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# Identification of the sites in MAP kinase kinase-1 phosphorylated by p74$^{raf-1}$

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**Many growth factors whose receptors are protein tyrosine kinases stimulate the MAP kinase pathway by activating first the GTP-binding protein Ras and then the protein kinase p74$^{raf-1}$. p74$^{raf-1}$ phosphorylates and activates MAP kinase kinase (MAPKK). To understand the mechanism of activation of MAPKK, we have identified Ser217 and Ser221 of MAPKK1 as the sites phosphorylated by p74$^{raf-1}$. This represents the first characterization of sites phosphorylated by this proto-oncogene product. Ser217 and Ser221 lie in a region of the catalytic domain where the activating phosphorylation sites of several other protein kinases are located. Among MAPKK family members, this region is the most conserved, suggesting that all members of the family are activated by the phosphorylation of these sites. A 'kinase-dead' MAPKK1 mutant was phosphorylated at the same residues as the wild-type enzyme, establishing that both sites are phosphorylated directly by p74$^{raf-1}$, and not by autophosphorylation. Only the diphosphorylated form of MAPKK1 (phosphorylated at both Ser217 and Ser221) was detected, even when the stoichiometry of phosphorylation by p74$^{raf-1}$ was low, indicating that phosphorylation of one of these sites is rate limiting, phosphorylation of the second then occurring extremely rapidly. Ser217 and Ser221 were both phosphorylated *in vivo* within minutes when PC12 cells were stimulated with nerve growth factor. Analysis of MAPKK1 mutants in which either Ser217 or Ser221 were changed to glutamic acid, and the finding that inactivation of maximally activated MAPKK1 required the dephosphorylation of both serines, shows that phosphorylation of either residue is sufficient for maximal activation.**

*Key words:* MAP kinase/MAP kinase kinase/oncogene/phosphopeptide/Raf/site-directed mutagenesis

## Introduction

One of the major signal transduction pathways by which growth factors initiate the proliferation and differentiation of cells leads to the activation of mitogen-activated protein (MAP) kinase (Sturgill and Wu, 1991). The activation of this enzyme requires its phosphorylation on a threonine and a tyrosine residue (Anderson *et al.*, 1990; Payne *et al.*, 1991), both catalysed by MAP kinase kinase (MAPKK) which is itself activated by phosphorylation (Gomez and Cohen, 1991) catalysed by one or more MAPKK kinases (MAPKKKs). Three mammalian MAPKKK activities have so far been identified, namely a homologue of the yeast protein kinases STE11 (*Saccharomyces cerevisiae*) and byr2 (*Schizosaccharomyces pombe*) (Lange-Carter *et al.*, 1993) and the proto-oncogene products c-Mos (which is found only in germ cells) (Nebreda *et al.*, 1993; Posada *et al.*, 1993) and p74$^{raf-1}$ (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). Evidence is accumulating that p74$^{raf-1}$ (and/or its close homologues A-Raf and B-Raf) is one of the MAPKKKs which is stimulated by growth factors that signal through receptor protein tyrosine kinases. For example, the overexpression of p74$^{raf-1}$ in COS-1 cells potentiates the activation of MAP kinase by growth factors (Howe *et al.*, 1992) and interfering mutants of Raf-1 block growth factor activation of MAP kinase (Howe and Marshall, 1993; Schaap *et al.*, 1993). *Drosophila* mutants defective in D-Raf are unable to signal through receptor protein tyrosine kinases that control a variety of developmental processes (Nishida *et al.*, 1988) and a *Drosophila* homologue of MAPKK was isolated as a dominant suppressor of the D-Raf mutant phenotype (Tsuda *et al.*, 1993), suggesting that D-Raf acts 'upstream' of MAPKK.

In the kinase cascade from the activation of p74$^{raf-1}$ to the activation of MAP kinase, only the mechanism by which MAP kinase is activated has been analysed in detail (Anderson *et al.*, 1990; Payne *et al.*, 1991). The mechanism(s) by which p74$^{raf-1}$ is activated by receptor tyrosine kinases is not completely understood, but appears to involve an interaction between the GTP-bound form of p21$^{ras}$ and the N-terminal non-catalytic domain of p74$^{raf-1}$ (van Aelst *et al.*, 1993; Vojtek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993). However, it does not appear that p74$^{raf-1}$ is activated allosterically by direct interaction with p21$^{ras}$-GTP, and the role of p21$^{ras}$ may be to recruit p74$^{raf-1}$ to the plasma membrane where it is activated by an unidentified mechanism (Traverse *et al.*, 1993). Even less is known about the mechanism of activation of MAPKK other than that it is via phosphorylation (Gomez and Cohen, 1991), probably on serine residues (Gomez *et al.*, 1992; Matsuda *et al.*, 1993). Furthermore it is unclear whether different MAPKKKs phosphorylate the same or distinct sites on

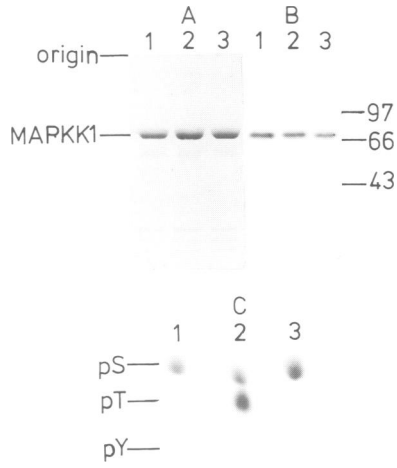


**Fig. 1.** Purification and phosphorylation of wild-type and mutant MAPKKs. GST−MAPKK-1 fusion proteins containing six histidine residues at their C-termini were purified from *E.coli* extracts, maximally activated by incubation with Mg[$\gamma$-$^{32}$P]ATP and p74$^{raf\text{-}1}$ and rechromatographed on glutathione−Sepharose to remove ATP and p74$^{raf\text{-}1}$ (see Materials and methods). The preparations were subjected to electrophoresis on 10% polyacrylamide gels in the presence of SDS and the gels were stained with Coomassie blue (**A**) and autoradiographed (**B**). Further samples of the MAPKK1 preparations were partially hydrolysed by incubation for 90 min at 110°C in 6 M HCl, electrophoresed on thin layer cellulose at pH 3.5 to resolve phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) (Nakielny *et al.*, 1992b) and autoradiographed (**C**). Lanes 1 show results with wild-type MAPKK1, lanes 2 results with MAPKK1(Thr221) and lanes 3 results with MAPKK1(Glu221). The positions of the molecular mass markers glycogen phosphorylase (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (43 kDa) are also marked.

MAPKK. Here, we identify the two serine residues on a mammalian MAPKK isoform that are phosphorylated *in vitro* by p74$^{raf\text{-}1}$FT1 and demonstrate that both serines are phosphorylated in NGF-stimulated rat phaeochromocytoma (PC12) cells.

## Results and discussion

### *Expression of MAPKK1 and its phosphorylation by p74$^{raf\text{-}1}$*

MAPKK1 was expressed as a fusion protein in *Escherichia coli* with glutathione S-transferase (GST) at the N-terminus and six histidine residues at the C-terminus, to permit the purification of the full length MAPKK1 protein by successive affinity chromatographies on glutathione−Sepharose and nickel-nitrilotriacetate-agarose. The preparations showed a major protein-staining band with the molecular mass (71 kDa) expected for this fusion protein (Figure 1A), and the purity estimated by densitometric analysis of the gels was 65%. All the wild-type and mutant MAPKK1 enzymes described below were isolated in similar amounts and purity (2−4 mg from a 25 l bacterial culture after correction for the presence of contaminants—see Figure 1A).

