Constitutive Activation of Mitogen-activated Protein Kinase-activated Protein Kinase 2 by Mutation of Phosphorylation Sites and an A-helix Motif*

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A recently described downstream target of mitogen-activated protein kinases (MAPks) is the MAPK-activated protein (MAPK) kinase 2 (Akt) which has been shown to be responsible for small heat shock protein phosphorylation. We have analyzed the mechanism of MAPK kinase 2 activation by MAPK phosphorylation using a recombinant MAPKAP kinase 2 fusion protein, p44MAPK and p38/40MAPK in vitro and by using an epitope-tagged MAPKAP kinase 2 in heat-shocked NIH 3T3 cells. It is demonstrated that, in addition to the known phosphorylation of the threonine residue carboxyl-terminal to the catalytic domain, Thr-317, activation of MAPKAP kinase 2 in vitro and in vivo is dependent on phosphorylation of a second threonine residue, Thr-205, which is located within the catalytic domain and which is highly conserved in several protein kinases. Constitutive activation of MAPKAP kinase 2 is obtained by replacement of both of these threonine residues by glutamic acid. A constitutively active form of MAPKAP kinase 2 is also obtained by deletion of a carboxyl-terminal region containing Thr-317 and the A-helix motif or by replacing the conserved residues of the A-helix. These data suggest a dual mechanism of MAPKAP kinase 2 activation by phosphorylation of Thr-205 inside the catalytic domain and by phosphorylation of Thr-317 outside the catalytic domain involving an autoinhibitory A-helix motif.

The network of mitogen-activated protein kinases is based on subsequent activation of protein kinases by phosphorylation (for a recent review, see Ref. 1). A major activator of the vertebrate MAP kinases is the phosphorylation of a kinase MEK, a dual specific kinase which itself is activated by protein kinases encoded by the proto-oncogenes ras1 or mos (2, 3) as well as by MEK kinase (4). In addition, stress-dependent signaling proceeds via parallel MAPK cascades leading to activation of further subgroups of MEKs and MAPks (5, 6). One of the MAPK subgroups is designated stress-activated protein kinases (SAPks) (7) and termed amino-terminal c-j un kinases (J Nks) (8, 9). Another distinct subgroup covers the p38MAP and p40MAPK, including the reactivating kinase (RK) (10–12), which are more similar to the yeast MAPK homologue HOG1 (13).

Signaling downstream of the MAPks proceeds by phosphorylation of several transcription factors and of at least two different MAPK-activated protein (MAPKAP) kinases, the different isoforms of ribosomal S6 kinase II (RSK, MAPKAP kinase 1) and the MAPKAP kinase 2. The latter enzyme has been shown to be activated by the MAPK ERK1 and ERK2 (14) and by the p38MAPK (RK) in vivo (11, 12). Interestingly, activation of this kinase seems to be correlated to the phosphorylation of a threonine residue in a MAP kinase recognition consensus sequence PXXTP located carboxyl-terminal to the catalytic domain of the enzyme (14). This would indicate a process of activation of MAPKAP kinase 2 different from other protein kinases, which are activated by phosphorylation within the catalytic domain in the vicinity of the putative substrate binding site (reviewed in Refs. 15 and 44).

In this article we use a recombinant glutathione S-transferase (GST)-MAPKAP kinase 2 fusion protein and various mutants to study the mechanism of activation of MAPKAP kinase 2 by p44MAPK (ERK1) and p38/40MAPK (RK) in vitro. Furthermore, we analyze the stress-dependent activation of MAPKAP kinase 2 in vivo by transfection experiments with an epitope-tagged enzyme and appropriate mutants in NIH 3T3 cells. We provide evidence that, in addition to the phosphorylation at Thr-317 outside the catalytic domain, activation of MAPKAP kinase 2 by ERK1 proceeds through phosphorylation of a second threonine residue Thr-205 inside the catalytic domain in vitro. Furthermore, the data presented indicate that there is no further regulatory residue in MAPKAP kinase 2 phosphorylated in vitro by p38/40 or in vivo as a result of heat shock. Different constitutively active mutants of MAPKAP kinase 2 are obtained by replacement of the threonine residues at the phosphorylation sites by glutamic acid, which mimics a negative phosphate charge. In addition, constitutive activation of MAPKAP kinase 2 is reached by mutations of the A-helix motif carboxyl-terminal to the catalytic domain. A model for the mechanism of dual regulation of MAPKAP kinase 2 activity by phosphorylation of Thr-205 inside the catalytic domain and by phosphorylation of Thr-317 outside the catalytic domain involving the A-helix motif is proposed.

