Xaa-Ser motifs (5, 6). However, few physiological substrates have been identified so far. Upon activation, MAPKAP-K1 is known to translocate from the cytosol to the nucleus (7), where one of its substrates in neuronal cells is believed to be the transcription factor CREB (reviewed in Ref. 8). Activation of CREB by MAPKAP-K1 is intriguing because MAPKs are highly expressed in post-mitotic neurons, where they are activated by agonists known to play a role in memory formation (9), and an important role for CREB phosphorylation in memory formation is well documented (10). Moreover, inactivating mutations in the gene encoding human MAPKAP-K1b (RSK2) are associated with Coffin-Lowry syndrome, a disease that results in severe mental retardation as well as progressive skeletal deformation (11). A role of the MAPK cascade in long-term facilitation in \textit{Aplysia} has also been reported recently (12).

MAPKAP-K1 is unusual in that the polypeptide contains two distinct protein kinase domains (13). The N-terminal kinase domain (NDT) is most similar to p70\textsuperscript{S6K} (almost 60% amino acid sequence identity) (14), whereas the C-terminal kinase domain (CTD) is most similar to phosphorylase kinase (40% amino acid sequence identity). The ability of MAPKAP-K1 to phosphorylate a variety of exogenous substrates is abolished by single amino acid replacements that inactivate the NTD (5, 15, 16). Inactivation of the CTD greatly reduces, but does not abolish, the phosphorylation of exogenous substrates (5, 15). These observations and the finding that the isolated CTD is capable of phosphorylating itself (16) suggest that the CTD may play a role in the activation of the NTD. Indeed, evidence that MAPKAP-K1 can be autophosphorylated by either the NTD or the CTD has been presented (15).

Several years ago, we identified a threonine in the CTD of MAPKAP-K1b (Thr\textsuperscript{774} in MAPKAP-K1a) as one of the residues phosphorylated by MAPKs \textit{in vitro} (17). However, others (15) found that the mutation of this residue to Ala in MAPKAP-K1c (RSK3) generated an enzyme that could still be activated in response to the epidermal growth factor. This finding prompted us to identify all the sites in MAPKAP-K1a (RSK1) that become phosphorylated \textit{in vivo}. Here we identify six phosphorylation...
sites in PMA-stimulated COS-1 cells, three of which are phosphorylated by MAPKs in vitro. Our results indicate that the MAPK-catalyzed phosphorylation of Ser^{164} and Thr^{574} is critical for activation of the NTD and CTD, respectively, and that the phosphorylation of Ser^{381}, catalyzed by the CTD, is also important for activation of the NTD.

EXPERIMENTAL PROCEDURES

**Materials**—Chemicals were the best grades available commercially and were purchased from BDH (Leicester, United Kingdom) or Sigma (Poole, UK). Tissue culture reagents, Taq DNA polymerase, and microcystin-LR were obtained from Life Technologies, Inc. (Paisley, UK); phosphate-free Dulbecco's modified Eagle's medium from ICN (Oxfordshire, UK); Nu-serum™ IV culture supplement from Stratex (Luton, UK); protein G-Sepharose from Pharmacia (Milton Keynes, UK); glutathione-agarose, PMA, chloroquine diphosphate, DEAE-dextran, and dimethyl sulfoxide from Sigma (Poole); phosphate-buffered saline (Dulbecco A) from Oxoid (Basingstoke, UK); sequencing-grade trypsin and chymotrypsin, monoclonal antibody 12CA5, and Pin A1 restriction enzyme from Boehringer (Lewes, UK). Immunoprecipitates were assayed for MAPK-K1 activity using the peptide S^{579}-G^{580} G^{581} (KEAEKRRGQIKARRKRLSAHRSGSQQK) (23). One unit of MAPK-K1 activity is that amount which incorporates 1 nmol of phosphate into the same peptide in 1 min.