The wild-type MAPKK1 preparations had extremely low, but nevertheless detectable, MAPKK activity which increased ~7000-fold upon incubation with Mg[$\gamma$-$^{32}$P]ATP and activated p74$^{raf\text{-}1}$ (Figure 2, Table I). Activation was paralleled by phosphorylation of the 71 kDa fusion protein (Figure 1B) which reached a plateau at ~1.1 mol/mol protein (Figure 2). Nearly all the phosphate was incorporated

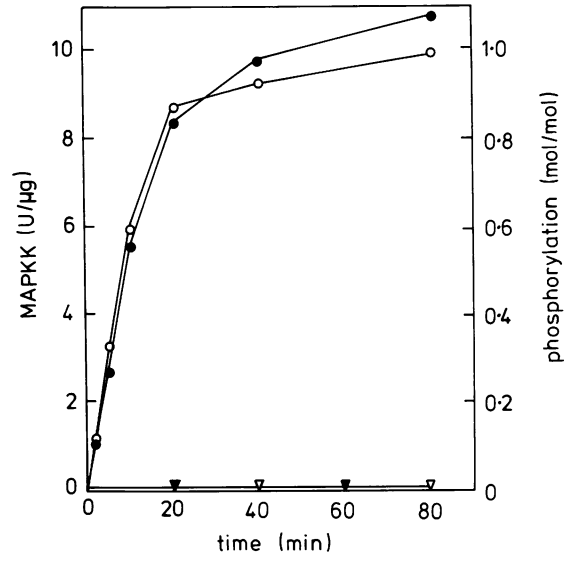


**Fig. 2.** Phosphorylation and activation of MAPKK1 by p74$^{raf\text{-}1}$. MAPKK1 (0.08 mg/ml) was incubated with Mg[$\gamma$-$^{32}$P]ATP and p74$^{raf\text{-}1}$ (8000 U/ml). Aliquots were removed at various times, and MAPKK1 was separated from ATP and p74$^{raf\text{-}1}$ by chromatography on glutathione−Sepharose and then analysed for phosphorylation (filled circles) and specific activity (open circles). Control experiments were carried out in which MAPKK1 was incubated with MgATP in the absence of p74$^{raf\text{-}1}$ and analysed for phosphorylation (open triangles) and activity (filled triangles).

**Table I.** Relative activities of wild-type and mutant forms of MAPKK1 before (−) and after (+) maximal activation by p74$^{raf\text{-}1}$

| Form of MAPKK | Phosphorylation (mol/mol) | Relative activity (−) | (+) |
|---|---|---|---|
| Wild-type | 1.1 | 1 | 7200 |
| Thr221 | 1.0 | 1 | 7200 |
| Thr217 | 1.0 | 1 | 2500 |
| Glu217 | 0.9 | 7 | 3600 |
| Glu221 | 0.8 | 30 | 2300 |
| Glu217/Glu221 | 0.2 | 38 | 38 |
| Ala217 | 0.9 | 0.05 | 75 |
| Ala221 | 0.3 | 0.02 | 0.04 |
| Ala207 | 1.1 | 0 | 0 |

The small amount of phosphate incorporated into the MAPKK1(Glu217, Glu221) mutant may result from non-specific low level phosphorylation of other sites by p74$^{raf\text{-}1}$ or trace contamination with another protein kinase, since the preparation was not homogeneous. The reason why wild-type MAPKK1 and the Thr217 and Thr221 mutants are not phosphorylated to 2 mol/mol is unknown. It is possible that some of the MAPKK molecules in these preparations are folded incorrectly and therefore not recognized by p74$^{raf\text{-}1}$.

into serine residues; only traces of phosphothreonine were detected and there was no phosphotyrosine (Figure 1C). No activation or phosphorylation of MAPKK1 occurred upon incubation with MgATP in the absence of p74$^{raf\text{-}1}$ (Figure 2).

### *Isolation of the tryptic peptide containing the residues on MAPKK1 phosphorylated by p74$^{raf\text{-}1}$*

The $^{32}$P-labelled MAPKK1 was oxidized with performic acid and digested with trypsin, and the tryptic peptide was partially purified on an iron-chelate column, which binds phosphopeptides relatively selectively (Anderson and Porath,

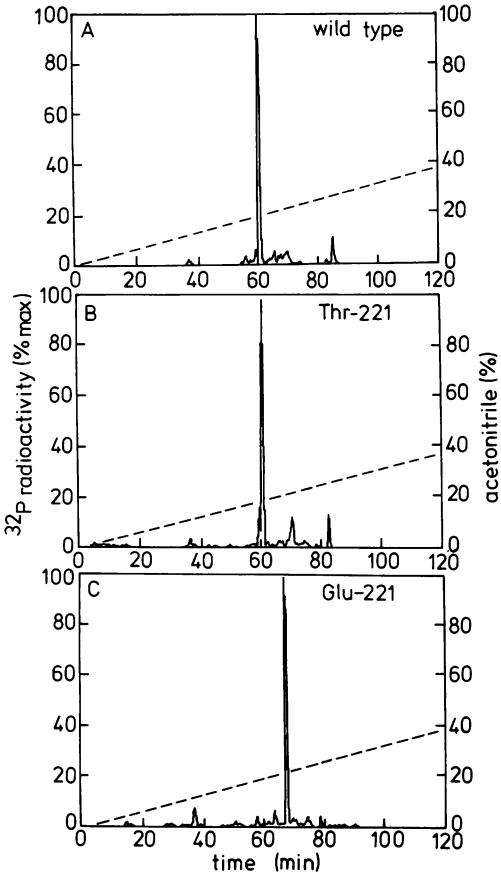


**Fig. 3.** Purification of the major tryptic peptide containing the residues on MAPKK1 phosphorylated during activation by p74$^{raf\text{-}1}$. MAPKK1 was maximally activated by incubation with Mg[$\gamma$-$^{32}$P]ATP and p74$^{raf\text{-}1}$ (Figure 2), oxidized and digested with trypsin (see Materials and methods). The digest was applied to a Vydac 218TP54 $C_{18}$ column (Separations Group, Hesperia, CA) equilibrated in 0.1% (v/v) trifluoroacetic acid (TFA), and the column developed with a linear gradient of acetonitrile (broken line) in 0.08% (v/v) 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. $^{32}$P radioactivity (full line) was recorded continuously with an on-line monitor. (**A**) Wild-type MAPKK1; (**B**), MAPKK1(Thr221); (**C**), MAPKK1 (Glu221). The major phosphopeptide isolated from either wild-type or mutant MAPKK1 was recovered in a yield of 30–50%.

1986). The $^{32}$P radioactivity eluted from this column was chromatographed on a $C_{18}$ column at pH 1.9 which revealed one major $^{32}$P-labelled peptide eluting at 20.5% acetonitrile (Figure 3A). In five separate experiments this peptide comprised between 30 and 50% of the applied $^{32}$P radioactivity. The peptide, which contained phosphoserine and no other phosphoamino acid (Figure 4), was purified to homogeneity by chromatography on the $C_{18}$ column at pH 6.5 (see legend to Figure 5). Its sequence commenced at residue 214 of MAPKK1 and two bursts of $^{32}$P radioactivity occurred after the fourth and eighth cycles of Edman degradation, suggesting that the serines at positions 217 and 221 were phosphorylated (Figure 5A). The tryptic cleavage between residues 213 and 214 was unexpected, because cDNA cloning of MAPKK1 from every mammalian source predicts that residue 213 is glutamine. Since the digestions were carried out at high proteinase concentrations, the anomalous cleavage may result from either an unusual tryptic cleavage or trace contamination with another proteinase. The peptide presumably terminates at Arg226.

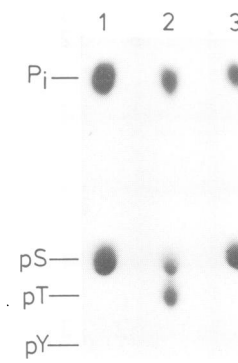


**Fig. 4.** Phosphoamino acid analysis of the major tryptic phosphopeptide from MAPKK1 phosphorylated during activation by p74$^{raf\text{-}1}$. The major phosphopeptides from Figure 3 were partially hydrolysed in 6 M HCl and electrophoresed on thin layer cellulose at pH 3.5 as in Figure 1. Abbreviations: pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine; $P_i$, inorganic phosphate. Lane 1, tryptic phosphopeptide from wild-type MAPKK1 (Figure 3A); lane 2, tryptic phosphopeptide from MAPKK1(Thr221) (Figure 3B); lane 3, tryptic phosphopeptide from MAPKK1(Glu221) (Figure 3C).

### *Phosphorylation and activation of MAPKK1 mutants*

In order to confirm that Ser217 and Ser221 were the sites phosphorylated by p74$^{raf\text{-}1}$ and to investigate their role in the activation of MAPKK1, site-directed mutants of MAPKK1 were generated. The phosphorylation of two sites in MAPKK1 upon incubation with p74$^{raf\text{-}1}$ suggests that either p74$^{raf\text{-}1}$ phosphorylates both serines, or that one serine is phosphorylated by p74$^{raf\text{-}1}$ and the other by MAPKK1 itself after it has been activated by p74$^{raf\text{-}1}$. To distinguish between these possibilities a 'kinase dead' MAPKK1 was made in which Asp207 of the Asp-Phe-Gly motif conserved in all protein kinases (Hanks *et al.*, 1988) was changed to Ala. p74$^{raf\text{-}1}$ phosphorylated this mutant MAPKK1 to the same stoichiometry as the wild-type enzyme, but the mutant protein was completely devoid of activity before or after phosphorylation (Table I). Tryptic digestion and $C_{18}$ chromatography revealed one major $^{32}$P-labelled peptide which coeluted with the phosphopeptide from the wild-type enzyme (data not shown) and amino acid sequencing established that it was phosphorylated at both Ser217 and Ser221 (Figure 5B). This experiment shows that Ser217 and Ser221 are both phosphorylated directly by p74$^{raf\text{-}1}$.