**EXPERIMENTAL PROCEDURES**

Site-directed Mutagenesis—Phosphorylation site and deletion mutants of MAPKAP kinase 2 were constructed by oligonucleotide-directed mutagenesis using the GST-MAPKAP kinase 2 Δ3B expression vector pGEX-SX-3-MK2-Δ3B (16) as double-stranded template and the Trans-
formal site-directed mutagenesis kit (Clontech). Mutations were verified by double-stranded plasmid DNA sequencing using Sequenase 2.0 (U.S. Biochemical Corp.). The carboxyl-terminal deletion mutant GST-fused by double-stranded plasmid DNA sequencing using Sequenase 2.0 LB medium containing 16 g/liter tryptone, 10 g/liter yeast extract, and purified fusion proteins was measured according to Ref. 17.

The deletion, this results in addition of the sequence SSGRIVTD to the GST-fusion protein in used to express the enzyme MAPKAP kinase 2 and its mutants as a treated with anisomycin (Sigma) at a final concentration of 10 μM. After an additional 90 min, cells were harvested and lysed, and GST-fusion protein was purified using glutathione-Sepharose 4B (Pharmacia) as described by the manufacturers. The concentration of purified fusion proteins was measured according to Ref. 17.

Partial Purification of p38 MAPK (RK) from Anisomycin-stimulated EAT Cells—1.5 × 10^6 Ehrlich ascites tumor (EAT) cells were treated with anisomycin (Sigma) at a final concentration of 10 μM for 20 min. Cells were washed 3 times in ice-cold phosphate-buffered saline, harvested, and resuspended in 5 ml of lysis buffer L (20 mM Tris acetate, pH 7.0, 0.1 mM EDTA, 1 mM EGTA, 1 mM NaVO_4, 10 mM β-glycerophosphate, 50 mM NaF, 5% Triton X-100, 1 mM benzamidine, 0.1% β-mercaptoethanol, 0.27 mM sucrose, 0.2 mM phenylmethylsulfonyl fluoride). After a 20 min incubation on ice, the lysate was prepared as the supernatant of a 13,000 rpm centrifugation, diluted with 10 ml of Mono Q buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.3 mM Na_3VO_4, 5% (v/v) glycerol, 0.03% (w/v) Brij 35, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.1% (v/v) β-mercaptoethanol) and loaded onto a Mono Q column (Pharmacia, column dimensions 5 × 0.5 cm). The column was developed with a linear gradient from 0 to 700 mM NaCl (40 ml) and 1-ml fractions were collected. 10-μl aliquots of the fractions were incubated with 1 μM recombinant GST-MAPKAP kinase 2.5 mM reaction mixture containing β-glycerophosphate, 1 mM EDTA, 10 mM magnesium acetate, 0.1 mM ATP, 0.1 μM okadaic acid, and 125 μM sodium orthovanadate for 30 min at 30°C. 10 μl of this reaction mixture were subsequently assayed for MAPKAP kinase 2 activity as described below. For determining endogenous MAPKAP kinase 2 activity, 4-μl aliquots of the fractions were assayed.

