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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 (MAPKAP) kinase 2 which has been shown to be responsible for small heat shock protein phosphorylation. We have analyzed the mechanism of MAPKAP kinase 2 activation by MAPK phosphorylation using a recombinant MAPKAP kinase 2-fusion protein, p44^{MAPK} and p38/40^{MAPK} *in vitro* and 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[1] kinases ERK1 and ERK2 has been identified as the protein kinase MEK, a dual specific kinase which itself is activated by protein kinases encoded by the proto-oncogenes *raf1* or *mos* (2, 3) as well as by MEK kinase (4). In addition, stress-dependent signaling seems to proceed 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 also termed amino-terminal c-Jun kinases (JNKs) (8, 9). Another distinct subgroup covers the p38^{MAPK} and p40^{MAPK}, 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 groups of 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) *in vitro* and by the p38/40^{MAPK} (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 P*X*TP 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 p44^{MAPK} (ERK1) and p38/40^{MAPK} (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-5X-3-MK2-Δ3B (16) as double-stranded template and the Trans-

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[1] The abbreviations used are: MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MAPKAP, MAPK-activated protein; MEK, MAP or ERK kinase; RK, reactivating kinase; RSK, ribosomal S6 kinase II; cAPK, catalytic subunit of the cAMP-dependent protein kinase; Cdk, cyclin-dependent kinase; EAT, Ehrlich ascites tumor; ERK, extracellular regulated kinase; GST, glutathione *S*-transferase; Hsp, heat shock protein.

former 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-MAPKAP kinase 2-ΔPC (Δ amino acids 315–383) was derived from pGEX-5X-3-MK2-Δ3B by digestion with *Ppu*MI and *Xho*I, subsequent filling to blunt ends by Klenow polymerase, and ligation. Apart from the deletion, this results in addition of the sequence SSGRIVTD to the carboxyl terminus.

Expression of Recombinant GST-MAPKAP Kinase 2 and Mutants—The *E. coli* expression vector pGEX-5X-3 (Pharmacia Biotech Inc.) was used to express the enzyme MAPKAP kinase 2 and its mutants as a GST-fusion protein in *Escherichia coli* SURE (Stratagene) cultivated in LB medium containing 16 g/liter tryptone, 10 g/liter yeast extract, and 5 g/liter NaCl. Expression was induced at an optical density $A_{600} = 1$ by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.1 mM. 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 p38/40^{MAPK} (RK) from Anisomycin-stimulated EAT Cells—1.5×10^8 Ehrlich ascites tumor (EAT) cells were treated with anisomycin (Sigma) at a final concentration of 10 μg/ml 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 Na_3VO_4, 10 mM β-glycerophosphate, 50 mM NaF, 5 mM pyrophosphate, 1% Triton X-100, 1 mM benzamidine, 0.1% β-mercaptoethanol, 0.27 M 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 Δ3B in a 25-μl reaction mixture containing 50 mM β-glycerophosphate, 0.1 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 p38/40^{MAPK} (RK) was achieved with a sheep antiserum against a carboxyl-terminal peptide from human RK (kindly provided by P. Cohen, Dundee). Immunoprecipitation of p38/40^{MAPK} (RK) was performed with a rabbit antiserum 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 p38/40^{MAPK} (RK)—1 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 pp44^{MAPK} (Biomol, purified from sea star) or 10 μl of the Mono Q fractions 13 and 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 μCi of [γ-^{33}P]ATP, and 10 μg of recombinant Hsp25 purified from *E. coli* (18). After 10 min at 30 °C, reactions were terminated by adding 8 μl of 4 × SDS sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis. ^{33}P-Labeled 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, Transfection, and Heat Shock Experiments, Immunoblotting, and Immunoprecipitation—The cDNA of mouse MAPKAP kinase 2 (19) was cloned into the *Kpn*I/*Bam*HI site of the eukaryotic expression vector pcDNA3 (Invitrogen) by a polymerase chain reaction strategy introducing the Myc epitope EQKLISEEDLG at the amino terminus of the protein using the oligonucleotide primer 5′-CGG GGT ACC ATG GAA CAG AAG CTC ATC AGC GAA GAG GAC CTA GGA GGC TCT CCG GGC CAG ACT CCG. The mutations of the phosphorylation sites were performed 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.