The conserved lysine in kinase subdomain 1 (residue 96 of MAPKK1) is frequently mutated to produce catalytically inactive protein kinases, but a MAPKK1(Met96) mutant was surprisingly found to possess 2% of the wild-type activity after maximal phosphorylation and activation (~200-fold) by p74$^{raf\text{-}1}$. Interestingly, this mutant could only be phosphorylated at Ser217 and not Ser221 (data not shown), demonstrating that its conformation is abnormal in the region recognized by p74$^{raf\text{-}1}$.

In order to assess the relative importance of the two phosphorylation sites to activation, further MAPKK1 mutants were produced in which Ser217 or Ser221 were changed to Glu, in order to try and mimic phosphorylation. These mutants had 7-fold (Glu217) and 30-fold (Glu221) higher activity than the dephosphorylated wild-type enzyme (Table I) and could be activated a further 500-fold (Glu217) and 80-fold (Glu221) by p74$^{raf\text{-}1}$, reaching 50% and 30% of the maximal activity of the wild-type enzyme. Tryptic

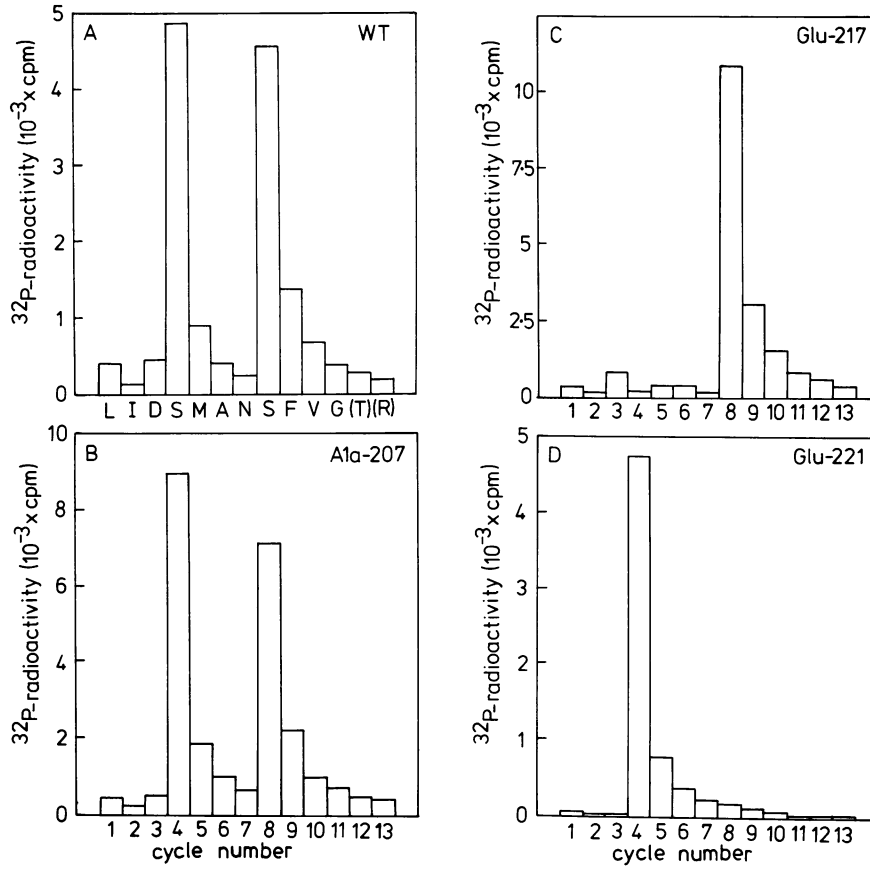


**Fig. 5.** Identification of the residues on MAPKK1 phosphorylated during activation by p74$^{raf-1}$. (**A**) The tryptic peptide from wild-type (WT) MAPKK1 (Figure 3A) was further purified by chromatography on the $C_{18}$ column equilibrated in 10 mM ammonium acetate pH 6.5 instead of 0.1% TFA. The single peak of $^{32}P$ radioactivity, which coincided with a peak of 214 nm absorbance, and eluted at 15% acetonitrile (50 pmol) was then analysed by conventional gas phase sequencing on an Applied Biosystems 470A/120A sequencer. The phenylthiohydantoin (Pth) amino acid identified after each cycle of Edman degradation is shown using the single letter code for amino acids. Pth-methionine was identified as Pth-methionine sulfone. The peptide is likely to terminate at Arg226, but neither this residue nor Thr225 (in parentheses) was identified by sequence analysis. A further sample of the peptide (1 pmol, 20 000 c.p.m.) was then sequenced after being coupled covalently to a Sequelon arylamine membrane (Stokoe *et al.*, 1992). $^{32}P$ radioactivity was measured after each cycle of Edman degradation to identify the positions of phosphorylated residues. (**B**) The tryptic peptide from the 'kinase dead' MAPKK1(Ala207) mutant in Figure 3C (2 pmol, 40 000 c.p.m.) was subjected to solid phase sequencing as in A. (**C**) and (**D**) show tryptic peptides from the Glu217 (2 pmol, 40 000 c.p.m.) and Glu221 (1 pmol, 20 000 c.p.m.) mutants analysed as in B.

digestion and $C_{18}$ chromatography of the $^{32}P$-labelled Glu217 mutant yielded one major phosphoserine-containing peptide which coeluted with the major phosphopeptide from the wild-type enzyme (data not shown), while the tryptic phosphopeptide from the Glu221 mutant eluted slightly later (Figures 3C and 4). As expected, the Glu217 mutant was only phosphorylated at Ser221 (Figure 5C) and the Glu221 mutant at Ser217 (Figure 5D). No phosphorylation or activation of MAPKK1(Glu217) or MAPKK1(Glu221) occurred upon incubation with MgATP in the absence of p74$^{raf-1}$, confirming that both residues are phosphorylated directly by p74$^{raf-1}$ and not by autophosphorylation. To show that Ser217 and Ser221 are the only sites phosphorylated by p74$^{raf-1}$, Ser217 and Ser221 were both changed to Glu. This mutant had a slightly higher activity than the dephosphorylated form of the Glu221 mutant and could not be phosphorylated or activated further by p74$^{raf-1}$ (Table I), which demonstrates that Ser217 and Ser221 are the only activating residues and are the major residues phosphorylated by p74$^{raf-1}$.

Interestingly, MAPKK1 mutants in which either Ser217 or Ser221 were changed to Ala had extremely low activity before or after phosphorylation by p74$^{raf-1}$ (Table I). The Ala217 mutant could be phosphorylated at Ser221 by p74$^{raf-1}$, but activity was only increased to 1% of the activated wild-type enzyme. The Ala221 mutant was phosphorylated poorly and was not activated by p74$^{raf-1}$ (Table I). This suggests that a small uncharged amino acid at these sites impairs the folding of MAPKK1, resulting in a catalytically deficient enzyme.

The combination of phosphopeptide sequencing and mutagenesis clearly shows that Ser217 and Ser221 are the sites in MAPKK1 phosphorylated by p74$^{raf-1}$. However, these experiments do not demonstrate whether phosphorylation of both sites is necessary for activation of MAPKK1. To address this issue further MAPKK1 mutants were made in which either Ser217 or Ser221 was changed to Thr, so that the state of phosphorylation of each site could be monitored by phosphoamino acid analysis. MAPKK1(Thr217) and MAPKK1(Thr221) were both phosphorylated by p74$^{raf-1}$ (Figure 1B) and at a similar rate and to a similar extent as the wild-type enzyme. The Thr221 mutant attained the same final specific activity and the Thr217 mutant 35% of the maximal activity of the wild-type enzyme (Table I). Phosphoamino acid analysis of the Thr221 mutant (Figure 1B) or the Thr217 mutant (data not shown)