Immunoblot detection of ERKs in the Mono Q fractions was performed using a mouse monoclonal pan ERK antibody (Transduction Laboratories, Lexington) and a secondary antibody conjugated to alkaline phosphatase (Promega). Western blot detection of p38 MAPK (RK) was achieved with a sheep antiserum against a carboxyl-terminal peptide from human RK (kindly provided by P. Cohen, Dundee). Immunoprecipitation of p38 MAPK (RK) was performed with a rabbit anti-serum raised against a carboxyl-terminal peptide from Xenopus Mpk2 as described in Ref. 11.

In Vitro Activation of GST-MAPKAP Kinase 2 Fusion Protein and Its Mutants by p44 MAPK (ERK1) and p38 MAPK (RK)—10 μM concentration of the purified recombinant fusion proteins GST-MAPKAP kinase 2 and its mutants were incubated in a 25-μl kinase reaction mixture containing 50 mM β-glycerophosphate, 0.1 mM EDTA, 4 mM magnesium acetate, 0.1 mM ATP, 0.1 μM okadaic acid, 125 μM sodium orthovanadate, and 5 ng of p44 MAPK (Biomol, purified from sea star) or 10 μl of the Mono Q fractions were 13 or 19 or the anti-Mpk2 immunoprecipitate for 30 min at 30°C. Control incubations omitting MAPKs to analyze the influence of autophosphorylation on the recombinant protein were always carried out.

Assay for MAPKAP Kinase 2 Activity—10-μl aliquots from the MAP kinase activation mixture or 4 μl of the Mono Q fractions were incubated in a kinase reaction mixture of a final volume of 25 μl, containing 50 mM β-glycerophosphate, 0.1 mM EDTA, 4 mM magnesium acetate, 0.1 mM ATP, 1.5 μl of (γ-32P)ATP, and 10 μl of recombinant Hsp25 purified by affinity chromatography. After 10 min of incubation at 37°C, reactions were terminated by adding 8 μl of 4× SDS sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis. 32P-Labelled proteins were detected by the Bio Imaging Analyzer BAS 2000 (Fuji) and Hsp25 labeling was quantified by photostimulated luminescence. Assay conditions were tested to guarantee a linear dependence of kinase activity determined on the assay time chosen.

Construction of Expression Vectors for Epitope-tagged MAPKAP Kinase 2—The cDNA of mouse MAPKAP kinase 2 (19) was cloned into the KpnI/ BamHI site of the eukaryotic expression vector pcDNA3 (Invitrogen) by a polymerase chain reaction strategy introducing the Myc epitope EKQLISEEDLG at the amino terminus of the protein using the oligonucleotide primer 5'-CGG GGT ACC ATG GAA CAG ACG TTC AGC GAA GAG GAC CTA GGA GAG GCC CGC ACT CCG. The mutations of the phosphorylation sites were confirmed with the Transformer site-directed mutagenesis kit (Clontech) as described above. Plasmids were transfected into NIH 3T3 cells by the LipofectAMINE Transfection Kit (Life Technologies, Inc.). Stable transfected cell lines were established by a 2-week selection with G418 (800 μg/ml). Heat shock treatment of cells was performed for 15 min at 43.5°C.

Immunoprecipitation was carried out after 15 min of incubation of 10^6 cells in 80 μl of lysis buffer L. Cell lysate was diluted with 500 μl of IP buffer (50 mM Tris-HCl, pH 7.4, 25 mM β-glycerophosphate, 25 mM NaF, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) and incubated with 25 μl of purified 9E10 antibody overnight at 4°C. Precipitation was achieved by adding 25 μl of a 1:1 (v/v) suspension of Protein A-Sepharose (Pharmacia) and a further incubation for 1 h at 4°C. Immunoprecipitate was washed four times with IP buffer, and the pellet was redissolved in 25 μl of MAPKAP kinase 2 reaction mixture and analyzed as described above.