**Immunoprecipitation of HA-MAPK-K1a from 32P-Labeled COS-1 Cells**—Cells transfected with HA-MAPK-K1a were washed three times in phosphate-free medium, incubated for 1 h, and washed twice more in the same medium. The cells were then incubated for 4 h with carrier-free (P^{32}) orthophosphate (1 μCi/ml) in the presence or absence of PD 98059. After appropriate stimulation, the cells were washed four times with ice-cold Dulbecco's modified essential medium and then lysed in 300 μl of lysis buffer (20 mM Tris-HCl, pH 7.5 (20 °C), 0.1% (by volume) 2-mercaptoethanol, 2 mM EDTA, 2 mM EGTA, 1% (by mass) Triton X-100, 0.25 mM NaCl, Complete™ protease inhibitor mixture (Boehringer), 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 μM microcystin-LR, and 1 μM soybean trypsin inhibitor (all at 1 μM concentration). The lysates were incubated for 30 min at 4 °C with protein G-Sepharose incubated with preimmune serum and centrifuged for 10 min at 13,000 × g, and the supernatant was added to protein G-Sepharose coupled to monoclonal antibody 12CA5, which recognizes the HA epitope. After agitating for 90 min at 4 °C, the complex was washed eight times with 1.0 ml of lysis buffer containing 0.5 mM NaCl and then twice with 50 mM Tris-HCl, pH 6.8, and 0.1% (by volume) 2-hydroxyethanethiol.

**Proteolytic Digestion of HA-MAPK-K1a—Immunoprecipitated** 32P-labeled HA-MAPK-K1a was denatured in SDS and resolved by electrophoresis on a 7.5% SDS-polyacrylamide gel. After autoradiography, the 86-kDa band corresponding to phosphorylated HA-MAPK-K1a was excised, eluted, precipitated, and oxidized as described (24). The protein was then incubated at 30 °C in 0.3 ml of 50 mM Tris-HCl, pH 8.0 (20 °C), and 0.1% (by volume) reduced Triton X-100 containing 5 μg of trypsin or chymotrypsin. After 3 h, another 5 μg of protease was added, and the suspension was left for a further 12 h. Guanidinium chloride (8 M) was added to a final concentration of 1 M to precipitate any residual SDS, and after standing on ice for 10 min, the sample was centrifuged for 10 min at 13,000 × g. The supernatant containing >90% of the 32P radioactivity was removed and fractionated as described below.

**Identification of Phosphorylation Sites**—A trypptic digest of 32P-labeled HA-MAPK-K1a obtained from 8 mg of cell lysate was first purified on a column of chelating Sepharose (Pharmacia) saturated with iron(III) chloride (25). The phosphopeptides were recovered (70% yield) by elution with water/ethanol/triethylamine, pH 10.5, dried, dissolved in 0.1% (by volume) trifluoroacetic acid, and applied to a 24 cm × 4 mm Vydac 218TP54 C_{18} column (Separations Group, Hesperia, CA) equilibrated with 0.1% (by volume) trifluoroacetic acid and connected to an on-line monitor for continuous measurement of 32P radioactivity. The column was developed with a linear acetonitrile gradient (0.33%/min) in 0.1% (by volume) trifluoroacetic acid. The flow rate was 0.8 ml/min, and fractions of 0.4 ml were collected. Typically, >90% of the 32P radioactivity applied to the column was recovered. Six major tryptic peptides (T1–T6) and chymotryptic peptides (C1–C6) were obtained. Peptide T1 was further purified by chromatography on a microbore C_{18} column equilibrated with 10 mM ammonium acetate, pH 6.5, and aliquots of peptides T1, T2, and T5 were then analyzed on an Applied Biosystems Model 476A sequencer to determine the amino acid sequence. Additional aliquots of peptides T1–T5 and chymotryptic peptides C1–C6 were coupled covalently to a Sequelon arylation membrane and analyzed on an Applied Biosystems Model 470A sequencer.

**32P radioactivity was measured after each cycle of Edman degradation.**

**Phosphorylation of GST-HA-MAPK-K1a with MAPK-K2 and MAPK**—Bacterially expressed GST-HA-MAPK-K1a was phosphorylated with recombinant MAPK-K2 and MAPK-2. The phosphorylation was efficiently and specifically inhibited by 20 mM microcystin-LR or 20 mM pyruvate, respectively. The procedure described above was followed for the separation of the peptides from the gel and subsequent sequencing. Additional aliquots of peptides T1–T5 and chymotryptic peptides C1–C6 were coupled to a Sequelon arylation membrane and analyzed on an Applied Biosystems Model 470A sequencer.