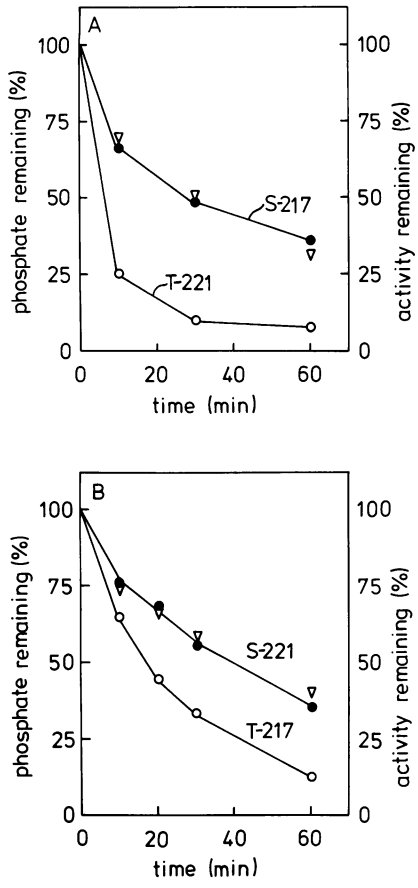


**Fig. 6.** Inactivation of MAPKK requires the dephosphorylation of residues 217 and 221. (**A**) $^{32}$P-labelled MAPKK1(Thr221) which had been maximally phosphorylated by p74$^{raf\text{-}1}$ and contained equal amounts of phosphate in Ser217 and Thr221 was incubated at 30°C with bacterially expressed PP1$\gamma$ (24 mU/ml) in Buffer B. At various times, aliquots were removed and assayed both for activity ($\nabla$) and for [$^{32}$P]phosphate released. Further aliquots were taken at each time point, hydrolysed for 2 h in 6 M HCl at 110°C and electrophoresed on thin layer cellulose at pH 3.5 as in Figure 1C to resolve phosphoserine from phosphothreonine. $^{32}$P radioactivity associated with each phosphorylated amino acid was counted and this information, in conjunction with the total $^{32}$P radioactivity released from the protein, was used to estimate the amount of phosphate released from Ser217 (filled circles, S-217) and Thr221 (open circles, T-221) at each time point. (**B**) shows the same experiment as (A) except that the MAPKK1(Thr217) mutant was used and the concentration of PP1 in the incubations was 12 mU/ml. The triangles show MAPKK1 activity, the open circles phosphate released from Thr217 and the closed circles phosphate released from Ser221. Similar results were obtained in A and B in 12 different experiments using three different times of acid hydrolysis (0.5, 1.0 and 2.0 h). No $^{32}$P radioactivity was released or activity lost if PP1$\gamma$ was omitted.

revealed similar amounts of phosphothreonine and phosphoserine, as would be expected if residues 217 and 221 are the major sites phosphorylated by p74$^{raf\text{-}1}$. Tryptic digestion of the Thr221 mutant yielded one major $^{32}$P-labelled peptide (Figure 3B) containing similar amounts of phosphoserine and phosphothreonine (Figure 4) which coeluted from the $C_{18}$ column with the phosphopeptide from the wild-type enzyme. Solid phase sequencing (see legend to Figure 5) showed that the phosphopeptides from the Thr217 and the Thr221 mutant were both phosphorylated at residues 217 and 221 (data not shown).

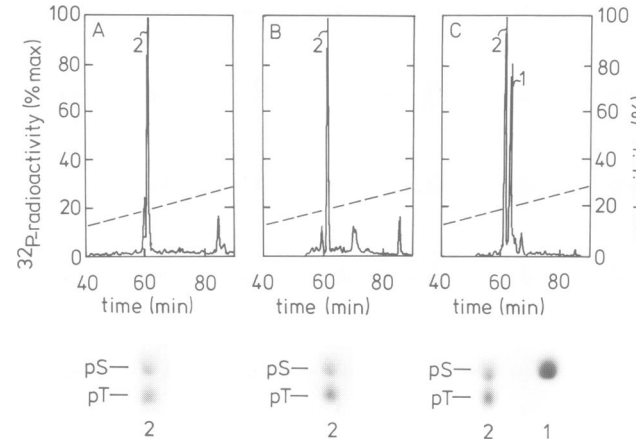


**Fig. 7.** MAPKK1 is phosphorylated at both residues 217 and 221 even when activation is only 10% of the maximal value. The MAPKK1(Thr221) mutant was activated with p74$^{raf\text{-}1}$ to 10% (**A**) and 100% (**B**) of the maximum value, oxidized with performic acid, digested with trypsin and chromatographed on a $C_{18}$ column as described in Materials and methods. The full line shows $^{32}$P radioactivity in arbitrary units and the broken line the acetonitrile gradient. The phosphoamino acid content of each major peak of $^{32}$P radioactivity was then analysed (see legend to Figure 1); the positions of phosphoserine (pS) and phosphothreonine (pT) are marked. The major peaks in A and B eluted at identical positions. They correspond to diphosphorylated derivatives (indicated as '2'), and not mixtures of the two possible monophosphorylated peptides, because the peptide phosphorylated at Ser217 alone (indicated as '1') is eluted 2 min later (**C**). Monophosphorylated peptide was generated by incubating fully activated MAPKK1(Thr221) for 20 min with 24 mU/ml PP1 which released 60% of the $^{32}$P radioactivity and then analysed as in A and B.

### *Relative rates of dephosphorylation and phosphorylation of residues 217 and 221*

MAPKK1(Thr221) and MAPKK1(Thr217) that had been maximally activated by p74$^{raf\text{-}1}$ were completely dephosphorylated and inactivated by incubation with either protein phosphatase $2A_1$ (PP2A) or protein phosphatase-1 (PP1), although PP2A was at least several hundred-fold more effective, as reported previously (Nakielny *et al.*, 1992a).

With MAPKK1(Thr221), PP1 dephosphorylated Thr221 preferentially; this finding was exploited to demonstrate that the rate of inactivation of MAPKK1(Thr221) correlated with the dephosphorylation of Ser217 (Figure 6A). However, with MAPKK1(Thr217) PP1 and PP2A both dephosphorylated Thr217 preferentially and inactivation now correlated with the dephosphorylation of Ser221 (Figure 6B). Taken together, these two experiments establish that the inactivation of MAPKK1 requires the dephosphorylation of both Ser217 and Ser221. The preference of PP2A and PP2C for phosphothreonine residues is well known from studies with synthetic peptide substrates (Donella-Deana *et al.*, 1990) and the present study suggests that this may also be true for PP1.

The tryptic peptide comprising residues 214–226 could either be a diphosphorylated derivative containing phosphate at both Ser217 and Ser221 or a mixture of monophosphorylated derivatives containing phosphate at either site (if the phosphorylation of these residues was mutually exclusive). The demonstration that both phosphates must be removed in order to inactivate MAPKK1 (Figure 6), implies that both residues are phosphorylated in each MAPKK1 molecule. If MAPKK1 was phosphorylated at either site, then inactivation would have correlated with overall dephosphorylation of the protein. Furthermore, when 60%

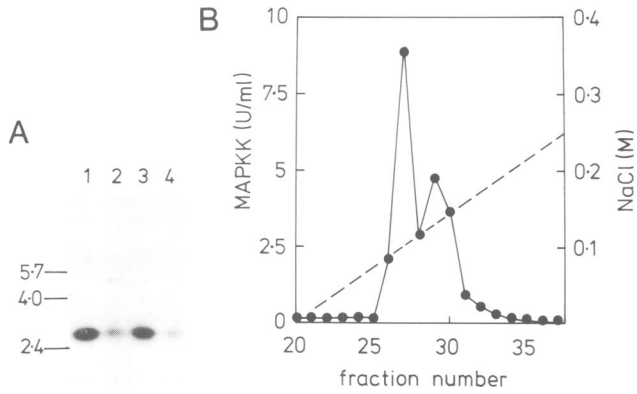


**Fig. 8.** MAPKK is phosphorylated at Ser217 and Ser221 in NGF-stimulated PC12 cells. (**A**) Lysates from $^{32}$P-labelled PC12 cells that had been stimulated for 10 min with NGF, and both peaks of MAPKK activity derived from chromatography on Mono Q (see B below) were immunoprecipitated with MAPKK1 antibody and digested with trypsin as described in Materials and methods. The digests were chromatographed on a $C_{18}$ column as in Figure 3 and the phosphoserine-containing peptide eluting at 20.5% acetonitrile was subjected to isoelectric focusing and autoradiography (Hardie and Guy, 1980). Lane 1 shows the diphosphorylated peptide comprising residues 214–226 of MAPKK1 obtained after phosphorylation of MAPKK1 by p74$^{raf-1}$ *in vitro* (Figure 3). Lanes 2 and 3 show the peptides obtained from the first and second peaks of active MAPKK in B which were generated by phosphorylating inactive MAPKK from PC12 cells with p74$^{raf-1}$. Lane 4 shows the peptide obtained after immunoprecipitation of MAPKK from lysates of NGF-stimulated PC12 cells. The isoelectric points of the markers Patent blue (2.4), acetylated cytochrome *c* (4.0) and azurin (5.7) are also shown. (**B**) Inactive MAPKK from the flowthrough fractions obtained by chromatography of PC12 cell lysates on Mono Q (see Materials and methods) was incubated for 30 min at 30°C with Mg[$\gamma$-$^{32}$P]ATP and p74$^{raf-1}$ (2000 MAPKKK U/ml) and rechromatographed on the Mono Q column. MAPKK activity (filled circles) was eluted as two peaks in fractions 26/27 and 29/30. The NaCl gradient is shown by the broken line.