Molecular Modeling of MAPKAP Kinase 2—The structure of the catalytic core of MAPKAP kinase 2 was modeled on the basis of the coordinates of the cAMP-dependent protein kinase (cAPK) at 2.7 Å (20), taken from the Brookhaven Protein Data Bank (8PDB entry 2cpp). The sequences were aligned with the best fit routine of the GCG package (Genetics Computer Group, Program Manual for the GCG Package, Version 7, April 1991, Madison, WI) giving an identity of 30%. The amino acids of MAPKAP kinase 2 were generated by mutating, deleting, and inserting residues with the program O (21) according to the alignment. The geometry of the model was improved iteratively with the energy minimization routine of X-PLOR (22) and optical inspection on the graphics screen. The positions of inserted residues were altered by local molecular dynamics calculations and subsequent energy minimization. The resulting model consists of residues 40 to 300 (the ATP-binding and catalytic domain) with root mean square deviations from target values of 0.009 Å for bond lengths and 2.92° for angles. No residue is in the disallowed region of the Ramachandran plot according to PROCHECK (23). The superposition of 74% of the C_α atoms on the start model gave a root mean square deviation of 0.68 Å. The A-helix was modeled in standard β-sheet conformation and fitted into the catalytic domain of MAPKAP kinase 2 by analogy to the position in cAMP-dependent protein kinase. The complex was then energy-minimized with backbone atoms kept fixed.
In Vitro Activation of MAPKAP Kinase 2 by p38/40

Constitutive Activation of MAPKAP Kinase 2 by T205E,T317E Mutations—To reinforce the notion that Thr-205 and Thr-317 both contribute to MAPKAP kinase 2 activation, we mutated these residues also to glutamate which can mimic the negative charge of the phosphate group. The appropriate T205E, T317E, and T205E,T317E mutants, and, as a control, the mutant T209E were expressed as GST-fusion proteins and their activity was analyzed before and after phosphorylation by ERK1 in vitro using Hsp25 as substrate (Fig. 2A). Mimicry of phosphorylation at both sites Thr-205 and Thr-317, but not at residue Thr-209, which is also located between subdomains VII and VIII and followed directly by a proline (not shown), leads to activation of MAPKAP kinase 2. The activity of the single mutants T205E and T317E is increased to about 5-fold compared to the wild type and could be stimulated further by ERK1 phosphorylation, indicating that the second, intact phosphorylation site contributes to activation. The activity of the double mutant T205E,T317E is increased to about 10–15-fold and could not significantly be further stimulated by incubation with MAPK (Fig. 2B), supporting the notion that Thr-205 and Thr-317 are the regulatory phosphorylation sites of MAPKAP kinase 2.

In Vitro Activation of MAPKAP Kinase 2 by p38/40MAPK—

Molecular Mechanism of MAPKAP Kinase 2 Activation

27215

Fig. 1. In vitro reconstitution of MAPKAP kinase 2 activation and subsequent Hsp25 phosphorylation in dependence of MAPKAP kinase 2 phosphorylation site mutations. A, schematic representation of the different recombinant forms of MAPKAP kinase 2 used. The fusion proteins GST-MAPKAP kinase 2 (GST-MK2) and the SH3-binding domain (3B) deletion mutant GST-MK2-3B show identical activation in the assay and are referred to as wild type protein (WT). Based on the wild type protein GST-MK2-3B, the phosphorylation site mutants T205A, T317A, and the double mutant T205A,T317A were constructed. (Catalytic, catalytic domain; NTS, nuclear translocation signal). B, analysis of the wild type form (WT) and phosphorylation mutants (T205A, T317A, T205A,T317A) of MAPKAP kinase 2 for their ability to phosphorylate Hsp25 in dependence on activation by pp44 ERK1 MAP kinase. C, sequence alignment of the region of MAPKAP kinase 2 containing the newly identified phosphorylation site Thr-205, to the region between subdomains VII and VIII of the catalytic core of serine/threonine protein kinases known to be phosphorylated and activated at similar sites (MAPKAP kinase 1, ERK2 Cdk2, cAPK). Phosphorylation sites are indicated by asterisks.
Until now, it is not completely understood which forms of the MAPKAP kinase 2 are responsible for MAPKAP kinase 2 activation. In vivo studies as shown in Fig. 3A, phosphorylation of Hsp25 by constitutively activated single and double mutants (T205E, T317E, and T205E.T317E) of MAPKAP kinase 2 in dependence on ERK1 phosphorylation. B, quantitative evaluation of enzymatic activity of the constitutive mutants compared to the wild type enzyme (WT) in dependence on ERK1 phosphorylation. \(^{32}P\) Labeled Hsp25 was detected by the BioImaging Analyzer BAS 2000 (Fuji), and labeling was quantified by photostimulated luminescence (PSL). The data represent the mean value of three independent experiments as shown in A.

