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Identification of novel phosphorylation sites required for activation of MAPKAP kinase-2

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MAP kinase-activated protein (MAPKAP) kinase-2 is activated *in vivo* by reactivating kinase (RK), a MAP kinase (MAPK) homologue stimulated by cytokines and cellular stresses. Here we show that *in vitro* RK phosphorylates human GST–MAPKAP kinase-2 at Thr25 in the proline-rich N-terminal region, Thr222 and Ser272 in the catalytic domain and Thr334 in the C-terminal domain. Using novel methodology we demonstrate that activation of MAPKAP kinase-2 requires the phosphorylation of any two of the three residues Thr222, Ser272 and Thr334. Ser9, Thr25, Thr222, Ser272, Thr334 and Thr338 became ^{32}P-labelled in stressed KB cells and labelling was prevented by the specific RK inhibitor SB 203580, establishing that RK phosphorylates Thr25, Thr222, Ser272 and Thr334 *in vivo*. The ^{32}P-labelling of Thr338 is likely to result from autophosphorylation. GST–MAPKAP kinase-2 lacking the N-terminal domain was inactive, but activated fully when phosphorylated at Thr222, Ser272 and Thr334 by p42 MAPK or RK. In contrast, full-length GST–MAPKAP kinase-2 was phosphorylated at Thr25 (and not activated) by p42 MAPK, suggesting a role for the N-terminal domain in specifying activation by RK *in vivo*. The mutant Asp222/Asp334 was 20% as active as phosphorylated MAPKAP kinase-2, and this constitutively active form may be useful for studying its physiological roles.

Keywords: cytokine/heat shock/MAPKAP kinase/MAP kinase/stress

Introduction

MAP kinase-activated protein (MAPKAP) kinase-2 was originally identified as an enzyme which was inactivated *in vitro* by protein phosphatase 2A (PP2A) and reactivated *in vitro* by the p42 or p44 isoforms of mitogen-activated protein (MAP) kinase (Stokoe *et al.*, 1992a). However, subsequent work has revealed that MAPKAP kinase-2 is activated *in vivo* by another MAP kinase homologue, variously termed reactivating kinase (RK) (Rouse *et al.*, 1994), p38 (Han *et al.*, 1994), p40 (Freshney *et al.*, 1994) and CSAID binding protein (CSBP) (Lee *et al.*, 1994a). RK is a component of a distinct protein kinase cascade that is activated by cellular stress, such as heat or osmotic shock (Rouse *et al.*, 1994), by bacterial endotoxin (LPS) (Han *et al.*, 1994; Lee *et al.*, 1994a) and by the cytokines interleukin-1 (IL1) and tumour necrosis factor α (TNFα) (Freshney *et al.*, 1994).

One physiological substrate of MAPKAP kinase-2 is heat shock protein 27 (HSP27) (Stokoe *et al.*, 1992b; Cuenda *et al.*, 1995), whose phosphorylation appears to stimulate polymerization of actin and so helps to repair the actin microfilament network, which is disrupted by cellular stress, thereby aiding cell survival (Lavoie *et al.*, 1995). A novel class of pyridinyl imidazoles (CSAIDs) such as SB 203580, has been identified, which inhibit RK but do not affect other MAP kinase homologues or protein kinases so far tested (Lee *et al.*, 1994a; Cuenda *et al.*, 1995). SB 203580 prevents activation of MAPKAP kinase-2 and phosphorylation of HSP27 by cellular stress, LPS or IL1, establishing that MAPKAP kinase-2 is an *in vivo* substrate of RK and that HSP27 is a physiological target of MAPKAP kinase-2 (Cuenda *et al.*, 1995). SB 203580 blocks production of IL1, IL6, IL8 and TNFα by LPS in monocytes (Lee *et al.*, 1994a,b). Thus the pathway regulates the synthesis as well as the actions of cytokines.

Human MAPKAP kinase-2 comprises 400 residues (Engel *et al.*, 1993; Stokoe *et al.*, 1993; Zu *et al.*, 1994). A proline-rich N-terminal domain is followed by the catalytic domain, most closely related to the family of calmodulin-dependent protein kinases, while the C-terminus contains a potential bipartite nuclear localization signal (Stokoe *et al.*, 1993). When MAPKAP kinase-2 from rabbit skeletal muscle was phosphorylated with p42 MAP kinase and digested with trypsin, one major phosphopeptide was detected after chromatography on a C_{18} column (Stokoe *et al.*, 1992a). Sequence analysis identified the site of phosphorylation as Thr334, which lies in a Pro-X-Thr-Pro motif, frequently found at residues phosphorylated by MAP kinase, just after the catalytic domain (Stokoe *et al.*, 1993). In order to determine its role in activation of MAPKAP kinase-2 we generated amino acid substitutions at Thr334. An aspartic acid substitution at this site generated an enzyme with constitutive activity *in vitro* and *in vivo*, but, surprisingly, this mutant could be further activated. Similarly, substitution of Thr334 by Ala generated an enzyme that could be activated *in vitro* and *in vivo*. These results suggested that MAPKAP kinase-2 is regulated by multisite phosphorylation and we therefore carried out experiments to identify these sites and to study their roles in activation.

Results

Mutants at Thr334 can be activated in vivo and in vitro

To investigate the role of phosphorylation at Thr334 in the activation of MAPKAP kinase-2, we generated aspartic

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Table I. Activities of wild-type and mutant forms of GST–MAPKAP kinase-2 (46–400)

Form of MAPKAP kinase-2	Activity (%)	
	–MAPK	+MAPK
1. Wild-type	<1	100
2. Ala222	<1	10
3. Ala272	<1	6
4. Ala334	<1	86
5. Ala222/Ala272	<1	<1
6. Ala222/Ala334	<1	<1
7. Ala272/Ala334	<1	<1
8. Asp222	0	14
9. Asp272	2	24
10. Asp334	6	60
11. Asp222/Asp272	<1	2
12. Asp222/Asp334	18	22
13. Asp272/Asp334	6	44

Activities were measured before (–MAPK) and after (+MAPK) incubation for 60 min at 30°C with MgATP and 5 U/ml p42 MAP kinase (MAPK), which activated the 'wild-type' enzyme maximally. Activities are presented as a percentage of the fully activated wild-type enzyme. Similar results were obtained in several different experiments.

and glutamic acid substitutions, to mimic phosphorylation, and an alanine substitution, to replace the phosphorylatable residue with an uncharged amino acid. In initial experiments the recombinant MAPKAP kinase-2 was expressed in bacteria as a glutathione S-transferase (GST) fusion protein lacking the proline-rich N-terminus, termed GST–MAPKAP kinase-2 (46–400). Both aspartic and glutamic acid substitutions at Thr334 led to elevated basal activity compared with wild-type enzyme, but, surprisingly, they could be further activated by incubation with p42 MAP kinase or RK (Table I). These results suggested that phosphorylation of Thr334 is involved in activation of MAPKAP kinase-2, but that other sites might also be involved. This conclusion was strengthened by analysis of a mutant in which Thr334 was changed to Ala. This mutant could be activated *in vitro* by p42 MAP kinase (Table I) or *in vivo* by expression in COS cells treated with sodium arsenite (Figure 1).

Identification of Thr222 and Ser272 as additional phosphorylation sites involved in the activation of MAPKAP kinase-2

In order to identify phosphorylation sites on MAPKAP kinase-2, we mapped the sites phosphorylated by RK or p42 MAP kinase *in vitro*, initially using GST–MAPKAP kinase-2 (46–400). Activation of this species by p42 MAP kinase (2 U/ml) reached a plateau after 20 min, when incorporation of phosphate had approached 4 mol/mol (Figure 2). More prolonged (60 min) incubation did not result in any further activation, although phosphorylation increased to over 7 mol/mol. After phosphorylation for 20 min, ATP was removed by gel filtration and the native enzyme incubated for 60 min with a high concentration of trypsin (1 mg/ml), which released 70% of the ^{32}P radioactivity as phosphopeptides soluble in 5% (by mass) trichloroacetic acid (TCA). Subsequent chromatography of the TCA-soluble radioactivity on a C_{18} column yielded four major peptides, termed T1, T2, T3 and T4c, eluting at 12, 14, 18 and 22% acetonitrile (Figure 3A). Peptides T1, T2 and T4c only contained phosphothreonine, while

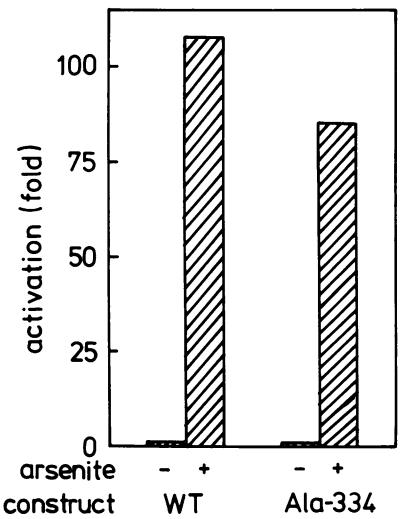

Fig. 1. MAPKAP kinase-2 (Ala334) is activated in COS cells following arsenite treatment. The full-length (amino acids 1–400) wild-type (WT) and Ala334 mutant DNA constructs of MAPKAP kinase-2, tagged at their C-termini with the myc epitope recognized by monoclonal antibody 9E10, were expressed in COS cells. The cells were incubated for 30 min with 0.5 mM sodium arsenite, the epitope-tagged MAPKAP kinase-2 immunoprecipitated from lysates with 9E10 and kinase activity measured with the peptide substrate (Stokoe *et al.*, 1992a). Similar results were obtained in three separate experiments.