For immunoblot detection of epitope-tagged MAPKAP kinase 2, 10^6 cells were lysed by boiling in SDS-electrophoresis loading buffer, applied directly to SDS-PAGE, and blotted onto nitrocellulose. Immunochemical detection was performed using monoclonal antibody 9E10 (European Collection of Animal Cell Culture, cell line 85102202) and an anti-mouse immunoglobulin secondary antibody conjugated to alkaline phosphatase (Promega).

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 (BPDB entry 2cpk). The sequences were aligned with the bestfit 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 α-helix 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.

RESULTS

In Vitro Activation of MAPKAP Kinase 2 and Its Phosphorylation Site Mutants by ERK1—We have analyzed the activation of mouse MAPKAP kinase 2 by MAP kinases using a recombinant GST-MAPKAP kinase 2 fusion protein and the p44^{MAPK} ERK1. The cDNA of mouse MAPKAP kinase 2 (19) was used to express the fusion protein GST-MAPKAP kinase 2 in *E. coli*. The nonphosphorylated purified GST-MAPKAP kinase 2 had no significant catalytic activity as judged by its ability to phosphorylate the small heat shock protein Hsp25 which is a dominant substrate for MAPKAP kinase 2 *in vitro* (24) and *in vivo* (25). As already shown for the native rabbit MAPKAP kinase 2 (14), *in vitro* phosphorylation of the recombinant mouse and human MAPKAP kinase 2 fusion protein by MAPK leads to activation of the enzyme (16, 26), indicating that the recombinant protein shares the mechanism of activation with the native enzyme. This has also been demonstrated to be true for a deletion mutant of the GST-MAPKAP kinase 2 where the 27 amino acids of the proline-rich SH3 binding motif are missing and which is, probably due to the missing GC-rich coding region, much better expressed in *E. coli* (16). This re-

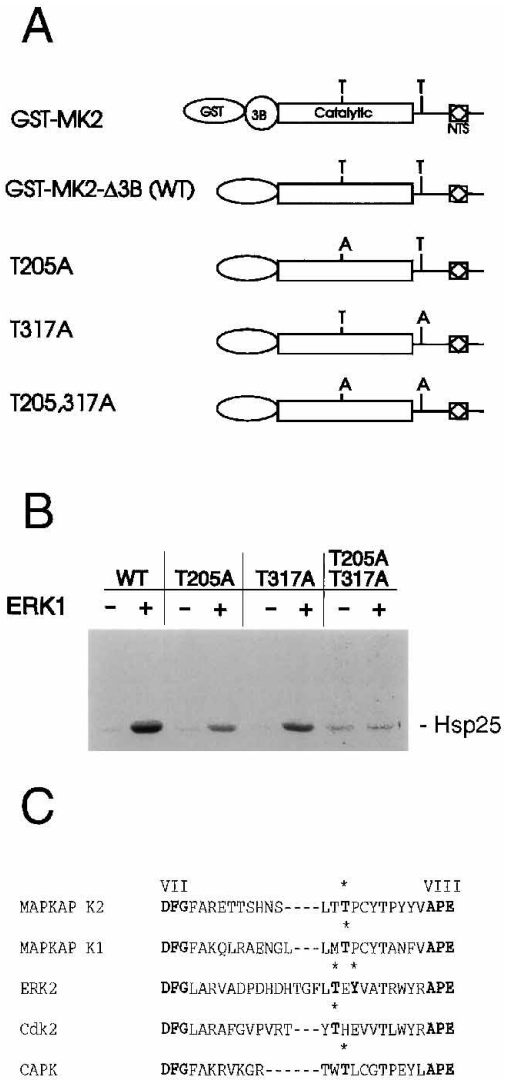

FIG. 1. ***In vitro* reconstitution of MAPKAP kinase 2 activation and subsequent Hsp25 phosphorylation in dependence of MAPKAP kinase 2 phosphorylation site mutations.** *A*, schematic representation of the different recombinant forms of MAPKAP kinase 2 used. The fusion proteins GST-MAPKAP kinase 2 (*GST-MK2*) and the SH3-binding domain (*3B*) deletion mutant GST-MK2-Δ3B show identical activation in the assay and are referred to as wild type protein (*WT*). Based on the wild type protein GST-MK2-Δ3B, 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,T 317A) 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*.

combinant fusion protein GST-MAPKAP kinase 2 Δ3B is used for the *in vitro* activation studies, since it has a K_m value very similar to the wild type enzyme and shows at least a 10-fold activation *in vitro*. This corresponds to the degree of MAPKAP kinase 2 activation *in vivo* (see below). To discern it from the different phosphorylation site and A-helix mutants described in the additional experiments, this recombinant form of MAPKAP kinase 2 is referred to as wild type MAPKAP kinase 2 (*cf.* Fig. 1*A*).