**Fig. 2.** Analysis of constitutively active forms of MAPKAP kinase 2. A, phosphorylation of Hsp25 by constitutively activated single and double mutants (T205E, T317E, and T205E.T317E) of MAPKAP kinase 2 in dependence on ERK1 phosphorylation. B, quantitative evaluation of enzymatic activity of the constitutive mutants compared to the wild type enzyme (WT) in dependence on ERK1 phosphorylation. \(^{32}P\) Labeled Hsp25 was detected by the BioImaging Analyzer BAS 2000 (Fuji), and labeling was quantified by photostimulated luminescence (PSL). The data represent the mean value of three independent experiments as shown in A.

**Fig. 3.** Activation of Epitope-tagged MAPKAP Kinase 2 in Transfected NIH 3T3 Cells after Heat Shock—To directly analyze the in vivo phosphorylation of MAPKAP kinase 2, we constructed vectors for expression of an epitope-tagged MAPKAP kinase 2 and its mutants which were transfected into NIH 3T3 cells. These vectors also contain the region which codes for the proline-rich amino-terminal SH3 binding motif of the kinase and which was deleted in the GST-MAPKAP kinase 2 used in the in vitro activation studies. Since heat shock is a potent inducer of MAPKAP kinase 2 in these cells and since it is known to stimulate both RK (11) and ERKs (47–48), we analyzed expression and activation of the transfected epitope-tagged enzyme after heat shock treatment by immunoblot detection and immunoprecipitation with an anti-Myc-tag antibody and subsequent kinase assay in the immunoprecipitate, respectively. The results (16), that the proline-rich SH3-binding domain does not alter the mechanism of MAPKAP kinase 2 regulation by phosphorylation.
nism underlying the regulation of MAPKAP kinase 2 activity by phosphorylation outside the catalytic domain at Thr-317 is not clear. To characterize this mechanism, we constructed carboxyl-terminal deletion mutants and analyzed their activity in dependence on pp44MAPK phosphorylation. Unexpectedly, the mutant ΔPC lacking the carboxyl-terminal region (amino acids 315–383), including the phosphorylation site Thr-317, shows significant enzymatic activity before phosphorylation by pp44MAPK (Fig. 5C). This indicates that the carboxyl terminus of the molecule contains an autoinhibitory domain which possibly may be regulated by phosphorylation at Thr-317. The recent description of a human isoform of MAPKAP kinase 2, which is probably the product of differential splicing and has a partially altered carboxyl terminus but is not constitutively active (26), restricts the location of a putative autoinhibitory domain to the carboxyl-terminal region which is homologous in both isoforms (amino acids 309 to 337 in mouse MAPKAP kinase 2). In this region, Zu et al. (32) have very recently...
identified an autoinhibitory domain of human MAPKAP kinase 2, which is proposed to act as a pseudosubstrate for MAPKAP kinase 2 by these authors (cf. Fig. 5A). However, our detailed analysis revealed a sequence motif within this region, which shows striking homology to the amphiphilic A-helix conserved in several protein kinases (33, 34) (Fig. 5B). To decide whether the autoinhibitory region of MAPKAP kinase 2 is based on the A-helix motif or on the similarity to a pseudosubstrate or on both, we mutated conserved residues of the A-helix and of the pseudosubstrate sequence proposed (cf. Fig. 5A and B). Since deletions of the core of the A-helix motif (Δ321–338, Δ326–333) lead to insolubility/instability of the recombinant protein (not shown), we mutated the A-helix by replacement of the central tryptophan residue with alanine (W332A) and by substitution of a further conserved lysine residue by a negatively charged glutamate residue K326E (cf. Fig. 5B). The pseudosubstrate sequence proposed in (32) is based on the conserved arginine residue in position 331. As we are aware of the strong preference of MAPKAP kinase 2 for the substrate sequence LXXRXS over LXXKXS (35), we mutated R331K to negatively affect the pseudosubstrate properties of this sequence. On the other hand, we changed the potential pseudosubstrate region in such a way to make this sequence an ideal substrate for MAPKAP kinase 2 by replacing K329L and D334S (cf. Fig. 5A). If this sequence would act as a pseudosubstrate, the residue Ser-334 should be phosphorylated as already known for other pseudosubstrates (36).