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Following stimulation of the cells with 80 nM PMA, the activity of MAPKAP-K1 was studied by transiently transfecting the cDNA into COS-1 cells, followed by immunoprecipitation of the HA-tagged MAPKAP-K1a. For these reasons, the mechanism of activation of MAPKAP-K1a could only be expressed in E. coli in very low yield, and could not be activated to a significant extent by p42 MAPK.

Phosphorylated at Ser222 and Ser733

Peptides T1, T2, and T5 were purified (see “Experimental Procedures”) or absence (○) of 50 μM PD 98059, the cells were stimulated with 80 nM PMA, and MAPKAP-K1 was immunoprecipitated from lysates using the C-terminal MAPKAP-K1 antibody and assayed. The activities are plotted as means ± S.E. for three experiments.

Results

Activation of MAPKAP-K1a in COS-1 Cells

MAPKAP-K1a could only be expressed in E. coli in very low yield, was highly degraded (see “Experimental Procedures”), and could not be activated to a significant extent by p42 MAPK. For these reasons, the mechanism of activation of MAPKAP-K1a was studied by transiently transfecting the cDNA into COS-1 cells, followed by immunoprecipitation of the HA-tagged enzyme from the cell lysates (see “Experimental Procedures”). Following stimulation of the cells with 80 nM PMA, the activity of transfected HA-MAPKAP-K1a increased 10–15-fold, peaking after 10–15 min, similar to the activation of the endogenous MAPKAP-K1 activity (Fig. 1). The rate of activation of the expressed and endogenous enzymes was strongly suppressed by PD 98059, an inhibitor of the activation of MAPK kinase-1 (3), indicating that the activation of MAPKAP-K1 is catalyzed by MAPKs. The failure of PD 98059 to suppress the PMA-induced activation of MAPKAP-K1 completely has been noted previously in Swiss 3T3 cells (3) and is likely to be explained by the finding that PD 98059 inhibits the activation of MAPK kinase by only 80–90%. Due to the enormous amplification potential of the MAPK cascade, the 10–20% residual MAPK kinase activity is sufficient to induce significant activation of MAPKAP-K1. However, the possibility that another PMA-activated PD 98059-insensitive kinase cascade may also contribute to the activation of MAPKAP-K1 cannot be excluded.

The activity of transfected HA-MAPKAP-K1a was 10-fold higher than the endogenous MAPKAP-K1a activity after stimulation with PMA (Fig. 1), even though the efficiency of transfection was only 30%. Thus, the intracellular concentration of HA-MAPKAP-K1a is far higher than that of the endogenous MAPKAP-K1. All the experiments presented below were carried out on HA-MAPKAP-K1 immunoprecipitated from cells stimulated for 15 min with PMA.

Recombinant HA-tagged MAPKAP-K1a Is Constitutively Phosphorylated at Ser222 and Ser733

We first wished to identify the residues in MAPKAP-K1a that were phosphorylated under basal conditions where MAPKAP-K1a was inactive. 32P-labeled COS-1 cells were therefore treated for 3 h with PD 98059 in the absence of PMA (Fig. 1), and phosphoamino acid analysis revealed that, under these conditions, inactive MAPKAP-K1a was phosphorylated only at serine residues. Following tryptic digestion and chromatography on a C18 column, two major 32P-labeled peptides were recovered (termed T1 and T2) that eluted at 18.5 and 24% acetonitrile, respectively (Fig. 2A). Both peptides were phosphorylated at serine, and a single burst of 32P radioactivity was observed after the fourth (peptide T1) and third (peptide T2) cycles of Edman degradation (data not shown). Further experiments described below established that peptides T1 and T2 corresponded to residues 730–736 and 220–237, respectively, and that in unstimulated cells treated with PD 98059, MAPKAP-K1a was phosphorylated at Ser222 in the “activation loop” of the NTD and at Ser733 four residues from the extreme C terminus.

PMA Induces the Phosphorylation of Thr360, Ser364, Ser381, and Thr574 in HA-MAPKAP-K1a

Evidence from Tryptic Digestion—To identify the residues that became phosphorylated when MAPKAP-K1a was activated, the experiments described above were repeated after stimulation of the COS-1 cells with PMA. Stimulation with PMA was accompanied by a 5–6-fold increase (three experiments) in the 32P labeling of MAPKAP-K1a with respect to cells incubated with PD 98059 in the absence of PMA (Fig. 2A). Phosphoamino acid analysis revealed that threonine residues became phosphorylated upon cell stimulation with PMA, although the majority of the phosphate was still present as phosphoserine (data not shown). Tryptic digestion and C18 chromatography revealed that stimulation with PMA not only caused a 2–3-fold increase in the phosphorylation of peptides T1 and T2 (three experiments), but also induced the 32P labeling of two new major peptides termed T4 (21.8% acetonitrile) and T5 (27.7% acetonitrile) along with several minor peptides termed T3 (13% acetonitrile), T6 (28.8% acetonitrile), and T7 (30% acetonitrile) (Fig. 2C).