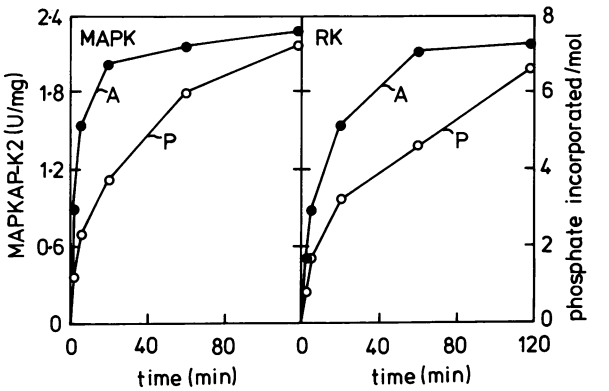

Fig. 2. Phosphorylation and activation of the catalytic domain of MAPKAP kinase-2 by p42 MAP kinase (MAPK) and RK. GST–MAPKAP kinase-2(46–400) (0.2 mg/ml) was incubated with Mg[γ-^{32}P]ATP and either 2 U/ml p42 MAP kinase or 200 U/ml RK and phosphorylation (P, open circles) measured at the times indicated. Activation of GST–MAPKAP kinase-2 (A, closed circles) was determined in parallel incubations using unlabelled ATP. There was no phosphorylation or activation of MAPKAP kinase-2 if p42 MAP kinase and RK were omitted. Similar results were obtained in several experiments with two different preparations of GST–MAPKAP kinase-2.

T3 was a phosphoserine-containing peptide (data not shown). The four peptides were further purified (see Materials and methods) and sequenced (Figure 4). T2 was the previously described tryptic peptide (residues 331–340) containing Thr334, while T1 was the same peptide phosphorylated at Thr338 as well as Thr334. Peptide T4c corresponded to residues 213–229 of MAPKAP kinase-2 resulting from a chymotryptic-like cleavage of the Tyr-Val peptide bond between residues 229 and 230. Thr222 was identified as the site of phosphorylation in T4c (Figure 4). Tryptic peptide T4 (residues 213–239), in which this chymotryptic split did not occur, was virtually absent in

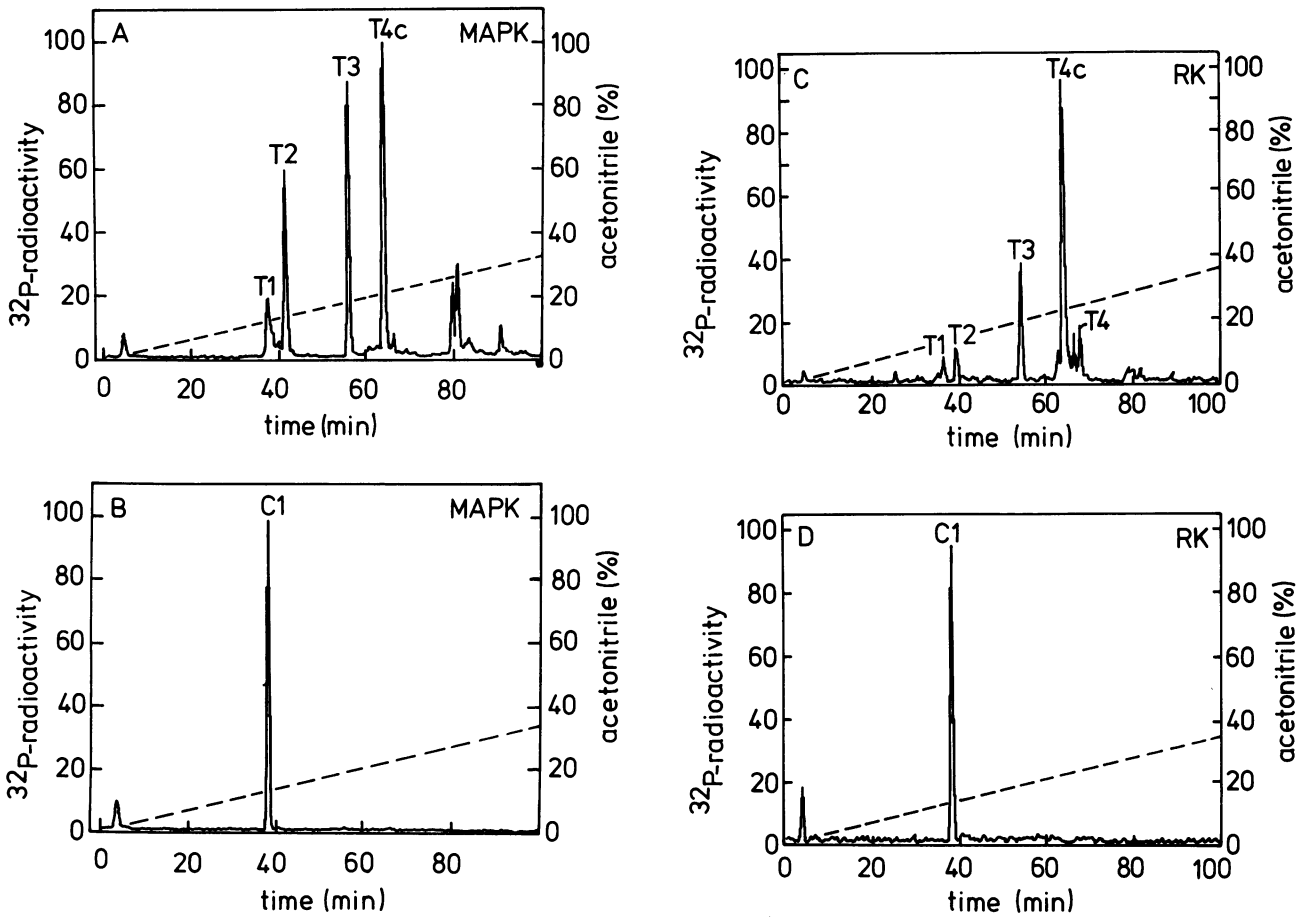

Fig. 3. Separation of tryptic and chymotryptic phosphopeptides from the catalytic domain of MAPKAP kinase-2. (**A**) GST–MAPKAP kinase-2 (46–400) was phosphorylated for 20 min with Mg[γ-^{32}P]ATP and 2 U/ml p42 MAP kinase (MAPK) and, after digesting the native enzyme with trypsin, TCA-soluble peptides were chromatographed on a C_{18} column equilibrated in 0.1% (by volume) TFA (see Materials and methods). The full line shows ^{32}P radioactivity detected with an on-line monitor and the broken line the acetonitrile gradient. (**B**) The TCA-insoluble peptides from tryptic digestion were solubilized by chymotryptic digestion and chromatographed on the C_{18} column as in (A). (**C** and **D**) As (A) and (B) except that MAPKAP kinase-2 was phosphorylated for 20 min with RK (200 U/ml) prior to tryptic and chymotryptic digestion.

this experiment, but its position is marked in later figures. The very high concentration of trypsin used to digest MAPKAP kinase-2 (1 mg/ml) was essential to release peptide T4/T4c and may explain why this phosphorylation site was not detected previously (Stokoe *et al.*, 1992a). Peptide T3 (Leu-Ile-Ser-Glu-Glu-Asp-Leu-Lys) corresponded to part of the myc epitope at the C-terminus of the construct and the serine was identified as the site of phosphorylation (data not shown).

The ^{32}P radioactivity present in the TCA pellet and not released by tryptic digestion (30%) was entirely associated with a serine residue(s) (data not shown) and could be solubilized by chymotryptic digestion. Subsequent C_{18} chromatography resolved a single peptide, C1, which accounted for 70% of the ^{32}P radioactivity solubilized (Figure 3B). This peptide was further purified (see Materials and methods) and corresponded to residues 270–276 of MAPKAP kinase-2, Ser272 being the site of phosphorylation (Figure 4). When Ser272 was mutated to Ala and the mutant phosphorylated by p42 MAP kinase, tryptic digestion now released all the ^{32}P radioactivity as TCA-soluble peptides, establishing that Ser272 was the only phosphorylation site in the TCA-insoluble fraction. Inspection of the sequence of MAPKAP kinase-2 reveals that the expected tryptic peptide containing Ser272 is very large (243–276) and extremely hydrophobic (Stokoe *et al.*, 1993), which presumably accounts for its insolubility in acid.