In order to understand the molecular mechanism underlying the activation of mouse MAPKAP kinase 2 by single MAPK phosphorylation carboxyl-terminal to the catalytic domain, we replaced the amino acid residue Thr-317, which is homologous to the only identified MAPK phosphorylation site of rabbit MAPKAP kinase 2 (14), by the amino acid alanine which cannot be phosphorylated. The mutation of this phosphorylation site led to decreased phosphorylation and activation of MAPKAP kinase 2 by ERK1 but, unexpectedly, the T317A mouse MAPKAP kinase 2 mutant (*cf.* Fig. 1*A*) could still be activated by ERK1 (Fig. 1*B*). This indicated that there is at least a second regulatory phosphorylation site of MAPKAP kinase 2 for ERK1. To identify further putative Ser/Thr phosphorylation sites of MAPKAP kinase 2, we compared primary sequences of MAPKAP kinase 2, MAPKAP kinase 1 ($p90^{RSK}$), ERK2, Cdk2, and cAPK. Fig. 1*C* demonstrates the conservation of threonine residues in the loop between subdomains VII and VIII of their catalytic core. These threonine residues are essential for the activation of these kinases: MAPKAP kinase 1 is known to be activated by ERK2 phosphorylation at the threonine residue which is nine residues amino-terminal to the subdomain VIII (APE) (27), MAPK ERK2 is phosphorylated by MEK1 both at the threonine and tyrosine in similar positions (28), and comparable regulatory phosphorylation sites are also present in Cdk2 or in cAMP-dependent protein kinase (29) (marked by *asterisks* in Fig. 1*C*). Interestingly, in both MAPKAP kinases 1 and 2, the equivalent threonine is followed by a proline, the minimum consensus sequence for phosphorylation by MAP kinases (30). Hence, a potential second phosphorylation site of MAPKAP kinase 2 could be Thr-205, the phosphorylation site equivalent to Thr-470, Thr-192, Thr-161, and Thr-197 of, respectively, MAPKAP kinase 1, ERK2, Cdk2, and cAPK. To experimentally prove whether this site is phosphorylated by MAPK, we substituted Thr-205 of mouse MAPKAP kinase 2 with alanine (*cf.* Fig. 1*A*) and analyzed the phosphorylation and activation of this mutant by ERK1. As shown in Fig. 1*B*, the T205A mutant shows an activation by ERK1 similar to the mutant T317A. There is still activation of the mutant by ERK1, but not to the same degree as in the wild type enzyme. Only the double mutant T205A,T317A, which shows a slightly increased basal activity, could not be activated by ERK1 (*cf.* Fig. 1*B*), indicating that both phosphorylation sites contribute to the *in vitro* activation of MAPKAP kinase 2 by ERK1 and that these sites seem to be the major regulatory phosphorylation sites of the enzyme.

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. 2*A*). 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. 2*B*), 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 $p38/40^{MAPK}$—