We then analyzed enzymatic activity of the different pseudosubstrate and A-helix mutants (Fig. 5C) and the phosphorylation of the K329L/D334S mutant (Fig. 5D). As seen in Fig. 5C, only the two mutants affecting conserved residues of the A-helix motif lead to constitutive activation of the enzyme indicating that the A-helix motif contributes to suppress MAPKAP kinase 2 activity. However, the higher constitutive activity of the W332A mutant compared to the K326E mutant may indicate the central structural role of this tryptophan residue within the A-helix. In contrast, mutants constructed to change the pseudosubstrate properties of this region do not influence kinase activity. Not even the alteration of the pseudosubstrate motif to an ideal substrate for MAPKAP kinase 2 does increase kinase activity. Furthermore, there is no increased autophos-
phorylation of the enzyme carrying the phosphorylatable Ser-334 in the potential pseudosubstrate sequence (Fig. 5D), although the corresponding peptide KKLERWSVK-amide is efficiently phosphorylated by the mutant K329L/D334S (data not shown). Taken together, these data strongly indicate that the autoinhibitory region of MAPKAP kinase 2 does not function as a pseudosubstrate. Hence, it could be assumed that the A-helix motif does not directly bind to the peptide acceptor site within the catalytic cleft of MAPKAP kinase 2, but acts auto-inhibitory by binding to some other region of the kinase. One potential binding region for the A-helix to the catalytic core could be the hydrophobic surface distal to the active site between the two lobes of the catalytic core as described for the A-helix of cAMP-dependent protein kinase (20).

**Molecular Modeling of the A-helix-Core Interaction in MAPKAP Kinase 2**—Molecular modeling was used to investigate whether the A-helix motif in MAPKAP kinase 2 could fill the hydrophobic region between the two lobes of the catalytic domain of the kinase as proposed for several other protein kinases (33). On the basis of the primary structure alignment and the three-dimensional structure of the catalytic subunit of the cAMP-dependent protein kinase (cAPK) (20), a model of the catalytic core of MAPKAP kinase 2 was constructed. In addition, a standard α-helix with the sequence of the A-helix of MAPKAP kinase 2 was built. Subsequently, the A-helix of MAPKAP kinase 2 was fitted into the catalytic domain of MAPKAP kinase 2 by analogy to the position of the A-helix in cAPK and the potential energy of the complex was minimized. As expected, the tryptophan residue in the A-helix of MAPKAP kinase 2 could be shown to fit into the hydrophobic pocket between the two lobes of MAPKAP kinase 2 (Fig. 6). In the model this tryptophan residue interacts by van der Waals contacts with several residues of the catalytic core. The major contribution to this interaction seems to come from the isoleucine residues Ile-103, Ile-163, and Ile-165 of the catalytic core. The major contribution to this interaction seems to come from the isoleucine residues Ile-103, Ile-163, and Ile-165 of the catalytic core of MAPKAP kinase 2 as seen in Fig. 6B. However, mutations of these residues to charged amino acids carried out to disturb the A-helix interaction with this region could not prove this model, since these mutations completely inactivate the enzyme (not shown), probably due to changes in the steric arrangements within the catalytic domain itself.