A 'kinase dead' mutant of MAPKAP kinase-2 (46–400) in which Asp207 of the conserved Asp-Phe-Gly motif was mutated to Ala was phosphorylated by p42 MAP kinase at Thr222, Ser272 and Thr334, although the rate of phosphorylation of Thr334 was very slow compared with the wild-type enzyme. The Ala207 mutant did not become phosphorylated at Thr338 or at the serine residue in the myc epitope, indicating that phosphorylation of the latter two residues results from autophosphorylation catalysed by MAPKAP kinase-2 itself (data not shown). The tryptic digest of wild-type GST–MAPKAP kinase-2 (46–400) phosphorylated by p42 MAP kinase contained a further ^{32}P-labelled peptide(s) eluting at 80 min (Figure 3A) whose amount varied from experiment to experiment. This peptide(s) was also absent after phosphorylation of the Ala207 mutant, suggesting that it represents a further site(s) of autophosphorylation.

The experiments described above were repeated using RK to activate GST–MAPKAP kinase-2 (46–400) instead of p42 MAP kinase. Maximal activation by RK after 60 min was accompanied by incorporation of 4–5 mol phosphate/mol protein (Figure 2B) and subsequent tryptic

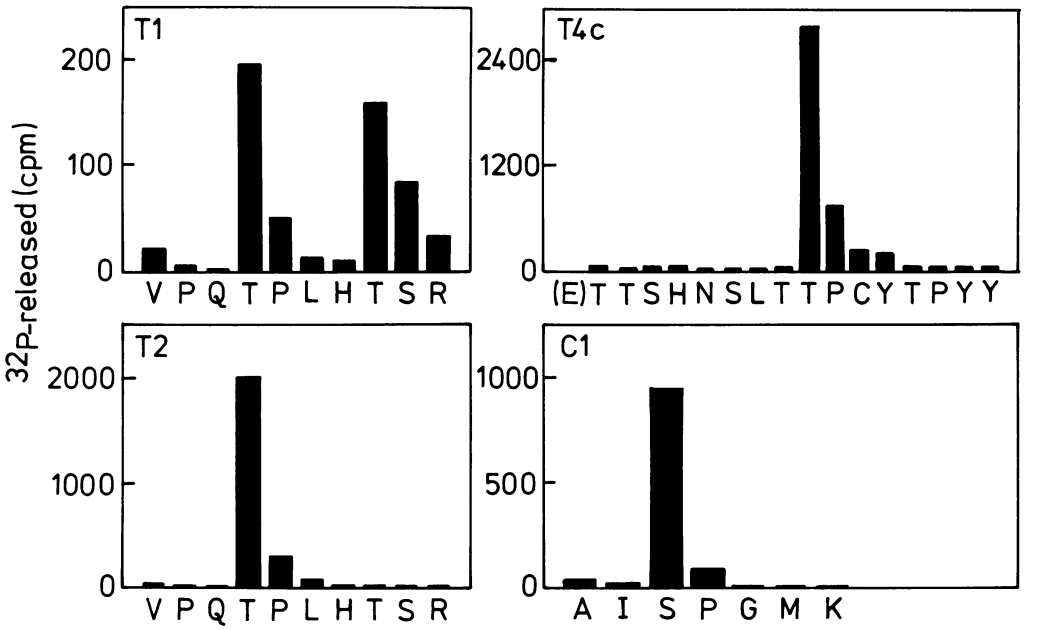

Fig. 4. Identification of the residues in the catalytic domain of MAPKAP kinase-2 phosphorylated by p42 MAP kinase. Peptides T1, T2, T4c and C1 from Figure 3 were first sequenced conventionally on an Applied Biosystems 476A sequencer to identify amino acid residues and then on an Applied Biosystems 470A sequencer by solid phase sequencing after coupling the peptide covalently to a Sequelon arylamine membrane via its C-terminal carboxylate group (Stokoe *et al.*, 1992a). The figure shows ^{32}P radioactivity released and amino acid residues identified after each cycle of Edman degradation. ^{32}P radioactivity coupled to the arylamine membrane was: T1, 1130 c.p.m.; T2, 3580 c.p.m.; T4, 3130 c.p.m.; C1, 1710 c.p.m. Peptide T4 was subjected to one cycle of Edman degradation before coupling to the arylamine membrane to remove the N-terminal glutamate residue. If this was not done, the peptide coupled to the arylamine membrane but its N-terminus became blocked due to cyclization of the glutamate to pyroglutamate, resulting in a poor recovery of ^{32}P radioactivity.

digestion again solubilized ~70% of the ^{32}P radioactivity. Chromatography of the TCA-soluble peptides demonstrated that the major peptides phosphorylated were T3 and T4c (Figure 3C) and sequence analysis confirmed that the sites of phosphorylation were the same as those phosphorylated by p42 MAP kinase, i.e. the serine residue in the myc epitope and Thr222 respectively (data not shown). However, under these conditions incubation with RK leads to considerably less labelling of peptides T1 and T2 (Figure 3C), showing that RK phosphorylates Thr334 in MAPKAP kinase-2 (46–400) less effectively than does p42 MAP kinase. Chymotryptic digestion of the TCA precipitate followed by C_{18} chromatography (Figure 3D) and sequence analysis established that Ser272 was also phosphorylated by RK. Thus with a GST fusion protein of MAPKAP kinase-2 that lacks the N-terminal proline-rich domain both RK and p42 MAP kinase phosphorylate Thr222 and Ser272, but only p42 MAP kinase phosphorylates Thr334 efficiently.

Roles of Thr222, Ser272 and Thr334 in the activation of MAPKAP kinase-2

In order to investigate the roles of individual phosphorylation sites in activation of MAPKAP kinase-2, GST–MAPKAP kinase-2 (46–400) was maximally activated by incubation with MgATP and p42 MAP kinase and then incubated with the catalytic subunit of PP2A. Dephosphorylation decreased MAPKAP kinase-2 activity by >95% (Table II), but only released ~50% of the covalently bound phosphate. No inhibition occurred in control incubations where PP2A was omitted or first pre-incubated with the inhibitor okadaic acid. Analysis of phosphopeptides after digestion with trypsin and chymotrypsin showed that

Table II. Activation of MAPKAP kinase-2 requires phosphorylation of any two of the three residues, Thr222, Ser272 and Thr334

Form of MAPKAP kinase-2	Activity (%)	
	+MAPK	+PP2A
Thr222/Ser272/Thr334	100	3
Ser222/Thr272/Thr334	98	4
Thr222/ Thr272/Ser334	97	0
Ser222/Ser272/Thr334	103	91
Thr222/Ser272/Ser334	93	87
Ser222/Thr272/Ser334	104	98

The activities of 'wild-type' (Thr222/Ser272/Thr334) and mutant forms of GST–MAPKAP kinase-2 (46–400) were measured after incubation for 20 min with MgATP and 20 U/ml p42 MAP kinase (activity +MAPK) and after subsequent dephosphorylation for 30 min with 10 mU/ml PP2A (activity +PP2A), which led to complete dephosphorylation of threonine residues (see Figure 5). Similar results were obtained in several different experiments.

PP2A treatment led to complete dephosphorylation of Thr222, Thr 334 and Thr338, while Ser272 and the serine residue in the myc epitope (peptide T3 in Figure 5A) remained phosphorylated. These experiments demonstrated that phosphorylation of Ser272 alone was insufficient to trigger the activation of MAPKAP kinase-2 and its resistance to dephosphorylation may explain why this phosphorylation site was not detected previously (Stokoe *et al.*, 1992a). The active form of MAPKAP kinase-2 isolated from rabbit skeletal muscle is probably fully phosphorylated at Ser272 and would therefore not become ^{32}P-labelled after inactivation with PP2A followed by reactivation with p42 MAP kinase.