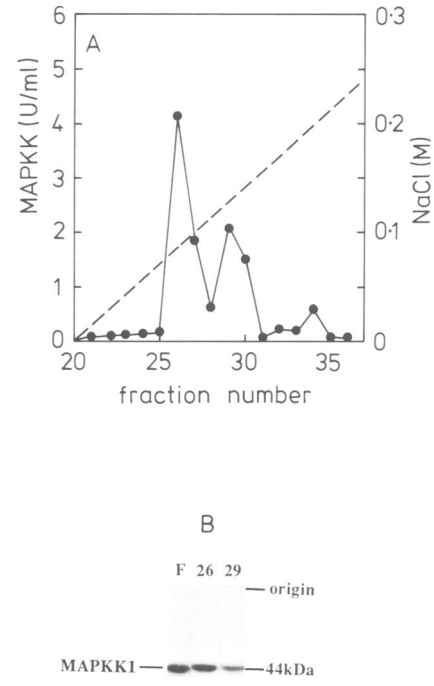


**Fig. 9.** Immunoprecipitation of different forms of MAPKK1 separated by chromatography of PC12 cell extracts on Mono Q. (**A**) $^{32}$P-labelled PC12 cells were stimulated with NGF for 40 min, and the lysates from 10 dishes ($6 \times 10^7$ cells) chromatographed on Mono Q as described in Materials and methods. Inactive MAPKK was present in the flowthrough fractions while MAPKK activity (filled circles) eluted as two peaks in fractions 26/27 and 29/30. The sodium chloride gradient is shown by the broken line. (**B**) 0.9 ml aliquots of the flowthrough fractions and fractions 26 and 29 from A were immunoprecipitated with MAPKK1 antibody as described in Materials and methods. The immunoprecipitates were electrophoresed on 10% SDS–polyacrylamide gels and autoradiographed.

of the phosphate was released from the Thr221 mutant with PP1, tryptic digestion yielded a phosphoserine-containing peptide eluting from the $C_{18}$ column 2 min later than the peptide containing both phosphothreonine and phosphoserine (Figure 7C). This peptide was phosphorylated at Ser217 because [$^{32}$P]phosphate was released after the fourth, but not after the eighth, cycle of Edman degradation (data not shown).

When MAPKK1(Thr221) was phosphorylated by p74$^{raf-1}$, phosphoamino acid analysis revealed similar amounts of phosphothreonine and phosphoserine at 10, 25, 50 and 100% activation (Figure 7). Tryptic digests of partially activated MAPKK1 revealed one major $^{32}$P-labelled peptide comigrating with the diphosphorylated derivative of the peptide 214–226, even when the degree of activation was only 10% (Figure 7A). This peptide was separated from the monophosphorylated derivative labelled at Ser217 alone, which elutes 2 min later (Figure 7C). These observations indicate that phosphorylation of one serine is rate-limiting, phosphorylation of the other then occurring extremely rapidly.

### *Ser217 and Ser221 are both phosphorylated in NGF-stimulated PC12 cells*

In order to investigate whether Ser217 and Ser221 are phosphorylated *in vivo*, the phosphorylation of MAPKK was studied in PC12 cells. When PC12 cells are stimulated with nerve growth factor (NGF), MAPKK is maximally activated after 5 min and this level of activity is maintained for at least 1 h (Traverse *et al.*, 1992). MAPKK1 was immunoprecipitated from the lysates of [$^{32}$P]orthophosphate-labelled PC12 cells stimulated with NGF. After SDS–PAGE, the major $^{32}$P-labelled 44 kDa protein (MAPKK1) was eluted, precipitated with trichloroacetic acid (TCA) and digested with trypsin. Chromatography on a $C_{18}$ column as in Figure 3 revealed a phosphoserine-containing $^{32}$P-labelled peptide eluting at the same position as the diphosphorylated peptide 214–226 from *in vitro* labelled MAPKK1. This peptide also comigrated in isoelectric focusing experiments with the diphosphorylated peptide 214–226 (Figure 8A), indicating that both Ser217 and Ser221 are phosphorylated in NGF-stimulated PC12 cells. The peptide was absent when MAPKK was immunoprecipitated from PC12 cells that had not been stimulated by NGF.

The peptide 214–226 is only a minor $^{32}$P-labelled peptide in tryptic digests of MAPKK from NGF-stimulated PC12 cells, because no more than 20% of the MAPKK is activated after stimulation by NGF (Saito *et al.*, 1994). Nevertheless, this low degree of activation is sufficient to activate fully the p42 and p44 isoforms of MAP kinase, as judged by gel-mobility shift experiments (D.Cross and P.Cohen, unpublished experiments). This emphasizes the high degree of amplification that may be inherent in this

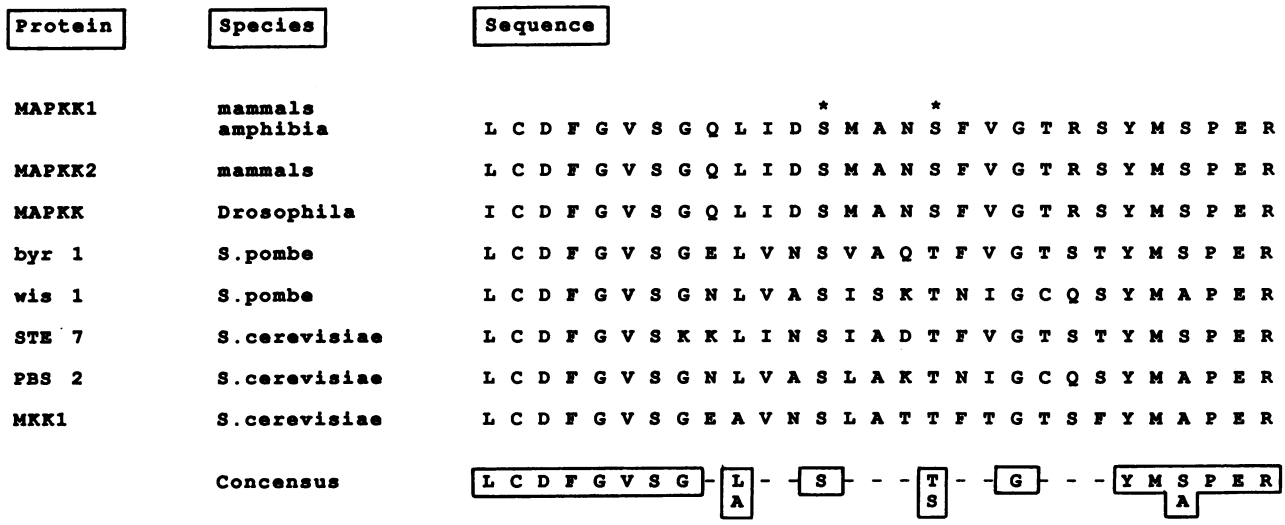


**Fig. 10.** The region of the catalytic domain that is most conserved among MAPKK family members contains the serines (*) that become phosphorylated during activation by c-Raf. Amino acid sequences are given in the following references: mammalian MAPKK1 (Ashworth *et al.*, 1992), mammalian MAPKK2 (Wu *et al.*, 1993), amphibian MAPKK (Kosako *et al.*, 1993), *Drosophila* MAPKK (Tsuda *et al.*, 1993), byr1 (Nadin-Davis and Nasim, 1988), wis-1 (Warbrick and Fantes, 1991), STE7 (Teague *et al.*, 1986), pbs2 (Boguslawski and Polazzi, 1987), MKK1 (Irie *et al.*, 1993). STE7 and byr1 lie in signal transduction pathways that coordinate the physiological changes which allow yeast cells to mate, while MKK1 lies downstream of a protein kinase C isoform in a pathway essential for cell integrity. wis1 is a mitotic control element in fission yeast, the disruption of which leads to a delay in mitosis, while PBS2 (in high copy number) confers resistance to the antibiotic polymyxin B (see Cohen, 1993; Nishida and Gotoh, 1993; for reviews).

protein phosphorylation cascade and suggests that only trace activation of MAPKKKs, such as p74$^{raf\text{-}1}$, may be sufficient to generate the MAPKK activity required for the full activation of MAP kinase. The low degree of activation of MAPKK explains why MAPKK immunoprecipitated from [$^{32}$P]orthophosphate labelled cells contains far less phosphoserine than phosphothreonine (Ahn *et al.*, 1993; Matsuda *et al.*, 1993; Saito *et al.*, 1994). Nearly all the $^{32}$P radioactivity bound covalently to MAPKK *in vivo* is attached to Thr291 and Thr385, the residues phosphorylated by MAP kinase *in vitro*, which are phosphorylated *in vivo* to a much higher stoichiometry than Ser217 and Ser221 (Saito *et al.*, 1994).