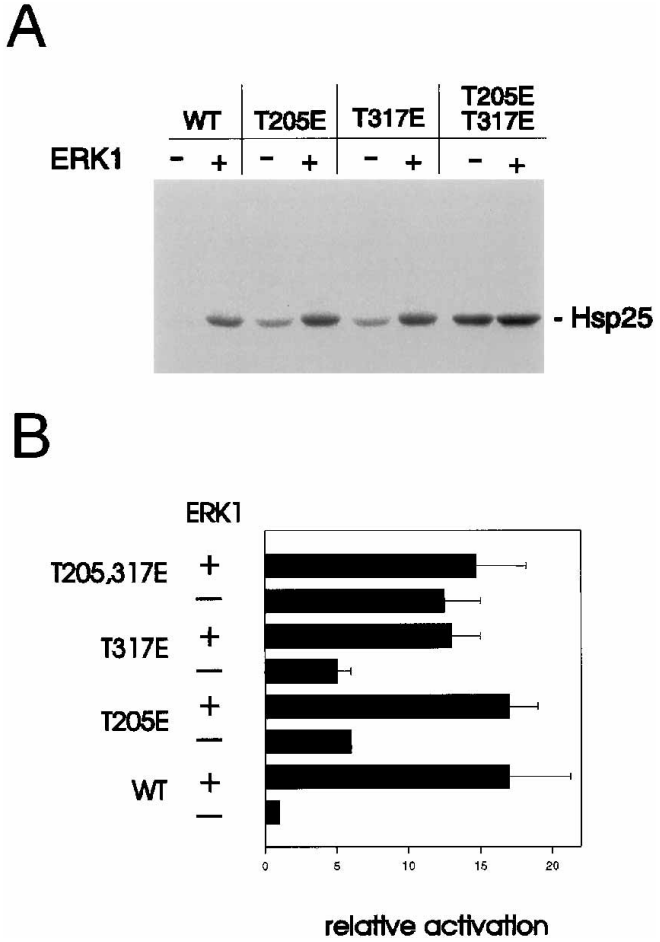

FIG. 2. **Analysis of constitutively active forms of MAPKAP kinase 2.** *A*, phosphorylation of Hsp25 by constitutively activated single and double mutants (T205E, T317E, and T205E,T317E) of MAPKAP kinase 2 in dependence on ERK1 phosphorylation. *B*, quantitative evaluation of enzymatic activity of the constitutive mutants compared to the wild type enzyme (WT) in dependence on ERK1 phosphorylation. ^{33}P-Labeled Hsp25 was detected by the Bio Imaging 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*.

Until now, it is not completely understood which forms of the MAPKs are responsible for MAPKAP kinase 2 activation *in vivo* and whether all these enzymes recognize and phosphorylate the same sites in MAPKAP kinase 2 (11–12, 25). Since there is growing evidence that the p38/40^{MAPK}, also designated as MAPKAP kinase 2 reactivating kinase (RK), is the major enzyme responsible for stress-induced activation of MAPKAP kinase 2 (6, 11–12), we also analyzed *in vitro* activation of MAPKAP kinase 2 by this enzyme. For this purpose, we stimulated Ehrlich ascites tumor (EAT) cells with the stress-signaling agonist anisomycin (31) and found about 10-fold activation of MAPKAP kinase 2 in these cells (Fig. 3*A*). The MAPKAP kinase 2 activator from the lysate of anisomycin-stimulated EAT cells was partially purified by Mono Q chromatography as described in Ref. 11. The chromatographic fractions were assayed both for MAPKAP kinase 2 and MAPKAP kinase 2 activator. In addition to a broad peak of MAPKAP kinase 2 activity eluting in the gradient between 100 mM and 200 mM NaCl as described for MAPKAP kinase 2 from interleukin 1-stimulated KB cells (12), a MAPKAP kinase 2 activator elutes from the Mono Q column in a very sharp peak at 350 mM NaCl (*Fraction 19* in Fig. 3*B*). This is exactly the same position in the gradient described for the p38/40^{MAPK} (RK) from arsenite-stimulated PC12 cells and heat shock-stimulated HeLa cells (11), indicating that this activity peak probably represents the p38/40^{MAPK} (RK) of EAT cells. The presence of p38/40^{MAPK} (RK) in fraction 19 was confirmed by Western blot analysis using an antiserum against a carboxyl-terminal peptide of human RK (not shown) and by immunoprecipitation with an anti-Mpk2 antiserum (11) and subsequent MAPKAP kinase 2 activation assay as described above (Fig. 3*C*). The presence of ERK2 in the peak fraction could be excluded, since Western blot detection of ERKs using the monoclonal pan ERK antibody (Transduction Laboratories, Lexington) clearly shows that these enzymes elute between fractions 8 and 14 (not shown).

We then analyzed activation of recombinant wild type MAPKAP kinase 2, the single mutants T205A and T317A, and the double mutant T205E,T317E by the p38/40^{MAPK} peak fraction. As seen in Fig. 3*D*, wild type MAPKAP kinase 2 can be stimulated by the p38/40^{MAPK} fraction in the same manner as by ERK1. This is also the case for ERK1/p38/40^{MAPK} stimulation of the single mutants T205A and T317A (not shown). In contrast, the constitutively active mutant T205E,T317E shows no changes in activity after treatment with the p38/40^{MAPK} fraction (Fig. 3*D*). These observations support the notion that regulation of MAPKAP kinase 2 by p38/40^{MAPK} (RK) *in vitro* proceeds also by dual phosphorylation and that there is no further regulatory phosphorylation site for p38/40^{MAPK} in MAPKAP kinase 2.