The selective dephosphorylation of residues Thr222 and

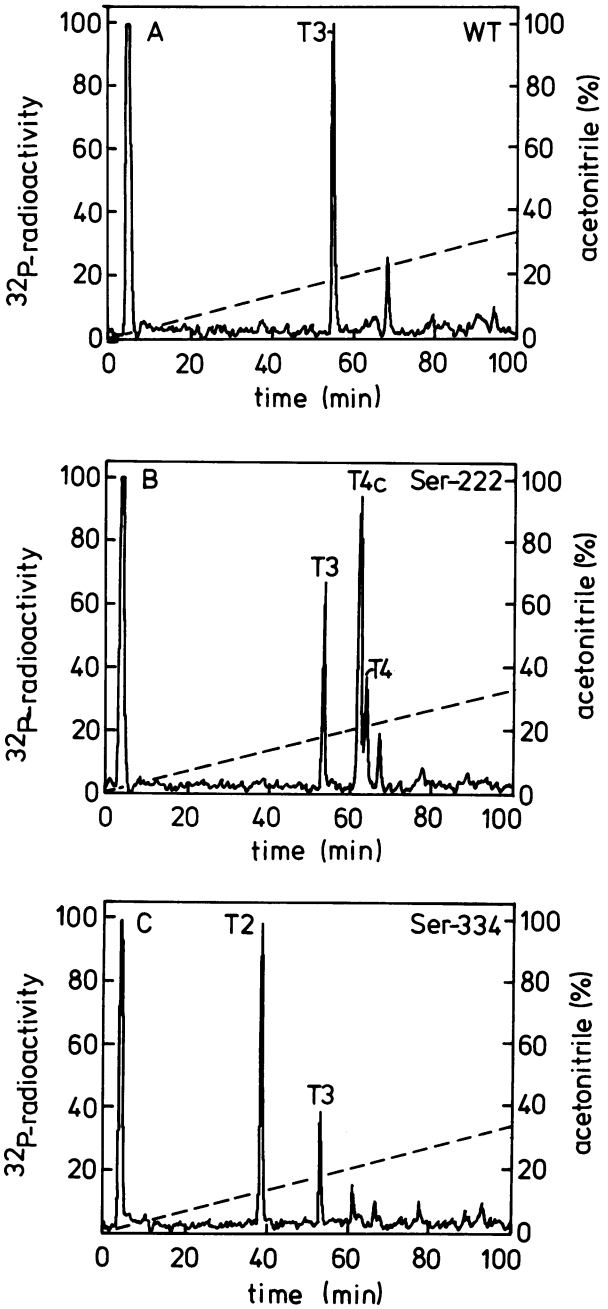

Fig. 5. The phosphothreonyl residues in MAPKAP kinase-2 are sensitive and the phosphoseryl residues resistant to PP2A. The wild-type, Ser222 and Ser334 mutants of GST–MAPKAP kinase-2 (46–400) were phosphorylated for 20 min with 20 U/ml p42 MAP kinase, then dephosphorylated for 30 min with 10 mU/ml PP2A, digested with trypsin and chromatographed on the C_{18} column as in Figure 3. (**A**) Wild-type (WT) GST–MAPKAP kinase-2. (**B**) Ser222 mutant. (**C**) Ser334 mutant. The ^{32}P radioactivity in the flow-through fractions is orthophosphate, resulting from dephosphorylation of the phosphothreonyl residues by PP2A.

Thr334 and the resistance of Ser272 to PP2A suggested a simple and elegant way of establishing the roles of the different phosphorylation sites in MAPKAP kinase-2. Thr222 and/or Thr334 were therefore mutated to Ser, and/or Ser272 was mutated to Thr in order to produce six forms of MAPKAP kinase-2 containing either one or two serine residues at the three sites of phosphorylation (see Table II). Each of these MAPKAP kinase-2 mutants was activated by p42 MAP kinase to the same level as the wild-type enzyme (Table II) and, following incubation with the catalytic subunit of PP2A, tryptic digestion and C_{18} chromatography established that every Thr→Ser mutation prevented dephosphorylation by PP2A. For example, in the Ser222/Ser272/Thr334 mutant Ser222 was not dephosphorylated, so that peptides T4c/T4 were now present after PP2A treatment as well as T3 (Figure 5B). Conversely, in the Thr222/Ser272/Ser334 mutant Ser334 was not dephosphorylated, so that peptide T2 was now present after PP2A treatment as well as T3 (Figure 5C).

Each mutant with a single serine residue (Ser222/Thr272/Thr334 and Thr222/Thr272/Ser334), like the wild-type Thr222/Ser272/Thr334 species, was almost completely inactivated by PP2A treatment (Table II), indicating that phosphorylation of residue 222 alone, residue 272 alone or residue 334 alone is insufficient for activation. In contrast, the three mutants which contained any combination of two serine residues at the three sites (Ser222/Ser272/Thr334, Thr222/Ser272/Ser334 and Ser222/Thr272/Ser334) remained fully active after PP2A treatment (Table II). These observations indicated that phosphorylation of any two of the three sites was sufficient for maximal activation.

In order to confirm these results by an independent method, Thr222, Ser272 and Thr334 were changed individually to alanine residues. Each of these mutants could be activated by p42 MAP kinase, although Ala222 and Ala272 could be activated to only 10 and 6% of the level of the wild-type enzyme respectively. In contrast, mutants containing Ala at two of the three positions could not be activated by p42 MAP kinase (Table I).

Further experiments were carried out in which Thr222, Ser272 and Thr334 were mutated to Asp, to see if the introduction of a negative charge at these positions could mimic the effect of phosphorylation. Mutation of Thr334 to Asp produced a form of MAPKAP kinase-2 with 6% of the activity of the fully phosphorylated wild-type enzyme, which could be activated a further 10-fold by p42 MAP kinase. However, its rate of activation was much slower than that of wild-type MAPKAP kinase-2 and full activation required prolonged incubation with higher concentrations of p42 MAP kinase. The Asp222 mutant was inactive and the Asp272 mutant had very slight activity, but both mutants could be activated by p42 MAP kinase to 14 and 24% of the level of maximally activated wild-type MAPKAP kinase-2. The Asp222/Asp334 double mutant had 20% of the activity of the maximally phosphorylated wild-type enzyme and could not be activated further by p42 MAP kinase.

Phosphorylation of MAPKAP kinase-2 containing the N-terminal proline-rich domain

The experiments described above were carried out using a GST construct containing the catalytic and C-terminal domains only (residues 46–400). In order to examine whether the N-terminal domain of MAPKAP kinase-2 plays a role in activation, a further GST fusion protein was constructed, GST–MAPKAP kinase-2 (5–400), which contained the proline-rich N-terminal domain as well as the catalytic and C-terminal domains, but lacked the myc epitope at the C-terminus. This protein could be activated by RK at a similar rate and to the same specific activity

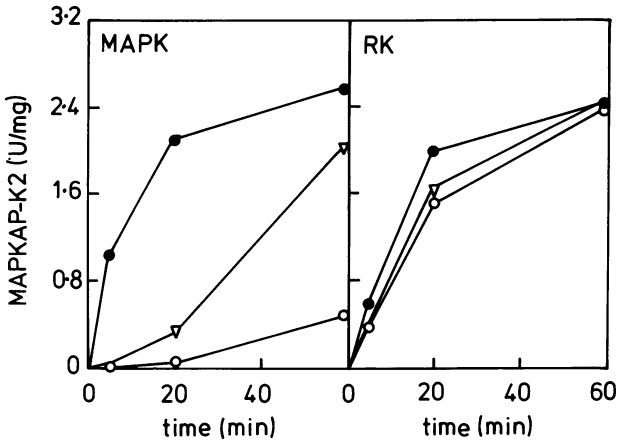

Fig. 6. Activation of GST–MAPKAP kinase-2 fusion proteins with and without the proline-rich N-terminal domain by p42 MAP kinase and RK. GST–MAPKAP kinase-2 (46–400) with a C-terminal myc epitope (closed circles), GST–MAPKAP kinase-2 (5–400) with a C-terminal myc epitope (open triangles) and GST–MAPKAP kinase-2 (5–400) lacking a myc epitope (open circles) were incubated with MgATP and either 2 U/ml p42 MAP kinase (MAPK) or 200 U/ml RK and activation measured at the times indicated. There was no activation of GST–MAPKAP kinase-2 if p42 MAP kinase or RK were omitted.

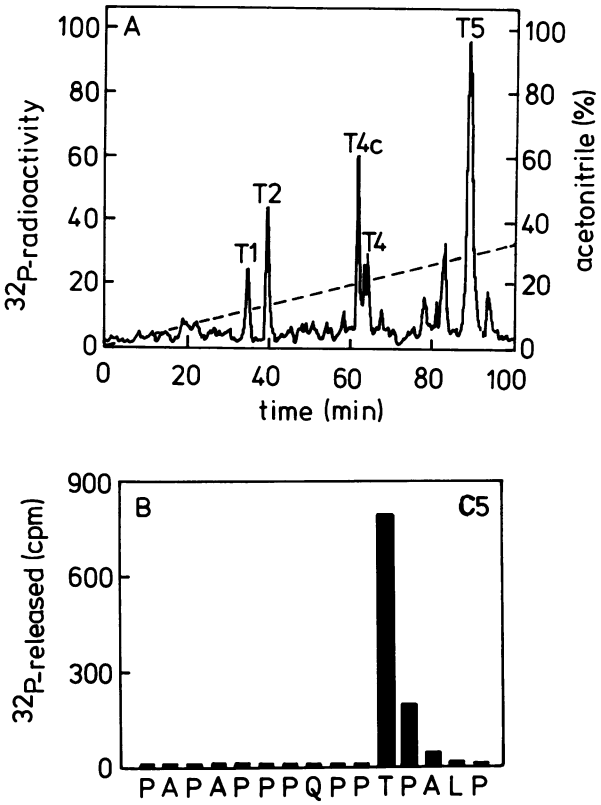

Fig. 7. RK and p42 MAP kinase phosphorylate a threonine residue in the N-terminal domain of MAPKAP kinase-2. (**A**) GST–MAPKAP kinase-2 (5–400) was phosphorylated for 20 min with 200 U/ml RK, digested with trypsin and chymotrypsin and chromatographed on the C_{18} column as in Figure 2. Peptide T3 is missing in this digest because the full-length GST–MAPKAP kinase-2 (5–400) construct lacks the myc epitope at the C-terminus (see Materials and methods). (**B**) Peptide T5 from (A) was sub-digested with chymotrypsin to cleave the Phe–Pro bond between residues 14 and 15 of MAPKAP kinase-2 (see Results). After rechromatography on the C_{18} column the chymotryptic peptide was sequenced as in Figure 3.

as GST–MAPKAP kinase-2 (46–400), lacking the N-terminal domain (Figure 6), but tryptic digestion and C_{18} chromatography revealed two interesting differences. First, Thr334 and Thr222 were phosphorylated to a similar extent (Figure 7A), in contrast to MAPKAP kinase-2 (46–400), where Thr334 was phosphorylated very poorly by RK (Figure 3C). Second, an additional phosphopeptide was present, termed T5 (Figure 7A). T5 was a phosphothreonine-containing peptide, although very small amounts of phosphoserine were also present (data not shown). Peptide T3 was absent, because GST–MAPKAP kinase-2 (5–400) did not contain the C-terminal myc epitope present in GST–MAPKAP kinase-2 (46–400).