Peptides T1, T2, and T5 were purified (see “Experimental Procedures”) and sequenced. Peptide T1 corresponded to resi-
confirmed by its comigration during C18 chromatography and by tryptic digestion (see "Experimental Procedures").

Peptide T7 was a phosphothreonine-containing peptide, and a single burst of radioactivity occurred after eight cycles of Edman degradation (Fig. 3E). The only threonine residues in MAPKAP-K1 located eight residues after an Arg or Lys residue are Thr519 and Thr574. Thr574 is located at the position equivalent to a Thr residue in rabbit MAPKAP-K1b phosphorylated by p42 MAPK in vitro (17). Further evidence presented below indicates that this residue is indeed the site of phosphorylation.

Evidence from Chymotryptic Digestion—To obtain further evidence that the assignment of phosphorylation sites was correct, 32P-labeled HA-MAPKAP-K1a from 32P-labeled COS-1 cells was digested with chymotrypsin and chromatographed on a C18 column. Two prominent 32P-labeled peptides termed C1 (17.9% acetonitrile) and C2 (18.5% acetonitrile) were isolated from cells incubated with PD 98059 in the absence of PMA (Fig. 4A). Peptide C1 contained phosphoserine, and 32P radioactivity was released after the first cycle of Edman degradation (data not shown). This is consistent with peptide C1 containing Ser220 and resulting from chymotryptic cleavage at Tyr221. Peptide C2 also contained phosphoserine, and 32P radioactivity was released after the fourth cycle of Edman degradation (data not shown). Moreover, peptide C2 coeluted with tryptic peptide T1 (Fig. 2C). These observations indicated that peptide C2 was phosphorylated at Ser733 and that it was generated by a pseudotryptic cleavage between Arg729 and Lys730.

The 32P labeling of peptides C1 and C2 was increased 2–3-fold when cells were stimulated with PMA in the absence of PD 98059, as expected from the results presented in Fig. 2. In addition, several new chymotryptic phosphopeptides were generated. Peptides C3 (13.5% acetonitrile) and C4 (14.2% acetonitrile) both contained phosphothreonine as well as phosphoserine, and in both cases, 32P radioactivity was released after the fourth and eighth cycles of Edman degradation. Based on additional evidence presented below, these peptides are most likely to have resulted from cleavage at Phe356 and to be phosphorylated at Thr360 and Ser364.

Peptide C5 (22.8% acetonitrile) also contained phosphoserine, and 32P radioactivity was released after four cycles of Edman degradation (data not shown). This is consistent with peptide C5 being generated by cleavage at Phe376 and to be phosphorylated at Thr360 and Ser364.

Peptide C6, a phosphothreonine-containing peptide, eluted at the same acetonitrile concentration as peptide-(566–581) phosphorylated at Thr574 by MAPK in vitro (17). Consistent with this assignment, 32P radioactivity was released after the ninth cycle of Edman degradation (Fig. 3F).

Evidence from Immunoblotting—To assign the sites of phosphorylation by an independent method, antibodies were produced that recognized MAPKAP-K1a only when it was phosphorylated at particular residues. The antisera were generated at much lower acetonitrile concentrations than peptide T4. In addition, peptide T4 comigrated during C18 chromatography and isoelectric focusing with a synthetic phosphopeptide comprising residues 363–377 and phosphorylated at Ser364. These results, and further experiments described below, established that Ser364 was phosphorylated in vivo in response to PMA.