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. Fig. 4*A* shows the level of expression of the epitope-tagged wild type MAPKAP kinase 2 and its mutants. In Fig. 4*B*, it can be seen that after heat shock treatment the activity of epitope-tagged wild type MAPKAP kinase 2 is increased. In contrast to that and to the slightly increased basal activity seen in the *in vitro* activation studies, the epitope-tagged double mutant T205A,T317A, although expressed to a relatively high level compared to the other epitope-tagged forms (Fig. 4*A*), shows no activity in these cells either before or after heat shock treatment. Furthermore, the epitope-tagged constitutively active mutant T205E,T317E shows an increased basal activity compared to the epitope-tagged wild type and could not be stimulated in its activity by heat shock. These results indicate that there is no further regulatory *in vivo* phosphorylation site in MAPKAP kinase 2. It also confirms, as expected from previous *in vitro* results (16), that the proline-rich SH3-binding domain does not alter the mechanism of MAPKAP kinase 2 regulation by phosphorylation.

Constitutive Activation of MAPKAP Kinase 2 by Carboxyl-terminal Deletion and Mutations in an Autoinhibitory A-helix-like Element Which Does Not Act as a Pseudosubstrate—Activation of MAPKAP kinase 2 as a result of phosphorylation of Thr-205 in the loop between subdomains VII and VIII can be understood as a direct steric influence on the substrate binding of the kinase (15, 44) (see "Discussion"). However, the mecha-