When lysates from NGF-stimulated PC12 cells are chromatographed on Mono Q, MAPKK activity is eluted as two peaks at 80–100 mM and 120–140 mM NaCl (Figure 9). The second peak accounts for less than 20% of the MAPKK activity after stimulation with NGF for 5 min, but its proportion rises to 40–50% after stimulation for 40 min (Traverse *et al.*, 1992). Both active forms of MAPKK are recognized as 44 kDa proteins in immunoblotting (data not shown) and immunoprecipitation experiments (Figure 9). The two peaks of MAPKK activity could also be generated by incubating the inactive enzyme present in Mono Q flowthrough fractions with Mg[$\gamma$-$^{32}$P]ATP and p74$^{raf\text{-}1}$ followed by rechromatography on Mono Q (Figure 8B). Furthermore, immunoprecipitation of the MAPKK from each peak followed by tryptic digestion generated the diphosphorylated peptide 214–226 (Figure 8A).

Both peaks of active MAPKK (and inactive MAPKK) isolated from NGF-stimulated PC12 cells are similarly phosphorylated at Thr291 and Thr385 (Saito *et al.*, 1994). The presence of Thr291 indicates that neither form of active MAPKK corresponds to the MAPKK2 isoform which lacks this residue (Otsu *et al.*, 1993; Wu *et al.*, 1993; Zheng and Guan, 1993). Either the two peaks of MAPKK may represent closely related isoforms, or the second peak may be derived from the first by an unidentified post-translational modification.

### *Potential significance of the dual phosphorylation of MAPKK1*

It is intriguing that the activation of MAPKK1 by p74$^{raf\text{-}1}$ is the mirror image of the activation of MAP kinase by MAPKK. The activation of MAP kinase requires the phosphorylation of both Thr183 and Tyr185 (in the 42 kDa isoform of MAP kinase) and is inactivated by the dephosphorylation of either residue (Anderson *et al.*, 1990). In contrast, the activation of MAPKK1 by p74$^{raf\text{-}1}$ appears to require the phosphorylation of either Ser217 or Ser221 and dephosphorylation of both these residues is necessary to inactivate the enzyme. However, our data do not exclude the possibility that phosphorylation of both Ser217 and Ser221 is required for activation, but that phosphorylation at either residue is sufficient to maintain the enzyme in its active conformation. The physiological significance of these observations is unclear, but it is possible that this unusual mechanism, coupled with the synergistic phosphorylation of Ser217 and Ser221, may help to promote significant activation of MAPKK, even under conditions where p74$^{raf\text{-}1}$ is only activated slightly.

Ser217 in MAPKK1 is totally conserved and Ser221 is either Ser or Thr in all known members of the MAPKK family from mammals, amphibians, insects and yeasts (Figure 10). Furthermore, a detailed comparison of these enzymes reveals that the sequence surrounding this region is the most conserved part of the whole molecule (Figure 10), suggesting that all MAPKK homologues may have analogous mechanisms of activation. It is also of interest that the serines in MAPKK1 phosphorylated by p74$^{raf\text{-}1}$ lie between protein kinase subdomains VII and VIII, the same region where the activating phosphorylations for a number

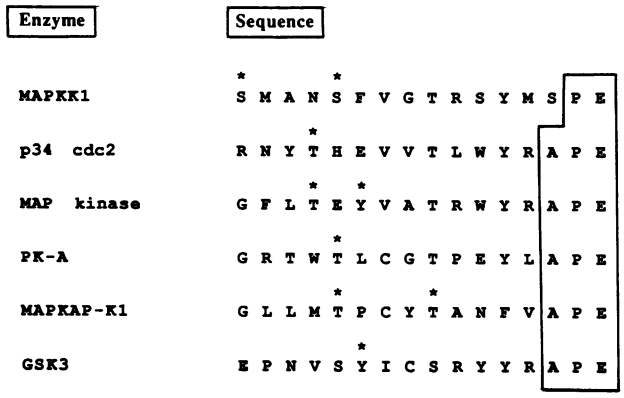


**Fig. 11.** The activating phosphorylation sites in MAPKK1 are located just before the Ala-Pro-Glu motif between subdomains VII and VIII, the region that contains phosphorylated residues essential for the activities of other protein kinases. Phosphorylated residues are marked with an asterisk. Abbreviations: MAPKAP kinase-1 (MAP kinase-activated protein kinase-1, also known as RSK-2); PK-A, cyclic AMP-dependent protein kinase; GSK3, glycogen synthase kinase-3. Amino acid sequences are given in the following references: MAPKK1 (Ashworth *et al.*, 1992), p34$^{cdc2}$ (Lee and Nurse, 1987), MAP kinase (Payne *et al.*, 1991), PK-A (Shoji *et al.*, 1983), MAPKAP kinase-1 (Sutherland *et al.*, 1993), GSK3 (Hughes *et al.*, 1993). The first threonine in MAPKAP kinase-1 is phosphorylated by MAP kinase and the second by autophosphorylation (Sutherland *et al.*, 1993). The DNA sequence of rabbit brain MAPKK1 has been deposited in the GenBank database under the accession number Z30163.

of other protein kinases are located (Figure 11). These include MAP kinase and MAP kinase-activated protein (MAPKAP) kinase-1, two enzymes that are immediately downstream of MAPKK1 in the same signal transduction pathway. However, this is not a universal rule for the activation of all protein kinases in the MAP kinase pathway, because in MAPKAP kinase-2 the threonine phosphorylated by MAP kinase lies C-terminal to the catalytic domain (Stokoe *et al.*, 1993).

A central issue in signalling by the Ras/Raf/MAPKK/MAP kinase pathway is which elements have multiple partners and substrates and thereby generate the branchpoints which allow growth factors to exert multiple effects on their target cells. It is clear that activation of either Ras or Raf is sufficient to transform Rat1a and C7 3T3 cells (Samuels *et al.*, 1993) or to cause the differentiation of phaeochromocytoma (PC12) cells (Wood *et al.*, 1993), suggesting that no branchpoints essential for these processes lie between Ras and Raf. The identification of MAPKK mutants that are slightly active in the absence of phosphorylation by p74$^{raf-1}$ (Table I) may therefore allow us to determine whether activation of MAPKK in the absence of stimulation by Raf or Ras is sufficient for biological responses.

p74$^{raf-1}$ is not the only activator of MAPKK1 in mammalian cells. It will therefore clearly also be important to determine whether other MAPKKKs (see Introduction) phosphorylate Ser217, Ser221 or both of these residues.

## Materials and methods

### *Materials*

Human protein phosphatase 1γ (PP1; Barker *et al.*, 1993) expressed in *E.coli* (Alessi *et al.*, 1993), protein phosphatase 2A$_1$ (PP2A) from rabbit skeletal muscle (Cohen *et al.*, 1988) (provided by Dr G.Moorhead at Dundee) were purified to homogeneity as described previously. One milliunit of PP1 or PP2A was that amount which catalysed the dephosphorylation of 1 nmol of glycogen phosphorylase in 1 min (Cohen *et al.*, 1988). The 42 kDa isoform of MAP kinase was expressed as a GST fusion protein (Stokoe *et al.*, 1992) and purified from *E.coli* extracts by affinity chromatography on glutathione–Sepharose (Stokoe *et al.*, 1992). Myelin basic protein was obtained from Gibco/BRL (Paisley, UK) and in the standard MAP kinase assay (Gomez *et al.*, 1990) gave a 5-fold higher activity than the Sigma product used previously. Trypsin (treated with tosylphenylalanylchloromethylketone) was obtained from the Worthington Biochemical Corporation (Freehold, NJ, USA) and [$^{32}$P]inorganic phosphate (catalogue number PBS 11) and [γ-$^{32}$P]ATP from Amersham International (Amersham, Bucks, UK). Okadaic acid was a generous gift from Dr Y.Tsukitani (Fujisawa Pharmaceutical Company, Tokyo).