Peptide T7 was also a phosphothreonine-containing peptide, and a single burst of radioactivity occurred after the second cycle of Edman degradation (Fig. 3D). There are three serine residues in human MAPKAP-K1a that occur two residues after trypsin-sensitive peptide bonds, namely Ser154, Ser364, and Ser631. The predicted tryptic phosphopeptides containing Ser154 (LSK) and Ser631 (IGSSK) would elute from C18 columns
in sheep against synthetic peptides from MAPKAP-K1a that were phosphorylated at Thr<sup>360</sup>, Ser<sup>364</sup>, or Ser<sup>381</sup>, and the phospho-specific antibodies were purified by affinity chromatography (see "Experimental Procedures"). All three antibodies recognized the 86-kDa HA-MAPKAP-K1a band from PMA-stimulated COS-1 cells, but did not recognize HA-MAPKAP-K1a from unstimulated cells that had been preincubated with PD 98059 (Fig. 5A). In the absence of PD 98059, Ser<sup>381</sup> was recognized weakly by the anti-phospho-Ser<sup>381</sup> antibody. The specificity of each antibody was confirmed in two ways: first, by the finding that the antibodies did not recognize HA-MAPKAP-K1a from PMA-stimulated cells if they were first incubated with the phosphopeptide immunogen (data not shown), and second, by the finding that mutation of a particular phosphorylation site to Ala or Glu prevented the relevant antibody from recognizing HA-MAPKAP-K1a (Fig. 5B). Interestingly, MAPKAP-K1a expressed in <i>E. coli</i> was phosphorylated at Ser<sup>381</sup>, and Ser<sup>381</sup> phosphorylation increased markedly following incubation with MAPK and MgATP (Fig. 5C).

**Effect of Mutagenesis of Phosphorylation Sites on the Activation of MAPKAP-K1**

The previous experiments identified two residues (Ser<sup>222</sup> and Ser<sup>733</sup>) that were partially phosphorylated in unstimulated cells and four residues (Thr<sup>360</sup>, Ser<sup>364</sup>, Ser<sup>381</sup>, and Thr<sup>574</sup>) that became phosphorylated in response to PMA. Thr<sup>360</sup>, Ser<sup>364</sup>, and Thr<sup>574</sup> are followed by proline residues, suggesting that they are likely to have been phosphorylated by MAPKs. Indeed, Thr<sup>574</sup> was identified previously as a residue phosphorylated by MAPK <i>in vitro</i> (17). The phosphorylation of Thr<sup>360</sup> and Ser<sup>364</sup> by MAPKs was confirmed by the finding that, after incubation of the bacterially expressed enzyme with MgATP and p42 MAPK <i>in vitro</i>, MAPKAP-K1a was recognized by the antibodies raised against these phosphorylated epitopes (Fig. 5C).

To investigate the role of each phosphorylation site in the activation of HA-MAPKAP-K1a, these Ser and Thr residues were initially mutated to Ala, and the mutants were transiently expressed in COS-1 cells. The PMA-induced activation of HA-MAPKAP-K1a was virtually abolished in the S364A and S381A mutants and suppressed by 85% in the S222A and T574A mutants (Fig. 6A). In contrast, the basal activation and the PMA-induced activation of HA-MAPKAP-K1a were hardly affected in the T360A and S733A mutants.

Rat MAPKAP-K1a contains three other threonine residues that are followed by proline, namely Thr<sup>613</sup>, Thr<sup>622</sup>, and Thr<sup>712</sup>. Although the evidence presented above indicated that these residues were not phosphorylated <i>in vitro</i>, Thr<sup>613</sup> and Thr<sup>622</sup> are conserved in every form of MAPKAP-K1 identified so far. However, their mutagenesis to Ala either individually (data not
shown) or in combination (Fig. 6) had little effect on the activation of MAPKAP-K1a by PMA. The third threonine (Thr712) is not conserved in the Drosophila homologue (26) and is therefore unlikely to be an important regulatory phosphorylation site. Ser364, Ser381, and Thr574 were also mutated to acidic residues to try to mimic the effect of phosphorylation by introducing a negative charge. The S364E mutant, like the S364A mutant, had greatly reduced basal activity in unstimulated cells. However, in contrast to the S364A mutant, the S364E mutant could still be activated by PMA, albeit to only 6% of the activity of the wild-type enzyme. These experiments suggested that the presence of a negatively charged residue at Ser364 was important for the activation of MAPKAP-K1a and indicated that the phosphorylation of at least one residue other than Ser364 was important for activation.

Unlike the S381A mutant, the basal activity of the S381E mutant was the same as that of the wild-type enzyme, and it could be activated to 25% of the level of the wild-type enzyme after stimulation with PMA. These experiments demonstrated that the phosphorylation of at least one residue other than Ser381 was important for activation.