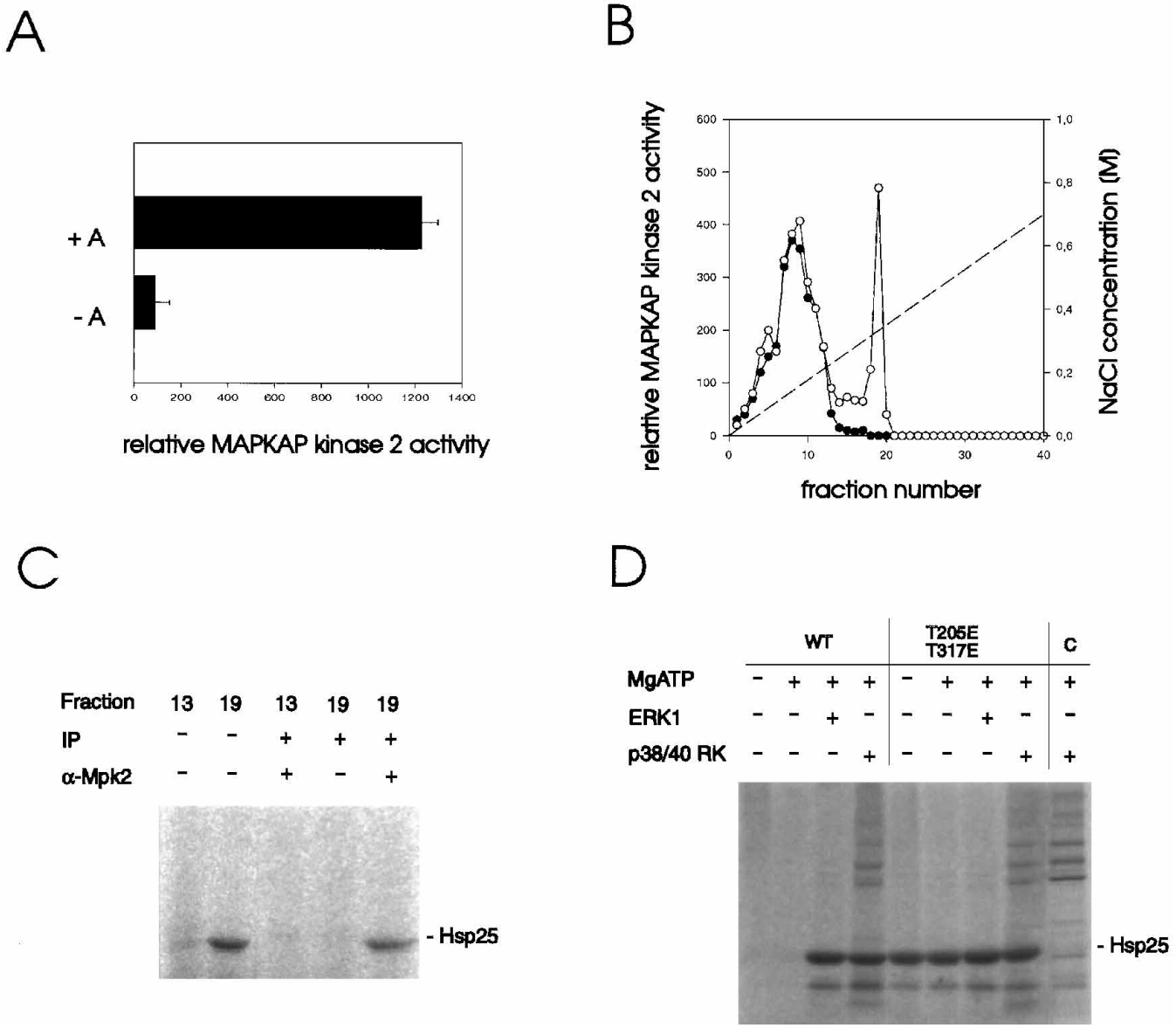

FIG. 3. **Partial purification of p38/40^{MAPK} (RK) from anisomycin treated EAT cells and *in vitro* reconstitution of MAPKAP kinase 2 activation by p38/40^{MAPK}.** *A*, stimulation of MAPKAP kinase 2 activity in EAT cells by treatment with 10 μg/ml anisomycin for 20 min (+*A*) compared to control EAT cells (−*A*). Activity shown was determined in unfractionated EAT cell lysates. *B*, Mono Q fractionation of cell lysates from anisomycin-stimulated EAT cells. Fractions were assayed for MAPKAP kinase 2 activators using recombinant GST-MAPKAP kinase 2 Δ3B and a subsequent assay for MAPKAP kinase 2 activity with Hsp25 as substrate (○). Since in this assay both MAPKAP kinase 2 activator and endogenous MAPKAP kinase 2 were measured, fractions were also analyzed in an assay omitting recombinant GST-MAPKAP kinase 2 Δ3B which detects MAPKAP kinase 2 activity only (●). In both assays, the same amount of protein from the Mono Q fractions was analyzed. The difference of both activity profiles clearly demonstrates an activator of MAPKAP kinase 2 eluting in fraction 19 at about 350 mM NaCl, probably corresponding to p38/40^{MAPK} (RK). *C*, detection of p38/40^{MAPK} (RK) in fraction 19 by immunoprecipitation of the enzyme with an anti-Mpk2 antiserum and subsequent *in vitro* reconstitution of MAPKAP kinase 2 activation using wild type MAPKAP kinase 2: *19*, peak fraction; *13*, control fraction containing ERKs. The *two left lanes* are controls using the fractions without prior immunoprecipitation. *D, in vitro* reconstitution of MAPKAP kinase 2 activation by ERK1 and peak fraction 19 using wild type MAPKAP kinase 2 (*WT*) and its phosphorylation mutant (T205E,T317E). *C* is a control assay where the *in vitro* reconstitution reaction with fraction 19 is carried out without adding recombinant MAPKAP kinase 2. A further control which excludes autophosphorylation and autoactivation of the recombinant MAPKAP kinase 2 during the *in vitro* reconstitution reaction is carried out by incubation of the recombinant enzyme in the presence of MgATP (+*MgATP*) omitting MAPKs (−*p38/40 RK*, −*ERK1*). As in the other *in vitro* reconstitution reactions, this autophosphorylation-permitting preincubation does not influence the enzymatic activity of the recombinant enzyme.