### *Expression of MAPKK1 in E.coli*

Rabbit MAPKK1 (Ashworth *et al.*, 1992) was initially expressed in *E.coli* as a GST fusion protein as follows. The 5′ end of the cDNA was generated by PCR so that a *Bam*HI site was present 16 bp in front of the ATG codon. The full-length cDNA was then ligated into *Bam*HI- and *Eco*RI-cut pGEX3X. This construct produces a fusion protein having the amino acids Gly-Ile-Pro-Arg-Ser-Ala between the factor X cleavage site encoded by pGEX3X and the initiating methionine of MAPKK1. When this fusion protein was expressed in *E.coli* and purified by affinity chromatography of the bacterial extracts on glutathione–Sepharose, the material that was eluted from the column with glutathione showed multiple protein-staining bands on SDS–PAGE, suggesting either that the GST-MAPKK1 had degraded or that translation had terminated prematurely. *In vitro* phosphorylation experiments indicated that only the most slowly migrating 71 kDa band, corresponding to the full-length fusion protein (but only accounting for ~5% of the protein), was phosphorylated by MAPKKK. To facilitate purification of full-length protein, six histidine residues were therefore introduced at the C-terminus of the fusion protein. The construct was transformed into the bacterial strain BL21/DES(pLysS), digested to completion with *Eco*RI and then partially digested with *Eag*I which cuts 330 bp and 10 bp before the termination codon. After gel purification the longer fragment was ligated to annealed oligonucleotides encoding the three C-terminal amino acids of MAPKK1, six histidine residues and a termination codon followed by an *Eco*RI site. The resulting construct, hereafter simply termed MAPKK1 was checked by DNA sequence analysis.

### *Purification of bacterially expressed MAPKK1*

Bacteria were grown at 37°C in a 25 l fermenter until the absorbance at 600 nm was 0.6. Isopropyl-β-D-thiopyranoside was then added to 0.5 mM to induce expression of MAPKK1 and the bacteria were grown for a further 2.5 h before centrifuging for 10 min at 4000 *g*. A bacterial pellet derived from 12.5 l of bacterial culture was resuspended at 4°C in 150 ml of 50 mM Tris–HCl pH 7.5 (20°C), 10 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM tosylphenylchloromethylketone and 1 mM benzamidine (Buffer A) containing 2 mM EDTA, 2 mM EGTA, 1% (w/v) Triton X-100 and 0.25 M NaCl and frozen by immersion in liquid nitrogen. After 5 min, the sample was thawed by immersion in cold water and 50 ml portions were sonicated for 4 min on ice. The suspension was centrifuged for 30 min at 28 000 *g* and the supernatant decanted, mixed at 4°C with 40 ml of glutathione–Sepharose equilibrated in Buffer A plus 0.03% (w/v) Brij-35. After mixing end over end for 30 min, the suspension was centrifuged for 5 min at 4000 *g*, the supernatant discarded and the resin washed five times with 200 ml of Buffer A containing 0.03% (w/v) Brij-35 and 0.125 M NaCl. The MAPKK1 was then eluted from the resin at ambient temperature with three 100 ml portions of Buffer A containing 0.03% (w/v) Brij-35 and 20 mM glutathione pH 8.0. The eluate was made 0.5 M in NaCl and 1 mM in imidazole–HCl pH 7.5 and applied to a 10 ml nickel-nitrilotriacetate-agarose column equilibrated in the same buffer. After washing with Buffer A containing 10 mM imidazole–HCl pH 7.5 and 0.5 M NaCl until the absorbance of the eluate at 280 nm was <0.01, the column was washed with 100 ml Buffer A containing 30 mM imidazole–HCl, 0.15 M NaCl, 0.03% (w/v) Brij-35 pH 7.5, and MAPKK1 eluted with 100 ml of Buffer A containing 300 mM imidazole–HCl pH 7.5, 0.15 M NaCl, and 0.03% (w/v) Brij-35. Fractions of 8 ml were collected and analysed by SDS–PAGE. The fractions containing highly purified MAPKK1 (~50 ml) were pooled and concentrated to 2.0 ml by ultrafiltration through an Amicon 30 membrane. The solution was diluted to 10 ml with Buffer A containing 0.1 mM EGTA and 0.03% Brij-35, reconcentrated to 2.0 ml and after repeating this procedure twice more the enzyme was stored in aliquots at −80°C. After this study was completed, we found that the yield of MAPKK1 could be increased from 2–4 mg to 100–200 mg per 25 l culture by inducing expression at 25°C

for 20 h and by decreasing the concentration of isopropyl-β-D-thiopyranoside to 0.03 mM.

### Mutagenesis of MAPKK1

Single-stranded sense DNA was prepared from MAPKK1 cloned into pBluescript. This was used as a template for site-directed mutagenesis using the Amersham system 2.1 and antisense mutagenic primers synthesized on an Applied Biosystems DNA synthesizer. Mutant clones were digested with *Bam*HI and *Asp*718 and the fragment was ligated into *Bam*HI- and *Asp*718-digested pGEX3XMAPKK1(His-tagged). The constructs were sequenced using an Applied Biosystems automated DNA sequencer before expression in bacteria and purification as described for wild-type MAPKK1.

### Purification of activated c-Raf from Sf9 cells

It has been reported that $p74^{raf\text{-}1}$ is activated in Sf9 insect cells when coexpressed with oncogenic forms of Ras (MacDonald *et al.*, 1993). Baculovirus vectors expressing $p74^{raf\text{-}1}$ containing six histidine residues at its C-terminus were constructed by adding appropriate linkers to the 3′ end of the $p74^{raf\text{-}1}$ cDNA coding sequence (pCMV $p74^{raf\text{-}1}$, *Sma*I and *Ava*I sites). In addition to the His tag this construct contained a thrombin recognition sequence immediately following the last amino acid of $p74^{raf\text{-}1}$. The $p74^{raf\text{-}1}$-His cassette was then excised by digestion with *Bam*HI and *Sma*I, blunt ended and cloned into the *Sma*I site of pAcC5. The presence of the insert and its orientation were verified by digestion with *Eco*RI and *Xba*I. In order to obtain large quantities of activated His-tagged $p74^{raf\text{-}1}$ Sf9 cells were grown as suspension cultures at 27°C in shaker flasks with an agitation of 50–100 r.p.m. When the density reached $2 \times 10^6$ cells/ml they were coinfected with the His-tagged $p74^{raf\text{-}1}$, H-RasV12 and $p56^{lck}$ recombinant baculoviruses at a multiplicity of infection of five for each virus. Approximately 48 h post-infection the cells were harvested, washed twice with ice-cold phosphate buffered saline and frozen on dry ice.

Frozen cell pellets (4 ml) were thawed on ice and lysed by the addition of 8 ml of ice-cold 20 mM Tris–HCl pH 7.5, 140 mM NaCl, 0.1% (v/v) 2-mercaptoethanol, 0.5% (w/v) sodium deoxycholate, 1% (w/v) Triton X-100, 10% (v/v) glycerol, 0.5 mM PMSF and 1 mM benzamidine. The lysate was centrifuged for 10 min at 14 000 *g* and the supernatant was decanted, made 0.5 M in NaCl and 1 mM in imidazole and purified on nickel-nitriliotriacetate-agarose as described for MAPKK1, except that the column was 2 ml and not 10 ml and the volumes of all buffers were 5-fold lower. The final preparation was concentrated to 4 ml (30 000 U/ml, dialysed against 50 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 0.03% (w/v) Brij-35 and 0.1% (v/v) 2-mercaptoethanol (Buffer B) containing 0.1 M NaCl and 10% (v/v) glycerol and 0.1 ml aliquots were quick frozen in liquid nitrogen and stored at −80°C. This procedure purified $p74^{raf\text{-}1}$ ~100-fold with an overall recovery of 60%. The final preparation, which had a sp. act. of 80 000 U/mg, was stable for at least 1 month at −80°C.

### Assay of MAPKK1

MAPKK1 was assayed using bacterially expressed $p42^{mapk}$ as described previously (Traverse *et al.*, 1992). One unit (U) of MAPKK1 activity was defined as that amount which increased the activity of MAP kinase by 1 U/min. One unit of MAP kinase was that amount which incorporated 1 nmol of phosphate into myelin basic protein per minute.

### Assay of $p74^{raf\text{-}1}$

$p74^{raf\text{-}1}$ was measured by its ability to activate MAPKK1. The assay was as described previously for MAPKKK from PC12 cells (Gomez *et al.*, 1992) except that $p74^{raf\text{-}1}$ was diluted to < 1 U/ml in Buffer B containing bovine serum albumin (1 mg/ml), and bacterially expressed MAPKK1 and MAP kinase were each included at 2 μM and the incubation was for 30 min instead of 20 min. One unit of $p74^{raf\text{-}1}$ was that amount which increased the activity of MAP kinase by 1 U/min in this assay. The activation of MAP kinase in this assay is directly proportional to the amount of $p74^{raf\text{-}1}$ added, up to a final $p74^{raf\text{-}1}$ concentration of 1 U/ml. The assay is extremely sensitive since 1 U/ml corresponds to only a 0.5% conversion of MAPKK1 to its activated form and a concentration of 0.1 U/ml can be quantified accurately.