After further purification (see Materials and methods) peptide T5 was found to start in the linker region between the GST and the N-terminal domain of MAPKAP kinase-2. Sub-digestion of T5 with chymotrypsin and chromatography on the C_{18} column as in Figure 3 revealed a single phosphothreonine-containing peptide (C5) at 25% acetonitrile. Sequencing of this peptide (Figure 7B) showed that it started at residue 15, due to abnormal cleavage of the Phe–Pro bond between residues 14 and 15 of MAPKAP kinase-2, and that Thr25 was the site of phosphorylation.

The initial rate of activation of GST–MAPKAP kinase-2 (5–400) by p42 MAP kinase was 100-fold slower than the rate of activation of GST–MAPKAP kinase-2 (46–400), in contrast to RK, which activated both constructs at similar rates (Figure 6). Similar results were obtained when the GST tags were removed by cleavage with thrombin (data not shown). Nevertheless, MAPKAP kinase-2 containing the N-terminal domain was phosphorylated by p42 MAP kinase and, after incubation for 20 min with 2 U/ml p42 MAP kinase, the extent of phosphorylation reached 0.8 mol/mol. GST–MAPKAP kinase-2 (5–400) phosphorylated by p42 MAP kinase was subjected to tryptic and chymotryptic digestion, C_{18} chromatography and peptide sequencing as described above. These studies established that Thr25 was the major site of phosphorylation and that phosphopeptides T1, T4 and C1 were absent (data not shown). Thus p42 MAP kinase fails to activate recombinant human MAPKAP kinase-2 (5–400) *in vitro* because it cannot phosphorylate Thr222, Ser272 or Thr 334.

The lack of activation of GST–MAPKAP kinase-2 (5–400) by p42 MAP kinase, as compared with GST–MAPKAP kinase-2 (46–400), could have been due to the presence of the proline-rich N-terminal domain or to the lack of the myc epitope. A further fusion protein was therefore expressed in which the myc epitope was added to the C-terminus of GST–MAPKAP kinase-2 (5–400). This fusion protein was also activated much more slowly than GST–MAPKAP kinase-2 (46–400), but not as slowly as GST–MAPKAP kinase-2 (5–400), lacking the myc epitope (Figure 6).

Phosphorylation of MAPKAP kinase-2 in vivo

Sodium arsenite mimics the effect of heat shock on gene expression (Johnston *et al.*, 1980) and HSP27 phosphorylation (Welch, 1985) and is a potent activator of MAPKAP kinase-2 in PC12 and KB cells (Rouse *et al.*, 1994; Cuenda *et al.*, 1995). ^{32}P-Labelled KB cells were therefore treated

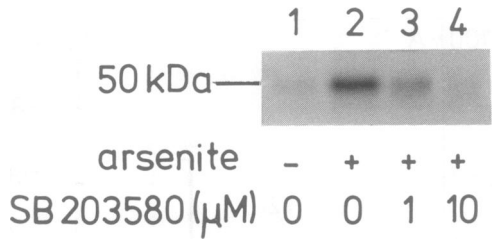

Fig. 8. MAPKAP kinase-2 is phosphorylated in human KB cells in response to sodium arsenite. Confluent KB cells were incubated for 16 h in the presence or absence of the indicated concentration of SB 203580, then ^{32}P-labelled as described under Materials and methods and incubated for 15 min in the absence (–, lane 1) or presence (+, lanes 2–4) of 0.5 mM sodium arsenite. Following cell lysis, MAPKAP kinase-2 was immunoprecipitated, denatured in SDS, electrophoresed on a 12.5% polyacrylamide gel and autoradiographed. Human MAPKAP kinase-2 migrated between bovine serum albumin (66 kDa) and ovalbumin (43 kDa), corresponding to the apparent molecular mass of 50 kDa reported by Freshney *et al.* (1994).

with 0.5 mM sodium arsenite and MAPKAP kinase-2 was immunoprecipitated from cell lysates. MAPKAP kinase-2 from control cells contained little covalently bound phosphate, but after treatment with arsenite for 15 min the ^{32}P-labelling of MAPKAP kinase-2 increased greatly (Figure 8). This increase in ^{32}P-labelling was prevented by pre-incubating KB cells with SB 203580 (Figure 8), a specific inhibitor of RK (Cuenda *et al.*, 1995; see also the Introduction).

^{32}P-Labelled MAPKAP kinase-2 was digested with trypsin and C_{18} chromatography revealed four major phosphopeptides, T1a, T2a, T4a and T5a (Figure 9A), the first three co-eluting with T1, T2 and T4c in Figure 3A. T1a, T2a and T4a contained phosphothreonine and no phosphoserine and ^{32}P radioactivity was released from each peptide after the expected cycles of Edman degradation (Figure 10A–C), demonstrating that Thr222, Thr334 and Thr338 are all phosphorylated *in vivo* after chemical stress. Chymotryptic digestion of the ^{32}P radioactivity which had not been solubilized by trypsin followed by incubation with performic acid to oxidize Met275 and chromatography on a C_{18} column revealed a single phosphoserine-containing peptide, C1a (Figure 9B), which co-migrated with the oxidized form of peptide C1. ^{32}P radioactivity was released from this peptide after the third cycle of Edman degradation (Figure 10D), establishing that Ser272 is also phosphorylated *in vivo* after arsenite treatment.

Peptide T5a (Figure 9A) contained similar amounts of phosphoserine and phosphothreonine (data not shown) and eluted from the C_{18} column at a similar position to peptide T5 in Figure 7A, suggesting that it might be the N-terminal peptide of MAPKAP kinase-2. MAPKAP kinase-2 does not have a free N-terminus and no ^{32}P radioactivity was released from the peptide after 25 cycles of Edman degradation. Peptide T5a was therefore digested with chymotrypsin to cleave the Leu–Ser bond between residues 2 and 3 of MAPKAP kinase-2 and the Phe–Pro bond between residues 14 and 15. The resulting phosphoserine- and phosphothreonine-containing peptides (C5a/C5b) were chromatographed on the C_{18} column and found to co-elute at the same acetonitrile concentration (25%) as the chymotryptic phosphopeptide containing Thr25. Because of the low levels of ^{32}P radioactivity

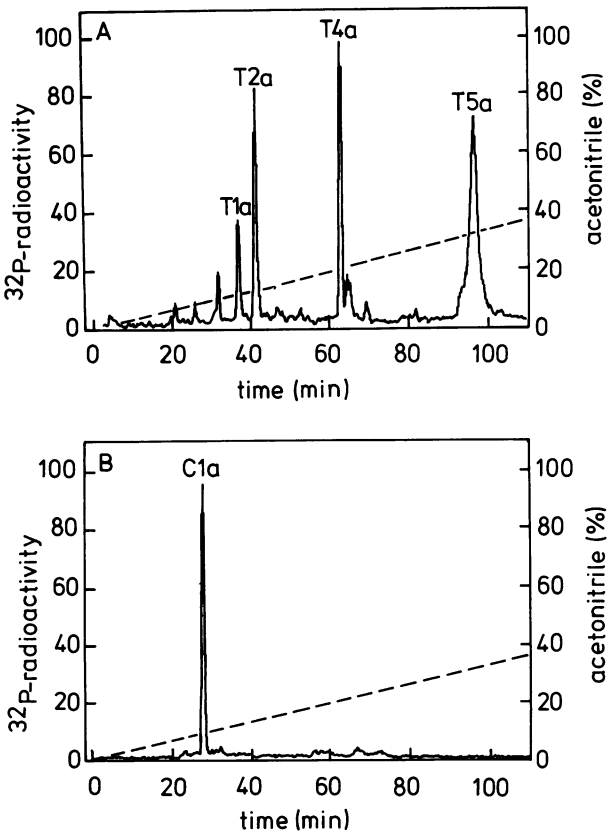

Fig. 9. Tryptic and chymotryptic phosphopeptides present in MAPKAP kinase-2 from arsenite-stimulated KB cells. (**A**) ^{32}P-Labelled MAPKAP kinase-2 immunoprecipitated from the lysates of eight dishes of arsenite-stimulated KB cells (Figure 8) was digested with trypsin (see Materials and methods) and the solubilized material chromatographed on a C_{18} column as in Figure 3. (**B**) The ^{32}P radioactivity not solubilized by trypsin was solubilized by chymotryptic digestion and, after performic acid oxidation, chromatographed on the C_{18} column as in (A).

obtainable from the *in vivo* phosphorylation experiments, C5a and C5b were sequenced as a mixture. The N-terminal sequence of peptide C5a is SNSQGQSP and the N-terminal sequence of C5b is PAPAPPPQPPTPALP. Bursts of ^{32}P radioactivity occurred after the seventh and eleventh cycles of Edman degradation (Figure 10E), indicating that the sites of phosphorylation were Ser9 and Thr25 respectively.