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 pp44^{MAPK} 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 pp44^{MAPK} (Fig. 5*C*). 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

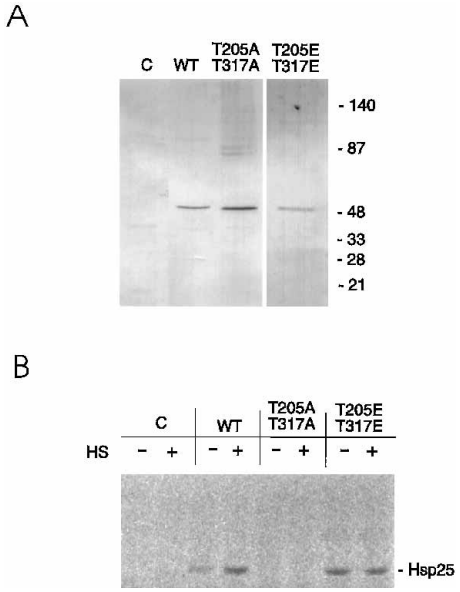

FIG. 4. **Expression of epitope-tagged MAPKAP kinase 2 and the phosphorylation site mutants T205A,T317A and T205E,T317E in NIH 3T3 cells and analysis of activity of epitope-tagged MAPKAP kinase 2 and its mutants before and after heat shock (*HS*).** *A*, Western blot detection of expression of wild type epitope-tagged MAPKAP kinase 2 (*WT*) and mutants using the anti-Myc antibody 9E10. As a control (*C*), lysate of NIH 3T3 cells which were transfected with the expression vector pcDNA3 is applied to Western blot analysis. *B*, MAPKAP kinase 2 activity assay after immunoprecipitation from cells transfected with pcDNA3 (*C*), wild type MAPKAP kinase 2, mutants T205A,T317A and T205E,T317E before (−) and after heat shock (+) using the anti-Myc antibody.