**DISCUSSION**

In this paper we identify a second regulatory phosphorylation site of MAPKAP kinase 2 and provide experimental evidence that stimulation of MAPKAP kinase 2 activity proceeds by MAPK phosphorylation at two different regulatory sites. The evidence came from the observation that single T205A and T317A MAPKAP kinase 2 mutants could still be activated by ERK1 and p38/40MAPK (RK) phosphorylation in vitro. Although there is a slight difference between the basal activity of the double mutant T205A,T317A in vitro (detectable) and in vivo (not detectable), which is probably due to the different expression systems, this mutant cannot be stimulated either by ERK1 phosphorylation in vitro or by the heat shock-stimulated forms of MAPKs in vivo. This finding indicates that both phosphorylation sites Thr-205 and Thr-317 are necessary for MAPKAP kinase 2 activation.

In a second approach we demonstrate that a constitutively active form of MAPKAP kinase 2 could be obtained as a result of mimicking the negative phosphate groups of phosphorylated Thr-205 and Thr-317 by replacement with glutamic acid. The finding that the fully constitutively active double mutant T205E,T317E cannot be further stimulated by ERK1 and p38/40MAPK (RK) phosphorylation in vitro and by heat shock treatment in NIH 3T3 cells gives independent support to the notion that these sites are the two major regulatory phosphorylation sites of MAPKAP kinase 2.

Our results demonstrate that the mechanism of activation of MAPKAP kinase 2 by ERK1 and p38/40MAPK (RK) is very similar and that MAPKAP kinase 2 activation by these enzymes proceeds with comparable efficiency in vitro. However, in PC12 and A431 cells, ERKs fail to activate MAPKAP kinase 2, whereas p38/40MAPK (RK) is a major activator for this enzyme (11, 12). An explanation for this discrepancy between in vitro and in vivo data could be a different subcellular location of ERKs and MAPKAP kinase 2 in these cells or a specific protein-protein interaction between MAPKAP kinase 2 and other unknown proteins, which prevent the contact to ERKs but facilitate the binding to p38/40MAPK (RK). The latter explanation would be in agreement with the idea of the existence of mammalian signal transduction particles tethered by "scaffolding proteins" analogous to the yeast protein STE5 (46).

The replacement of regulatory phosphorylation sites by negatively charged residues from aspartate and glutamate to constitutively active protein kinases has recently been used in the case of the MAPK kinase MEK1 (37–40). Using this approach, it was possible to restore MEK activity independent of the upstream kinases and to analyze the role of activated MEK.
in growth, differentiation, and oncogenic transformation. The constitutively active form of MAPKAP kinase 2, which is in mitogenic signal transduction downstream of the bifurcation point of the MAPKs, will now open further ways to analyze the cellular role of MAPKAP kinase 2, as well as the role of the phosphorylation of its major substrate, the small mammalian heat shock protein.

The identification of the phosphorylation sites of MAPKAP kinase 2 yields new insight into the mechanism of the regulation of protein kinase activity. The phosphorylation site identified in this report, Thr-205, in the loop between subdomains VII and VIII of the catalytic domain is homologous to regulatory phosphorylation sites of several other protein kinases involved in mitogenic signal transduction (cf. Fig. 2) and places the regulation of MAPKAP kinase 2 in one line with the emerging common mechanism of activation of many protein kinases. These phosphorylation sites are in the activation loop of the kinase and could regulate the accessibility of the substrate binding sites and/or the relative location of the amino- and carboxyl-terminal lobes of the catalytic core, leading to correct alignment of the different catalytic residues of these kinases (44).