Unlike the S381A mutant, the basal activity of the S381E mutant was the same as that of the wild-type enzyme, and it could be activated to 25% of the level of the wild-type enzyme after stimulation with PMA. These experiments demonstrated that the phosphorylation of at least one residue other than Ser381 was important for activation of the NTD.

The T574D mutant, unlike the T574A mutant, had the same basal activity as wild-type MAPKAP-K1a and could be activated to 80% of the level of the wild-type enzyme after stimulation with PMA. This indicated that a negatively charged residue at position 574 was important for the activation of MAPKAP-K1a and also that phosphorylation of at least one residue other than Thr574 was critical for activation of the NTD.

Effect of Inactivating the Kinase Domains on the Phosphorylation of MAPKAP-K1a in COS-1 Cells

To investigate the roles of the NTD and CTD in phosphorylating the residues in MAPKAP-K1a that were not followed by proline (Ser222, Ser381, and Ser733), the phosphorylation of these sites was investigated using additional mutants of MAPKAP-K1a in which either the NTD or the CTD had been inactivated. The D558A mutant, in which the CTD should be inactivated, decreased the basal and PMA-stimulated activities of MAPKAP-K1a by 85%. Significantly,
the basal and PMA-stimulated activities of the D558A/T574A double mutant were identical to those of the single mutants (Fig. 7).

The $^{32}$P labeling of Ser$^{364}$ (peptide T4), Thr$^{574}$ (peptide T7), or Ser$^{222}$ (peptide T1) was not affected significantly in the D558A mutant, but this mutation greatly reduced the $^{32}$P labeling of Ser$^{381}$ (peptide T5) (Fig. 8). The reduction in phosphorylation of Ser$^{381}$ was confirmed by immunoblot experiments (Fig. 8C). Similar results were obtained with the T574A mutant (data not shown).

Inactivation of the NTD caused some decrease in the $^{32}$P labeling of Ser$^{381}$ (peptide T1); had little effect on the $^{32}$P labeling of Ser$^{364}$ (peptide T4), Ser$^{381}$ (peptide T5), and Thr$^{574}$ (peptide T7); but greatly reduced the $^{32}$P labeling of Ser$^{222}$ (peptide T2) (Fig. 8B). The lack of effect on Ser$^{381}$ phosphorylation was confirmed by immunoblotting (Fig. 8C).

**DISCUSSION**

In this paper, we have identified six residues in MAPKAP-K1a that are phosphorylated in COS-1 cells. One of these (Ser$^{222}$) is located in the NTD, one (Thr$^{574}$) in the CTD, three (Thr$^{360}$, Ser$^{364}$, and Ser$^{381}$) in the intervening sequence between the NTD and CTD, and one (Ser$^{381}$) in the C-terminal tail (Fig. 9). Thr$^{360}$, Ser$^{364}$, and Thr$^{574}$ lie in the minimum consensus sequence for phosphorylation by MAPKs ((Ser/Thr)-Pro), are phosphorylated by MAPKs in vitro (Fig. 5 and Ref. 17), and become phosphorylated in response to an agonist (PMA) that activates MAPKs in vivo (Figs. 2–4). We therefore conclude that these three residues are phosphorylated by MAPKs in vivo. The phosphorylation of the other three serine residues is also reduced/increased in response to PMA, but these residues are not followed by proline (Fig. 9), indicating that they are targeted by distinct protein kinases. We argue below that Ser$^{381}$ is phosphorylated by the CTD of MAPKAP-K1a and that Ser$^{222}$ and probably Ser$^{381}$ are phosphorylated by the NTD (Fig. 9).