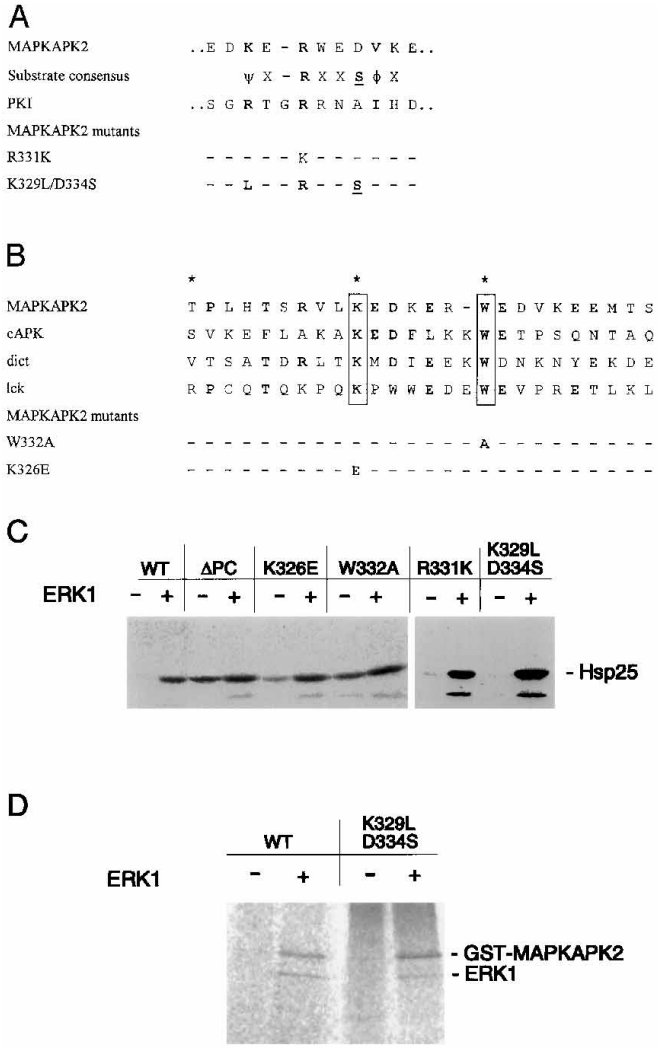

FIG. 5. **Characterization of the autoinhibitory region of MAPKAP kinase 2.** *A*, sequence alignment of the autoinhibitory region of MAPKAP kinase 2, the MAPKAP kinase 2 consensus substrate motif and the protein kinase A pseudosubstrate PKI as supposed in Ref. 32. In the consensus substrate motif, ψ is a large hydrophobic residue (F > L > V), and ϕ represents a hydrophobic or acidic residue (35). Matching residues are in *bold letters*. The phosphorylated serine residue is *underlined*. The appropriate MAPKAP kinase 2 mutants, which should have altered pseudosubstrate properties, R331K and K329L/D334S, are indicated. *B*, sequence alignment of the putative A-helix motif of MAPKAP kinase 2 with the A-helix of the catalytic subunit of mammalian (cAPK) and *Dictyostelium discoideum* (*dict*) cAMP-dependent protein kinase and of the protein tyrosine kinase lck (*lck*). The phosphorylation site Thr-317 of MAPKAP kinase 2 and the conserved tryptophan and lysine residues of the A-helix are indicated by *asterisks*. The appropriate MAPKAP kinase 2 mutants, which should have an altered A-helix motif, W332A and K326E, are indicated. *C*, analysis of the wild type form (*WT*), deletion mutant (Δ*PC*; Δ amino acids 315–383), pseudosubstrate mutants R331K, K329L/D3345 and A-helix mutants (*W332A*, *K326E*) of MAPKAP kinase 2 for their ability to phosphorylate Hsp25 in dependence on activation by pp44 ERK1 MAP kinase. *D*, assay for autophosphorylation of MAPKAP kinase 2 and mutant K329L/D334S. As a control, MAPKAP kinase 2 and the mutant were incubated in the presence of ERK1 leading to phosphorylation of MAPKAP kinase 2 and to autophosphorylation of ERK1.

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. 5*A*). 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. 5*B*). 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. 5, *A* 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. 5*B*). 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 L*X*R*XX*S over L*X*K*XX*S (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. 5*A*). 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. 5*C*) and the phosphorylation of the K329L/D334S mutant (Fig. 5*D*). As seen in Fig. 5*C*, 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. 5*D*), although the corresponding peptide KK**L**E**R**WE**S**VK-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 autoinhibitory 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 of MAPKAP kinase 2 as seen in Fig. 6*B*. 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.

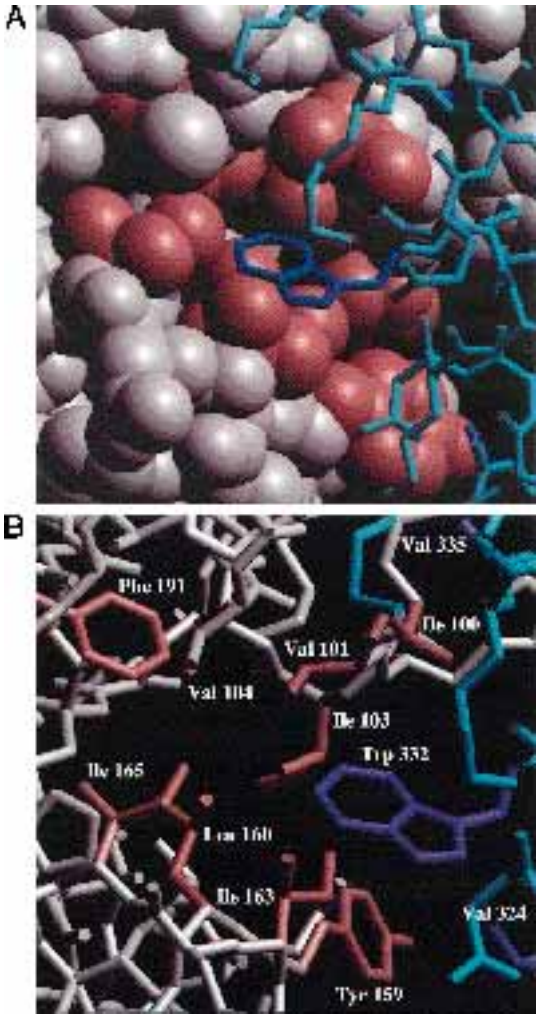

FIG. 6. **Molecular modeling of the interaction of the A-helix with the catalytic domain of MAPKAP kinase 2.** The different colors help to identify the side chains of the hydrophobic region (*pink*) in the catalytic domain (*gray*) and the tryptophan and valine side chains (*blue*) of the A-helix (*green*). The plot was drawn with SETOR (45). *A*, space-filling model of the catalytic domain to give an impression of the hydrophobic surface. *B*, a closer view with labeled side chains using the same coloring mode.

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 p38/40^{MAPK} (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 p38/40^{MAPK} (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 p38/40^{MAPK} (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 p38/40^{MAPK} (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 p38/40^{MAPK} (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 activate 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 intrasteric 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 regulative 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 carboxyl-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 CVLKEDKERWEDVK and a GST-fusion protein containing the carboxyl-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 tryptophan 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 tryptophan 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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