MAPKAP kinase-2 was immunoprecipitated from KB cells pre-incubated with 10 μM SB 203580 prior to stimulation with arsenite (Figure 8, lane 4), digested with proteinases and analysed as in Figure 9. These experiments showed that SB 203580 had suppressed phosphorylation of peptides T1a, T2a, T4a, T5a and C1a almost completely.

Discussion

Sodium arsenite mimics the effects of heat shock on gene expression (Johnston *et al.*, 1980) and HSP27 phosphorylation (Welch, 1985) and triggers the activation of RK and MAPKAP kinase-2 within minutes in several cell lines, without activating p42 MAP kinase (Rouse *et al.*, 1994). In this study we found that arsenite induced phosphorylation of MAPKAP kinase-2 at six residues in KB cells, namely Ser9 and Thr25 in the N-terminal domain, Thr222

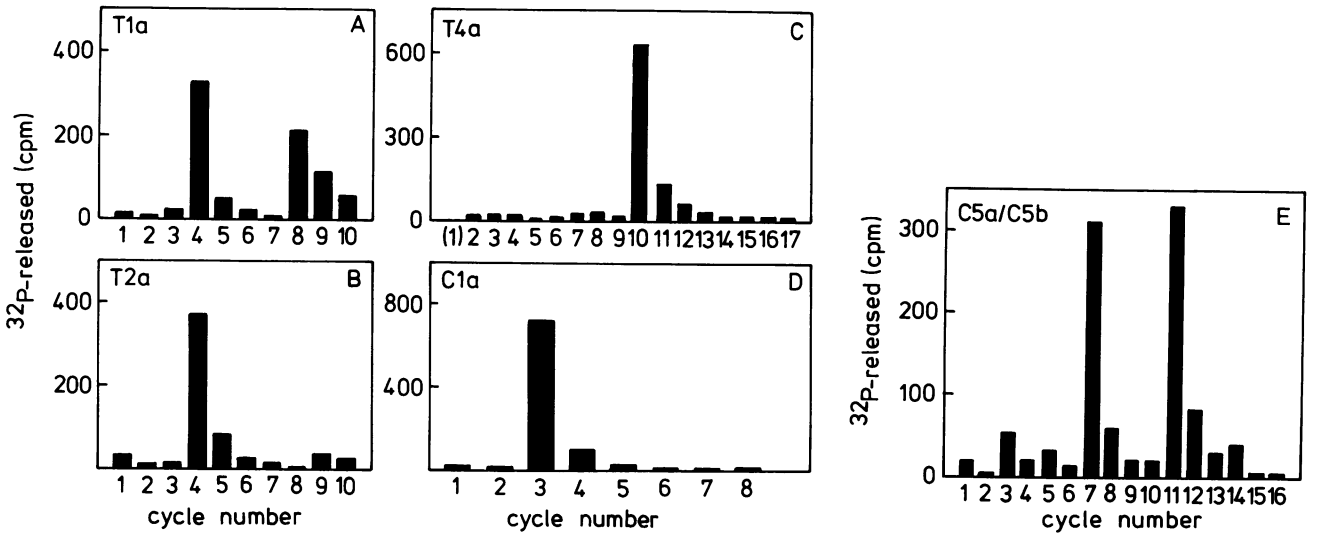

Fig. 10. Identification of the *in vivo* sites of phosphorylation on MAPKAP kinase-2 from arsenite-stimulated KB cells. Peptides T1a (**A**), T2a (**B**), T4a (**C**) and C1a (**D**) from Figure 9 and a mixture of peptides C5a and C5b (**E**) obtained by chymotryptic sub-digestion of peptide T5a from Figure 9 were each subjected to solid phase sequencing as in Figure 4. The ^{32}P radioactivity released after each cycle of Edman degradation is shown. ^{32}P radioactivity coupled to the Sequelon membrane was: T1a, 1830 c.p.m.; T2a, 1180 c.p.m.; T4a, 3475 c.p.m.; C1a, 1730 c.p.m.; C5a/C5b, 1600 c.p.m. Peptide T4a was subjected to one cycle of Edman degradation before coupling to the arylamine membrane to remove the N-terminal glutamate residue. If this was not done the peptide coupled to the arylamine membrane, but its N-terminus became blocked, presumably due to the cyclization of the glutamate residue to pyroglutamate, resulting in a very poor recovery of ^{32}P radioactivity.

and Ser272 in the catalytic domain and Thr334 and Thr338 in the C-terminal domain (Figures 9 and 10). Apart from Thr338, all the sites are followed by proline, an obligatory requirement for phosphorylation by MAP kinase homologues. Moreover, Thr25, Thr222, Ser272 and Thr334 were all found to be phosphorylated by RK *in vitro* (Figures 4 and 7) and SB 203580, a specific inhibitor of RK which suppresses activation of MAPKAP kinase-2 by arsenite in KB and PC12 cells (Cuenda *et al.*, 1995), prevented arsenite-induced phosphorylation of MAPKAP kinase-2 (Figure 8). These results establish that Thr25, Thr222, Ser272 and Thr334 are all phosphorylated by RK *in vivo*. Thr338 became phosphorylated when wild-type MAPKAP kinase-2 was incubated with MgATP and RK *in vitro* (Figure 7), but not when a MAPKAP kinase-2 mutant devoid of catalytic activity was phosphorylated. This indicates that phosphorylation of Thr338 is probably catalysed by MAPKAP kinase-2 itself, subsequent to activation by RK. In the presence of SB 203580, MAPKAP kinase-2 is not activated and therefore does not phosphorylate Thr338. Ser9 is followed by proline and its phosphorylation *in vivo* is prevented by SB 203580. However, Ser9 did not become phosphorylated to a significant extent *in vitro* when GST–MAPKAP kinase-2 (5–400) was incubated with MgATP and RK, even if the GST tag was removed by cleavage with thrombin prior to phosphorylation. However, the N-terminal sequence of the thrombin-cleaved protein is not identical to human MAPKAP kinase-2 and further work is needed to establish whether Ser9 is phosphorylated by RK *in vivo* or by another protein kinase.

Thr222 is situated between sub-domains VII and VIII, in a region where the phosphorylation events that activate many other protein kinases take place (Figure 11). Thr222 is located nine residues N-terminal of the conserved Ala-Pro-Glu motif, the same position as one of the serine residues whose phosphorylation (by c-Raf) activates MAP kinase kinase-1 (Alessi *et al.*, 1994) and the threonine residue whose phosphorylation (by p42 MAP kinase) activates MAPKAP kinase-1 (Sutherland *et al.*, 1993). Indeed, the amino acid sequence surrounding Thr222 is remarkably similar to that surrounding the threonine in MAPKAP kinase-1 (Figure 11), perhaps explaining why they are both activated by MAP kinase homologues.

The N-terminal domain of MAPKAP kinase-2 contains many proline residues, including two Pro-Pro-Pro-X-Pro-Pro motifs, which in other proteins are known to interact with SH3 domains. Indeed, it has been reported that the N-terminal domain of MAPKAP kinase-2 is recognized by the SH3 domain of c-abl in Far Western blotting experiments (Plath *et al.*, 1994). However, while the N-terminus of MAPKAP kinase-2 may interact with another protein(s), the present work has suggested two alternative functions for this domain. First, Thr334 was phosphorylated poorly by RK unless the proline-rich domain was present (compare Figures 3C and 7), suggesting that the N-terminus is required to maintain MAPKAP kinase-2 in a conformation that allows RK to phosphorylate Thr334. Phosphorylation of Thr334 is clearly very sensitive to alterations in the conformation of MAPKAP kinase-2, because mutation of Asp207 to Ala greatly reduced the rate at which it was phosphorylated by p42 MAP kinase, without affecting the rate of phosphorylation of Thr222 or Ser272 significantly. Second, the proline-rich domain prevented activation of recombinant (bacterially expressed) human MAPKAP kinase-2 by p42 MAP kinase *in vitro*, without affecting activation by RK significantly (Figure 6). This may explain, at least in part, why activation of MAPKAP kinase-2 *in vivo* is catalysed by RK and not by p42/p44 MAP kinases. However, the situation *in vivo* may be more complex, because MAPKAP kinase-2 preparations isolated from rabbit skeletal muscle (Stokoe *et al.*, 1992a), rat PC12 cells and human A431, HeLa and KB cells (Rouse *et al.*, 1994; Y.N.Doza, unpublished results) can be activated by p42 MAP kinase; indeed, it is this finding that gave MAPKAP kinase-2 its name. The