The effect of the T574A mutation on the activation of MAPKAP-K1a on its activation by PMA. COS-1 cells were transiently transfected with DNA constructs expressing wild-type HA-MAPKAP-K1a or HA-MAPKAP-K1a with the following amino acid mutations: S222A, S364A, S381A, T574A, T613A/T622A, S733A, S364E, S381E, and T574D. Cells were incubated for 15 min in the presence or absence of 80 nM PMA. Each mutant was immunoprecipitated from the lysates and assayed. The results are expressed as fold-activation relative to the specific activity of PMA-stimulated wild-type HA-MAPKAP-K1a from unstimulated COS-1 cells (0.2 ± 0.05 units/mg) and are plotted as means ± S.E. for at least five experiments. The specific activity of wild-type HA-MAPKAP-K1a from PMA-stimulated cells was 8-fold higher than that of any of the three mutants.
MAPKAP-K1a by PMA was similar to that produced by inactivation of the CTD, with either mutation reducing the PMA-induced activation of the NTD by 85%, whereas combining both mutations had no further effect (Figs. 6 and 7). Similar results were obtained by Bjarbaek et al. (15) using the MAPKAP-K1c (RSK3) isoform. These investigators found that changing Thr to Ala (the residue equivalent to Thr274 in MAPKAP-K1a) and inactivating the CTD by changing Lys to Ala reduced basal and epidermal growth factor-stimulated activation to a similar extent. Fischer and Blenis (16) also showed that the CTD expressed alone was capable of undergoing a slow autophosphorylation reaction that was enhanced 7-fold after incubation with MAPK and MgATP. Taken together, these results strongly suggest that the MAPK-catalyzed phosphorylation of Thr triggers activation of the CTD. The T574D mutant had a very low activity in unstimulated cells, but could still be activated in response to PMA (Fig. 6), indicating that the presence of an acidic residue at position 574 can substitute for the phosphorylated threonine, at least partially.

The T574A mutation (data not shown) or inactivation of the CTD greatly reduced the PMA-stimulated phosphorylation of Ser, whereas inactivation of the NTD had no effect on the PMA labeling of this site (Fig. 8). These observations indicate that the phosphorylation of Ser is catalyzed by the CTD in vivo after it has been activated by the phosphorylation of Thr. Two further lines of evidence indicate that the phosphorylation of Ser is catalyzed by the CTD. First, Vik and Ryder (27) have shown that Ser is a major site that becomes phosphorylated when native MAPKAP-K1 is incubated with MgATP in vitro. These investigators expressed wild-type Xenopus MAPKAP-K1b (termed S6KII) and the isolated NTD and CTD of avian MAPKAP-K1 in SF9 cells. They found that the native enzyme and the isolated CTD, but not the isolated NTD, underwent a slow intermolecular autophosphorylation reaction in vitro. Second, we have shown that bacterially expressed MAPKAP-K1a is partially phosphorylated at Ser (presumably as a result of autophosphorylation) and that the phosphorylation of this residue increases upon incubation with p42 MAPK and MgATP (Fig. 5C). The low level of phosphorylation induced by PMA after transfection of the D558A mutant of MAPKAP-K1a, in which the CTD should be inactive (Fig. 8), is presumably catalyzed by the endogenous MAPKAP-K1 isoforms, which are present in vivo at much lower concentrations.

The S381A mutation virtually abolished the PMA-induced activation of MAPKAP-K1a, suggesting that the phosphorylation of this residue is critical for activation of the NTD. The lack of phosphorylation of Ser may also explain why the isolated NTD lacking the CTD displays very low activity after transient transfection in COS cells (15). In contrast, the S381E mutation was still activated by PMA, albeit to only 25% of the level of the wild-type enzyme (Fig. 6); however, the S381E mutant was not constitutively active in unstimulated cells. These results not only demonstrate the importance of a negatively charged residue at position 381 for the activation of MAPKAP-K1a, but also indicate that phosphorylation of an additional residue is required to activate the NTD. This residue appears to be Ser because its mutation to Ala abolished the activation of MAPKAP-K1a by PMA (Fig. 6). The S384E mutant was not active in unstimulated cells, but could be activated by PMA to ~10% of the level of the wild-type enzyme (Fig. 6). This suggests that a negative charge at residue 384 is necessary for Ser phosphorylation to be able to induce any activation. The T360A mutant had no effect on the activation MAPKAP-K1a by PMA (Fig. 6), and the role of Thr phosphorylation remains to be determined.