### Maximal phosphorylation and activation of MAPKK1 by $p74^{raf\text{-}1}$

The reaction was initiated by adding purified activated $p74^{raf\text{-}1}$ (250 μl, 30 000 U/ml) to 750 μl of Buffer B containing 0.08 mg/ml MAPKK1, 10 mM magnesium acetate, 0.1 mM [$\gamma$-$^{32}$]ATP ($1-3 \times 10^6$ c.p.m./nmol), and 1 μM okadaic acid which had been preincubated for 3 min at 30°C. At various times, 0.2 ml aliquots were removed, added to an ice-cold suspension of 0.03 ml of 0.2 M EDTA pH 7.5 and 0.075 ml of glutathione–Sepharose (Pharmacia) equilibrated in Buffer B and mixed end over end for 45 min at 2°C. After centrifugation for 1 min at 14 000 *g*, the supernatant was discarded, and free [$\gamma$-$^{32}$P]ATP was eliminated by washing the resin repeatedly with 0.5 ml portions of Buffer B containing 0.1 M NaCl. The $^{32}$P-labelled MAPKK1 was then eluted (60–70% recovery) with three successive 0.4 ml aliquots of Buffer B containing 0.1 M NaCl and 20 mM glutathione pH 8.0. Phosphorylation stoichiometries were determined using the molecular mass of 71 kDa for the MAPKK1 fusion protein and protein concentrations determined by amino acid analysis. Each aliquot was also diluted 200-fold into ice-cold Buffer B containing 1 mg/ml bovine serum albumin and assayed for MAPKK1 activity as described above.

### Isolation of the major tryptic peptide from MAPKK1 containing the residues phosphorylated during activation by $p74^{raf\text{-}1}$

$^{32}$P-labelled MAPKK1 (0.32 mg) that had been phosphorylated for 15 min at 37°C with $p74^{raf\text{-}1}$ (8000 U/ml) was freed from ATP and $p74^{raf\text{-}1}$ by chromatography on glutathione–Sepharose as described above and precipitated by addition of 0.025 vols of 100% (w/v) TCA. The suspension was centrifuged for 15 min at 14 000 *g*, the supernatant discarded, and the pellet washed three times with diethyl ether to remove TCA and dried. The $^{32}$P-labelled MAPKK1 was dissolved in 0.5 ml of ice-cold performic acid, made prior to use by mixing 0.45 ml of 98% (w/v) formic acid with 0.05 ml of 30% (w/v) hydrogen peroxide for 60 min at room temperature. After standing for 60 min at 0°C to oxidize cysteine residues to cysteic acid and methionine to methionine sulfone, 5 ml of water was added, and the solution was frozen and lyophilized. These residues were oxidized to avoid peptide heterogeneity resulting from partial oxidation. The pellet was resuspended in 1.0 ml of *N*-methylmorpholine acetate pH 8.0, and 0.01 ml of 10 mg/ml tosylphenylalanylchloromethylketone-treated trypsin added. After incubation for 16 h at 37°C, a further 0.01 ml of 10 mg/ml trypsin was added and the incubation continued for a further 5 h at 37°C. 0.06 ml of 100% (w/v) TCA was added, and after standing on ice for 10 min, the suspension was centrifuged for 10 min at 14 000 *g*. The supernatant, which contained ~98% of the $^{32}$P radioactivity, was extracted three times with 2.0 ml of diethyl ether to remove TCA, made 0.1% (v/v) in acetic acid and added to 0.4 ml of an iron-chelating Sepharose resin (Anderson and Porath, 1986) equilibrated in 0.1% (v/v) acetic acid. The resin was mixed end over end for 15 min at room temperature, then centrifuged for 0.5 min at 14 000 *g*, and the supernatant discarded. The resin was then washed 15 times with 1.0 ml of 0.1% acetic acid and the $^{32}$P radioactivity was eluted in 80% yield by extracting the resin three times with 0.5 ml portions of water:ethanol:triethylamine pH 10.5 (9:9:2). The eluate was lyophilized and further purified as described in Results.

### Preparation of antisera to MAPKK1

GST-MAPKK1 (0.5 ml, 1.0 mg/ml) without the six C-terminal histidine residues was emulsified with 0.5 ml of complete Freund's adjuvant and injected intramuscularly into a rabbit below the scapula and under the legs in the area of the lymph nodes. After 31 days, a further 0.1 mg of protein was mixed with 0.5 ml of incomplete Freund's adjuvant and injected as before. The rabbit was bled after a further 11 days and after adsorbing with GST–Sepharose to remove antibodies against GST, the serum used for immunoblotting and immunoprecipitation experiments.

### $^{32}$P-labelling of phaeochromocytoma (PC12) cells

The cells were cultured as described (Gomez *et al.*, 1990) and then washed twice with Krebs-Ringer-HEPES (KRH) buffer (25 mM HEPES pH 7.4 containing 0.125 M NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 5.6 mM glucose) which had been prewarmed to 37°C. Each 6 cm diameter dish of cells was then incubated for 160 min at 37°C with 2.5 ml of KRH buffer containing 1.0 mCi of [$^{32}$P]inorganic phosphate and stimulated for 10 min with NGF (100 ng/ml) (Gomez and Cohen, 1991). The buffer was removed by aspiration, the cells washed twice with ice-cold KRH buffer, then lysed with 0.4 ml of ice-cold 20 mM Tris–acetate pH 7.3 containing 0.27 M sucrose, 1% (w/v) Triton X-100, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.1 mM PMSF and 1 mM benzamidine.

### Mono Q chromatography of lysates from $^{32}$P-labelled PC12 cells

Lysates prepared from 10 dishes of $^{32}$P-labelled cells ($6 \times 10^7$ cells) were concentrated from 4 ml to 1 ml by centrifugation through a Centricon 10 membrane, rediluted to 4 ml in lysis buffer and reconcentrated to 1 ml; this process was repeated twice more to remove $^{32}$P-labelled phosphate and metabolites. The solution was then diluted to 10 ml in 50 mM Tris–HCl pH 7.3, 2 mM EDTA, 2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol, 0.03%(w/v) Brij-35, 0.3 mM sodium orthovanadate (Buffer C) and applied to a $5 \times 0.5$ cm column of Mono Q equilibrated in Buffer

C containing 0.1 mM PMSF and 1.0 mM benzamidine. After washing with a further 10 ml of Buffer C, the column was developed with a 40 ml linear salt gradient to 0.7 M NaCl at a flow rate of 1.0 ml per min and fractions of 1.0 ml were collected.

### *Immunoprecipitation of MAPKK*

The lysates from $^{32}$P-labelled PC12 cells or Mono Q fractions were centrifuged for 10 min at 13 000 *g* (4°C) and the supernatants incubated for 90 min at 4°C with 0.03 ml of protein A–Sepharose in 20 mM sodium phosphate pH 7.5 containing 0.15 M NaCl and centrifuged for 1 min at 13 000 *g* to remove any protein binding non-specifically to protein A–Sepharose. The supernatant was added to a 0.06 ml aliquot of antibody-conjugated protein A–Sepharose and after 2 h at 4°C, the suspension was again centrifuged for 1 min at 13 000 *g*. The supernatant was removed, incubated for a further 2 h with antibody-conjugated protein A–Sepharose and the suspension recentrifuged. Finally, the supernatant from the second immunoprecipitation was incubated overnight with a third aliquot of antibody-conjugated protein A–Sepharose. The MAPKK was imunoprecipitated quantitatively after these three treatments with protein A–Sepharose-conjugated antibody. Each immunoprecipitate was washed four times with Buffer C containing 0.4 M NaCl, then denatured in 1% (w/v) SDS, heated for 5 min at 100°C and subjected to SDS–PAGE on 10% gels. The gels were dried and autoradiographed.

### *Digestion of immunoprecipitated MAPKK with trypsin*

The immunoprecipitated MAPKK1 was subjected to SDS–PAGE and the $^{32}$P-labelled 44 kDa MAPKK1 band was excised and homogenized in 5 vols of 25 mM *N*-ethylmorpholine acetate pH 7.7. SDS and 2-mercaptoethanol were added to final concentrations of 0.1% (w/v) and 5% (v/v) respectively and, after heating for 5 min at 100°C, the suspension was incubated for a further 12 h at 37°C, then centrifuged for 5 min at 13 000 *g*. The supernatants (containing 50–70% of the $^{32}$P radioactivity in the gel slice) were removed and made 5% in TCA, the suspension was centrifuged for 5 min at 13 000 *g* and the supernatant was discarded. The precipitate was washed three times with ether to remove TCA, dried and then oxidized with performic acid and digested with trypsin as described for MAPKK1 phosphorylated *in vitro* by p74$^{raf-1}$.

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