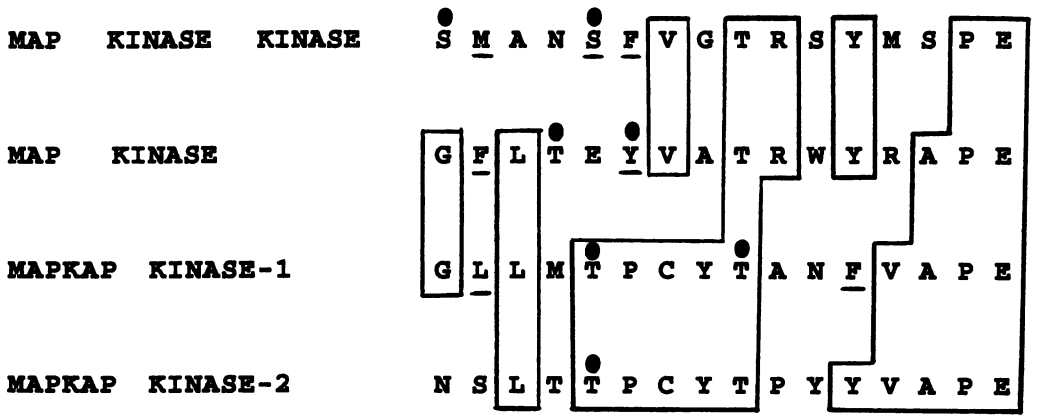

Fig. 11. The activating phosphorylation sites in MAP kinase kinase-1, MAP kinase and MAPKAP kinases are all located just N-terminal of the 'APE motif', between sub-domains VII and VIII. Identities are boxed and conservative substitutions underlined. Phosphorylation sites are denoted by filled circles. The threonine in MAPKAP kinase-1 located five residues N-terminal of the APE motif appears to be a site of autophosphorylation, while that located nine residues N-terminal of the APE motif is phosphorylated by p42/p44 MAP kinases (Sutherland *et al.*, 1993). The threonine in MAPKAP kinase-2 located nine residues N-terminal of the APE motif is one of the three residues whose phosphorylation is required for activation (see text for details).

MAPKAP kinase-2 extracted from mammalian cells would therefore appear to contain a modification(s) which suppresses the negative effect of the proline-rich N-terminus on activation by p42 MAP kinase *in vitro*. For example, it is possible that phosphorylation of Ser9 might overcome the negative effect of the proline-rich domain on activation by p42 MAP kinase.

The GST–MAPKAP kinase-2 (46–400) preparations contained two bands staining for protein with apparent molecular masses of 65 and 62 kDa. Both proteins had the same N-terminal sequence (corresponding to GST), but only the 65 kDa band contained the myc epitope, demonstrating that the 62 kDa species was truncated at the C-terminus. Intriguingly, only the 65 kDa species could be phosphorylated by either RK or p42 MAP kinase (see Materials and methods). We have found that the 62 kDa species terminates at residue 374, presumably as a result of proteolytic cleavage of the Lys–Ile bond between residues 374 and 375 (N.Morrice, unpublished results), and therefore lacks the putative nuclear localization sequence. The C-terminal 26 residues of MAPKAP kinase-2 may therefore play a critical structural role in permitting phosphorylation by RK.

Studies with synthetic phosphopeptides have shown that PP2A dephosphorylates threonine in preference to serine residues (Donella-Deana *et al.*, 1990) and we have exploited this property to elucidate the role of three phosphorylation sites in the regulation of MAPKAP kinase-2 activity. Mutating Thr222 or Thr334 to Ser made these residues resistant to the catalytic subunit of PP2A, while changing Ser272 to Thr made this residue susceptible to PP2A, yet had little or no effect on the rate or extent of activation of GST–MAPKAP kinase-2 (46–400) (Table II). The extreme resistance of each phosphoserine residue to dephosphorylation may be explained by the presence of a C-terminal proline in each case, which is known to greatly suppress the dephosphorylation of synthetic peptides by PP2A (Donella-Deana *et al.*, 1990). We have also found that PP2A dephosphorylates the two phosphothreonine residues, but not the two phosphoserine residues, in histone H1 which are phosphorylated by cyclin-dependent protein kinases; all four residues are followed by proline (M.Sola and P.Cohen, unpublished results). The methodology we have introduced in this paper may therefore be of general application, and especially useful in elucidating the roles of phosphorylation sites in proteins that are physiological substrates for MAP kinases, cyclin-dependent protein kinases and other 'proline-directed' protein kinases.

Our studies demonstrated that maximal activation of MAPKAP kinase-2 was achieved when any two of the three residues 222, 272 and 334 were phosphorylated. On the other hand, phosphorylation of any one residue was insufficient to achieve significant activation (Table II). These conclusions were supported by more conventional mutagenesis experiments in which these residues were changed to alanine, conversion of any two of the three residues to Ala being required to abolish activation (Table I). GST–MAPKAP kinase-2 (5–400) was phosphorylated specifically at Thr25 by p42 MAP kinase, demonstrating that phosphorylation of this residue alone is not sufficient to activate MAPKAP kinase-2. Prior phosphorylation of Thr25 by p42 MAP kinase also had no effect on the rate of activation of GST–MAPKAP kinase-2 (5–400) by RK (I.A.Leighton and P.Cohen, unpublished results).

In summary, we have identified the residues in human MAPKAP kinase-2 which are phosphorylated by RK *in vitro*, demonstrated that they become phosphorylated *in vivo* when human KB cells are chemically stressed and found that the N-terminal proline-rich domain may be important in suppressing activation of MAPKAP kinase-2 by p42 MAP kinase *in vivo*. We have used a novel methodology to reveal that activation of MAPKAP kinase-2 is extremely unusual, in that it requires the phosphorylation of any two of the three residues Thr222, Ser272 and Thr334, but further work is needed to understand the reason for this complexity. We are currently using the cell permeant RK inhibitor SB 203580 to identify physiological roles of the RK pathway. Cell transfection studies, exploiting the constitutively active forms of MAPKAP kinase-2 that we have generated, may help to define which cellular functions of the RK pathway are mediated by MAPKAP kinase-2.

Materials and methods

Materials

The *Xenopus* homologue of RK (XMpk2) activated by the *Xenopus* MAP kinase kinase homologue termed XMEK2 (Doza *et al.*, 1995) was kindly provided by Dr A.R.Nebreda (ICRF, Clare Hall Laboratories, Herts, UK). One unit of RK was that amount which increased the activity of MAPKAP kinase-2 by 1 mU/min (Rouse *et al.*, 1994). Bacterially expressed p42 MAP kinase was purified and activated with MAP kinase kinase-1 (Stokoe *et al.*, 1992a). One unit of p42 MAP kinase was that amount that catalysed the incorporation of 1 nmol phosphate/min into myelin basic protein. The catalytic subunit of PP2A was purified from bovine heart by Dr R.Mackintosh at Dundee as for the rabbit muscle enzyme (Cohen *et al.*, 1988) and anti-MAPKAP kinase-2 antibodies were affinity purified (Cuenda *et al.*, 1995). SB 203580 was a gift from Drs J.Lee and P.Young (Smith Kline Beecham, Philadelphia, PA) and okadaic acid a gift from Dr Y.Tsukitani (Fujizawa Pharmaceutical Co., Tokyo, Japan). Myelin basic protein was from Gibco-BRL (Paisley, UK), trypsin from Worthington (Freehold, NJ), chymotrypsin (sequencing grade) from Boehringer-Mannheim (Lewes, UK) and protein G–Sepharose from Pharmacia (Milton Keynes, UK). The specific peptide inhibitor of cAMP-dependent protein kinase (PKI) was synthesized by Mr F.B.Caudwell at Dundee.

Expression and site-directed mutagenesis of human MAPKAP kinase-2

In order to obtain a recombinant protein lacking the N-terminal proline-rich domain (Stokoe *et al.*, 1993), a fusion protein comprising residues 46–400 of human MAPKAP kinase-2 with a GST tag at the N-terminus and a C-terminal myc epitope (GHMEQKLISEEDLK) recognized by monoclonal antibody 9E10 (Evan *et al.*, 1985) was constructed and expressed from the vector pGEX2T using *Escherichia coli* JM 83 as host. GST–MAPKAP kinase-2 (46–400) was isolated on GSH–agarose following induction of bacteria with 30 μM IPTG at 30°C for 20 h. About 10 mg purified protein was obtained from a 1 l bacterial culture. The purified wild-type protein and all the mutants generated by site-directed mutagenesis showed two bands on SDS–polyacrylamide gels with apparent molecular masses of 65 and 62 kDa that stained with similar intensity. The N-terminal sequences of the 65 and 62 kDa species were identical (corresponding to that of GST), but only the upper band was recognized by antibody 9E10 in immunoblotting experiments, demonstrating that the 62 kDa species is truncated at the C-terminus. In contrast, both bands were recognized by another anti-peptide antibody raised against residues 356–371. Only the upper 65 kDa band was phosphorylated by p42 MAP kinase or RK and specific activities and stoichiometries of phosphorylation were corrected to take this into account. Two further GST–MAPKAP kinase-2 fusion proteins containing the N-terminal proline-rich domain [GST–MAPKAP kinase-2 (5–400)] were also expressed in *E.coli*, one of which lacked the C-terminal myc epitope. After chromatography on GSH–agarose ~1 mg purified protein was obtained from a 1 l bacterial culture. The preparations showed a major 67 kDa band on SDS–polyacrylamide gels and minor bands of lower molecular mass whose relative amounts were estimated by densitometric analysis of the gels. Only the upper 67 kDa band was phosphorylated by p42 MAP kinase or RK and specific activities were corrected for the presence of the lower molecular mass impurities. GST–MAPKAP kinase-2 (5–400) and GST–MAPKAP kinase-2 (46–400) were made 0.03% (by mass) in Brij-35, dialysed against 20 mM MOPS, pH 7.5, 0.1 mM EDTA, 0.1% (by volume) 2-mercaptoethanol, 50% (by volume) glycerol and stored unfrozen at –20°C. Under these conditions the proteins were stable for at least 6 months.