The second regulatory phosphorylation site of MAPKAP kinase 2, Thr-317, has been identified outside the catalytic domain, indicating an indirect influence of this phosphorylation on the catalytic properties of the kinase. Besides the direct activation of protein kinases through phosphorylation within the catalytic domain, several cases of regulation of protein kinase activity by intracellular inhibition of catalytic activity due to autoinhibitory "pseudosubstrate" regions have been described. These autoinhibitory domains could be regulated by allosteric factors such as calcium/calmodulin (CaM) in the case of the CaM-dependent kinases or phospholipid diacylglycerol in the case of protein kinase C and, probably, also by phosphorylation (for reviews see Refs. 42 and 43). A second, recently described common sequence motif of protein kinases which has a reguative potential is the amphiphilic A-helix (33, 34). The A-helix has been described originally as a stabilizing element of protein kinase structure which binds to a hydrophobic pocket present in most protein kinases between the two lobes of the catalytic core on the surface opposite to the catalytic cleft opening (44).

In this paper we first provide evidence that an A-helix can act as an autoinhibitory element in MAPKAP kinase 2. Deleting the carboxy-terminal region containing the A-helix motif and even changing the conserved tryptophan and lysine residues of the A-helix led to activation of the MAPKAP kinase 2, indicating that the presence of a functional A-helix can suppress the activity of the enzyme. This is in agreement with the recent finding that an amphiphilic A-helix-like motif can also suppress the catalytic activity of the protein kinase MEK (39). The mechanism by which the A-helix inhibits the kinase activity and by which phosphorylation may regulate this inhibition is still unclear. An action of the A-helix as a pseudosubstrate for the kinase seems unlikely, since alteration of the conserved arginine residue of the pseudosubstrate motif and modification of this motif to an ideal substrate does not influence kinase activity or its autophosphorylation. However, it seems likely that this mechanism is based on complex intramolecular interactions, since an A-helix motif-derived peptide CVLKEDKER-WEDVK and a GST-fusion protein containing the carboxy-terminal part of MAPKAP kinase 2 were not able to specifically inhibit MAPKAP kinase 2 activity of the wild type protein purified from rabbit muscle (generous gift of P. Cohen, Dundee) or of the constitutively active A-helix deletion mutant ΔPC.²

By molecular modeling, we have shown a possible interaction of the A-helix motif of MAPKAP kinase 2 with the catalytic core. Interestingly, even in a protein kinase without an A-helix motif, as in the MAPK ERK2, the hydrophobic pocket between the lobes opposite to the catalytic cleft is filled by hydrophobic residues of the non-core sequences which are located approximately 30 residues downstream to the subdomain XI (41, 44). This distance is similar to the distance of the cryptophasic residue of the A-helix from the subdomain XI in MAPKAP kinase 2 and supports the notion that the A-helix of MAPKAP kinase 2 could also bind to this hydrophobic pocket between the lobes. Although binding of the A-helix of MAPKAP kinase 2 to other regions of the enzyme could not be excluded, molecular modeling supports binding of the A-helix to the hydrophobic pocket between the two lobes of MAPKAP kinase 2 predominantly based on interaction of the central cryptophasic residue of the A-helix. Hence, a mechanism proposed to contribute to the regulation of MAPKAP kinase 2 is the binding of the A-helix to the hydrophobic pocket between the two lobes which could affect catalysis. Phosphorylation of Thr-317 at the proposed beginning of the A-helix may destabilize the A-helix itself and/or its binding to the hydrophobic cleft and by that activates MAPKAP kinase 2. Further studies to resolve the phosphorylation-dependent three-dimensional structure of MAPKAP kinase 2 will prove whether the proposed model describes the molecular mechanism underlying MAPKAP kinase 2 activation.

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Molecular Mechanism of MAPAP Kinase 2 Activation

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