Ser and Ser were partially phosphorylated in unstimulated cells pretreated with PD 98059 under conditions where transfected HA-MAPKAP-K1a was inactive. This demonstrated that the phosphorylation of Ser and Ser per se was insufficient for activation. The PMA-induced activation of MAPKAP-K1a was also unaffected in the S733A mutant. However, the S222A mutation suppresses PMA-induced activation of MAPKAP-K1a by 90% (Fig. 6), and the equivalent mutation in MAPKAP-K1c reduces the epidermal growth factor-stimulated activation of this isoform to a similar extent (15). Moreover, the phosphorylation of Ser was greatly decreased under another condition where PMA-induced activation was...
suppressed, i.e. after inactivation of the CTD (Fig. 8A). Thus, the presence of phosphate at Ser222 may also be important for the activation of MAPKAP-K1. Ser222 lies in the position equivalent in the NTD to Thr574 in the CTD, with these residues lying just N-terminal to the conserved Ala-Pro-Glu motif in subdomain VIII of each kinase domain, whereas Ser733 is four residues from the C terminus. Thr260, Ser264, and Ser381 are located between the two kinase domains. B, residues equivalent to Ser222, Ser264, and Ser381 in MAPKAP-K1 and the sequences surrounding these sites are conserved in p70S6K, protein kinase B (PKB), and protein kinase C (PKC). Thr260 in MAPKAP-K1 is also present in p70S6K. Identical residues in all four kinases are shown in boldface type, and phosphorylation sites are underlined and are in boldface type.

In summary, we have shown that two independent MAPK-catalyzed phosphorylation events are required to activate the NTD and CTD of MAPKAP-K1α. Activation of the CTD requires the MAPK-catalyzed phosphorylation of Thr574, allowing it to phosphorylate Ser381. The phosphorylation of Ser381 and the MAPK-catalyzed phosphorylation of Ser364 appear to be critical for the activation of the NTD, which may then enhance the phosphorylation of Ser222 in the NTD and Ser733 in the CTD. Although more work is necessary to understand why the activation of MAPKAP-K1α is so complex and to define the role of each phosphorylation event precisely, it is intriguing that Ser222, Ser364, and Ser381 lie in positions analogous to residues whose phosphorylation is important for the activation and/or stability of other growth factor-stimulated protein kinases, namely p70S6K (29, 30), protein kinase B (31), and protein kinase C (32, 33) (Fig. 9B). In MAPKAP-K1α, the PMA-induced increase in Ser222 phosphorylation appears to be mediated by the NTD, but the equivalent residue in protein kinase B is phosphorylated by 3-phosphoinositide-dependent protein kinase-1 (34, 35). It would clearly be of considerable interest to investigate whether 3-phosphoinositide-dependent protein kinase-1 can phosphorylate the equivalent residues in MAPKAP-K1α, p70S6K, and protein kinase C. As in MAPKAP-K1α, the residues equivalent to Ser364 are always followed by proline (Fig. 9B), but only in MAPKAP-K1α is there evidence that phosphorylation is catalyzed by the MAPKs of the “classical” MAPK cascade. The protein kinase(s) that phosphorylate this residue in p70S6K, protein kinase B, and protein kinase C are unknown. p70S6K, but not protein kinase B or protein kinase C, also contains a residue equivalent to Thr260 in MAPKAP-K1α (Fig. 9B), and this site is known to be phosphorylated in vivo (29, 30). The residues equivalent to Ser381 all lie in a Phe-Xaa-Xaa-Phe-(Ser/Thr)-(Phe/Tyr) motif (Ref. 36 and Fig. 9B). In MAPKAP-K1α, Ser381 is phosphorylated by the CTD, but in p70S6K, protein kinase B, and protein kinase C, it is phosphorylated by a distinct protein kinase(s) that has not yet been identified. As suggested by Vík and Ryder (27), it is also possible that the CTD does not just phosphorylate the NTD in vivo and that it has additional physiological substrates, such as

**Fig. 9. Location of the major in vivo sites of phosphorylation in HA-MAPKAP-K1α.** A, Ser222 and Thr574 are both located nine residues N-terminal to the conserved Ala-Pro-Glu motif in subdomain VIII of each kinase domain, whereas Ser733 is four residues from the C terminus. Thr260, Ser264, and Ser381 are located between the two kinase domains. B, residues equivalent to Ser222, Ser264, and Ser381 in MAPKAP-K1α and the sequences surrounding these sites are conserved in p70S6K, protein kinase B (PKB), and protein kinase C (PKC). Thr260 in MAPKAP-K1α is also present in p70S6K. Identical residues in all four kinases are shown in boldface type, and phosphorylation sites are underlined and are in boldface type.
In addition, activation of the CTD might be triggered by distinct signal transduction pathways and occur independently of the activation of the NTD.

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