For expression in mammalian cells wild-type and mutant MAPKAP kinase-2 containing amino acid residues 1–400 and a C-terminal myc epitope were constructed in a pEXV3 expression vector. These constructs were transfected into COS cells (Howe *et al.*, 1992), exogenous MAPKAP kinase-2 immunoprecipitated with monoclonal antibody 9E10 and MAPKAP kinase-2 activity measured with the standard peptide substrate (Stokoe *et al.*, 1992a).

Site-directed mutagenesis was carried out by polymerase chain reaction and all mutations verified by DNA sequencing.

Phosphorylation and activation of MAPKAP kinase-2 by p42 MAP kinase and RK

The incubations contained 25 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 1.0 μM PKI, 0.2 mg/ml MAPKAP kinase-2, 2 U/ml p42 MAP kinase or 200 U/ml RK, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$ and 0.1 mM ATP. The reactions were initiated with MgATP after pre-incubating the other components for 3 min at 30°C. At various times aliquots were removed and assayed for MAPKAP kinase-2 activity (Stokoe *et al.*, 1992a). One unit of MAPKAP kinase-2 was that amount which phosphorylated 1 μmol peptide KKLNRTLSVA/min. In order to determine the extent of phosphorylation of MAPKAP kinase-2, parallel incubations were carried out in which unlabelled ATP was replaced by [γ-^{32}P]ATP (10^6 c.p.m./nmol). At various times aliquots of the reactions were added to 1 ml 5% TCA and, after standing on ice for 5 min, the suspensions were centrifuged for 3 min at 13 000 *g*. The supernatants were discarded, the pellets washed three times with 25% TCA and then analysed by Cerenkov counting. Phosphorylation stoichiometries were determined using the calculated molecular masses of each fusion protein and concentrations determined by quantitative amino acid analysis of the purified proteins.

Proteolytic digestion of ^{32}P-labelled MAPKAP kinase-2 and purification of phosphopeptides

MAPKAP kinase-2 (0.2 mg) was phosphorylated as described above and reactions terminated after 20 min by addition of Na–EDTA, sodium pyrophosphate and NaF to final concentrations of 5, 20 and 50 mM respectively. The solution (1.1 ml) was applied to a 20 ml column of Sephadex G-50 equilibrated in 50 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 100 mM NaCl, 0.1% (by volume) 2-mercaptoethanol, 0.03% (by mass) Brij-35, 5% (by volume) glycerol to separate ^{32}P-labelled MAPKAP kinase-2 from [γ-^{32}P]ATP. The native [^{32}P]MAPKAP kinase-2 was incubated for 1 h at 30°C with trypsin (1 mg/ml), the reaction terminated by addition of 0.05 vol 100% TCA and, after 5 min, the suspension was centrifuged for 5 min at 13 000 *g*. The supernatant was applied to a 24 cm × 4 mm Vydac 218TP54 C_{18} column (Separations Group, Hesperia, CA) equilibrated in 0.1% (by volume) trifluoroacetic acid (TFA) and connected to an on-line monitor for continuous measurement of ^{32}P radioactivity. The column was developed with a linear acetonitrile gradient in 0.1% (by volume) TFA with an increase in acetonitrile concentration of 0.33%/min. The flow rate was 0.8 ml/min and fractions of 0.4 ml were collected. The TCA pellet was washed three times with 5% TCA and three times with diethyl ether, dried and resuspended in 50 μl 100 mM Tris–HCl, pH 7.8, 10 mM $CaCl_2$. Chymotrypsin (0.5 μl, 10 mg/ml) was added and the suspension incubated for 4 h at 30°C. A further 0.5 μl chymotrypsin was added and the suspension incubated for a further 12 h at 30°C. TCA (50 μl, 10%) was added and, after standing on ice for 5 min, the suspension was centrifuged for 2 min at 13 000 *g*. The supernatant, containing >90% of the ^{32}P radioactivity, was then chromatographed on the C_{18} column as described above.

Phosphopeptides were further purified by chromatography, using either the same C_{18} column equilibrated in 10 mM ammonium acetate, pH 6.5, instead of 0.1% TFA (peptides T1, T2, T3 and T5), or a much smaller C_{18} column (15 cm × 0.2 mm) equilibrated in 0.1% TFA (peptides T4 and C1). The latter column was developed with a linear acetonitrile gradient in 0.1% TFA with an increase in acetonitrile concentration of 0.25%/min. The flow rate was 0.2 ml/min and peptides absorbing at 214 nm were collected manually, as they were eluted.

Phosphoamino acid analysis

Peptides were dried, hydrolysed for 90 min at 110°C in the presence of 6 M HCl, redried and dissolved in pyridine acetate, pH 3.5, containing unlabelled phosphotyrosine, phosphothreonine and phosphoserine. Phosphoamino acids were separated by electrophoresis for 40 min at 1000 V on thin layer cellulose plates and their positions visualized by staining with ninhydrin and autoradiography.

Dephosphorylation of MAPKAP kinase-2 by PP2A

Phosphorylated MAPKAP kinase-2 was diluted with an equal volume of 50 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 1 mg/ml bovine serum albumin, 0.1% (by volume) 2-mercaptoethanol and incubated for 30 min at 30°C with the catalytic subunit of PP2A (10 mU/ml). At various times the incubations were made 0.5 μM with respect to okadaic acid to inhibit PP2A and an aliquot assayed for MAPKAP kinase-2 activity. The remainder was digested with trypsin and chymotrypsin and chromatographed on the 24 cm × 4 mm Vydac C_{18} column as described above.

^{32}P-Labelling of KB cells and immunoprecipitation of MAPKAP kinase-2

Confluent 6 cm dishes of KB cells were washed twice in phosphate-free medium (ICN, Oxon, UK), incubated for 2 h in phosphate-free medium containing 10% phosphate-free fetal calf serum (dialysed against 20 mM HEPES, pH 7.2) and then incubated for 2–5 h with carrier-free

[^{32}P]phosphate (0.25–3 mCi/ml). After appropriate stimulation, the cells were washed once with 3 ml ice-cold phosphate-buffered saline (Oxoid, Hampshire, UK), then lysed in 300 µl 20 mM Tris–HCl, pH 7.0, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1% (by mass) Triton X-100, 10 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.1% (by volume) 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride (lysis buffer). The lysate was centrifuged at 4°C (10 min, 13 000 *g*) and the supernatant from one dish was incubated for 2 h with 30 µl MAPKAP kinase-2 antibody coupled to 5 µl protein G–Sepharose. The protein G–Sepharose–antibody–MAPKAP kinase-2 complex was washed three times with lysis buffer containing 0.5 M NaCl and once with lysis buffer without 0.5 M NaCl.

Proteolytic digestion of in vivo phosphorylated MAPKAP kinase-2

The immunoprecipitated MAPKAP kinase-2 was denatured in 10 µl 1% (by mass) SDS at 100°C and electrophoresed on a 12.5% polyacrylamide gel. After autoradiography the 50 kDa band corresponding to human MAPKAP kinase-2 was excised and the gel piece homogenized in 5 vol 25 mM *N*-ethylmorpholine HCl, pH 7.7, containing 0.1% (by mass) SDS and 0.5% (by volume) 2-mercaptoethanol. The suspension was incubated for 12 h at 37°C on a shaker and then centrifuged for 5 min at 13 000 *g*. The supernatant (containing 80–90% of the ^{32}P radioactivity) was removed, made 1 mg/ml with respect to bovine serum albumin and protein precipitated by addition of 0.2 vol 100% TCA. The suspension was centrifuged for 2 min at 13 000 *g*, the supernatant discarded and the pellet washed three times with 1 ml 20% (by mass) TCA and three times with ice-cold acetone and dried. The pellet was resuspended in 50 µl 50 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 5% (by volume) glycerol, 0.1% (by volume) 2-mercaptoethanol, 0.03% (by mass) Brij-35, 0.1% (by mass) reduced Triton X-100 and trypsin added to a concentration of 1 mg/ml. After 3 h at 30°C, the digest was centrifuged for 5 min at 13 000 *g* and the supernatant, containing 70% of the ^{32}P radioactivity, chromatographed on a Vydac C_{18} column as described for MAPKAP kinase-2 phosphorylated *in vitro*. The ^{32}P radioactivity not solubilized with trypsin was incubated for 1 h at 4°C with 0.1 ml performic acid. After dilution to 0.5 ml with water and lyophilization, the pellet was digested with chymotrypsin and chromatographed on the C_{18} column.

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