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The threonine residues in MAP kinase kinase 1 phosphorylated by MAP kinase in vitro are also phosphorylated in nerve growth factor-stimulated rat phaeochromocytoma (PC12) cells

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Abstract

The residues on MAP kinase kinase-1 (MAPKK1) phosphorylated by MAP kinase in vitro have been identified as Thr-291 and Thr-385. Both threonines are phosphorylated in PC12 cells and the ^{32}P-labelling of each residue increases after stimulation with nerve growth factor (NGF). The results establish that MAPKK1 is a physiological substrate for MAP kinase. The two active forms of MAPKK that are resolved by Mono Q chromatography of PC12 cell extracts are both phosphorylated at Thr-291 and Thr-385, demonstrating that neither species is the MAPKK2 isoform which lacks Thr-291.

Key words: MAP kinase; MAP kinase kinase; Growth factor; Phosphopeptide; Amino acid sequence

1. Introduction

MAP kinase kinase (MAPKK) is a key component of the mitogen-activated protein (MAP) kinase cascade, a signal transduction pathway of central importance in mediating the actions of growth factors that cause cells to proliferate or to differentiate [1,2]. MAPKK is a 'dual specificity' protein kinase which activates MAP kinase by phosphorylating a threonine and a tyrosine residue [3,4], and is itself activated by phosphorylation [5] catalysed by one or more MAP kinase kinase kinases (MAPKKKs). A MAPKKK which is activated after cell stimulation by growth factors is the proto-oncogene p74^{raf-1} which phosphorylates the MAPKK isoform, termed here MAPKK1 [6–9], at Ser-217 and Ser-221 [10]. Only the diphosphorylated derivative of MAPKK1 is detected, even at low levels of phosphorylation by p74^{raf-1}, indicating that phosphorylation of one serine residue is rate limiting, phosphorylation of the second then occurring extremely rapidly. Furthermore, dephosphorylation of both Ser-217 and Ser-221 is required to inactivate MAPKK1, suggesting that phosphorylation of either serine residue is sufficient for maximal activation. The unusual properties of this system may provide a mechanism for achieving significant activation of MAPKK1, even under conditions where p74^{raf-1} is only activated slightly. Ser-217 and Ser-221 are both phosphorylated in vivo when phaeochromocytoma (PC12) cells are stimulated with nerve growth factor [10].

Phosphoamino acid analysis of MAPKK immunoprecipitated from ^{32}P-labelled *Xenopus* oocytes [11] and EGF-stimulated human A431 cells [12], has revealed the presence of much more phosphothreonine than phosphoserine, indicating that the enzyme is phosphorylated in vivo at sites distinct from those phosphorylated by p74^{raf-1}. Here we identify the threonine residues in MAPKK1 phosphorylated in nerve growth factor (NGF)-stimulated rat phaeochromocytoma (PC12) cells and show that they correspond to the residues phosphorylated by MAP kinase in vitro.

2. Materials and methods

2.1. Materials

The p42 isoform of murine MAP kinase (p42^{mapk}) was expressed in *E. coli* as a glutathione transferase fusion protein, activated with MAPKK from rabbit skeletal muscle [13] and assayed as described previously [14]. One unit of MAP kinase was that amount of activity which catalysed the incorporation of 1.0 nmol of phosphate into myelin basic protein in one min. Inactive rabbit MAPKK1 was expressed in *E. coli* as a fusion protein, with glutathione transferase at its N-terminus and six histidine residues at its C terminus, and purified by successive affinity chromatographies on glutathione-Sepharose and nickel-nitrilotriacetate-agarose [10]. Antisera to MAPKK1 were raised in rabbits [10] and the sources of other materials are given in [10].

2.2. Phosphorylation of MAPKK1 by MAP kinase

The incubations (1.0 ml) contained 25 mM Tris-HCl pH 7.0, 0.1 mM

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EDTA, 0.1 mM EGTA, 0.02% (w/v) Brij 35, 5% (v/v) glycerol (Buffer A), 0.2 mg (2.8 nmol) of MAPKK1, 10 mM magnesium acetate, 0.1 mM [γ^{32}P]ATP (1.7×10^6 cpm/nmol) and MAP kinase (5 U/ml). After incubation for 1 h at 30°C, the solution was concentrated to 0.05–0.1 ml by centrifugation through a Centricon 30 membrane, diluted with 1.0 ml of Buffer A and reconcentrated. This procedure was repeated four times until [γ^{32}P]ATP comprised < 5% of the ^{32}P-radioactivity.

2.3. Digestion of ^{32}P-labelled MAPKK1 with thermolysin and purification of thermolytic phosphopeptides

0.1 mg of native ^{32}P-labelled MAPKK1 in 0.2 ml of Buffer A (phosphorylated by MAP kinase) was incubated for 60 min at 37°C with 1.0 mg of thermolysin in 0.2 ml of 20 mM Tris-HCl pH 7.5 containing 4 mM $CaCl_2$ and the reaction terminated with 50 μl of 20% (w/v) TCA. After standing in ice for 5 min, the suspension was centrifuged for 5 min at $13,000 \times g$ to pellet the thermolysin and undigested MAPKK1, and the supernatant (containing > 90% of the ^{32}P-radioactivity) was removed, diluted with two volumes of 0.1% (v/v) trifluoroacetic acid, and chromatographed on a Vydac C_{18} 218TP54 column (Separations Group, Hesperia, CA, USA) equilibrated in 0.1% (v/v) trifluoroacetic acid pH 1.9. The column was developed with a linear acetonitrile gradient with an increase in acetonitrile concentration of 0.33% per min. The flow rate was 0.8 ml/min and fractions of 0.4 ml were collected. ^{32}P-labelled peptides were pooled and rechromatographed on the C_{18} column in an identical manner except that 0.1% trifluoroacetic acid pH 1.9 was replaced by 10 mM ammonium acetate pH 6.5.

2.4. Digestion of immunoprecipitated MAP kinase kinase with thermolysin

PC12 cells were labelled with ^{32}P-inorganic phosphate and MAPKK1 was immunoprecipitated from the cell lysates or Mono Q fractions and analysed by SDS/PAGE as described [10]. The ^{32}P-labelled 44 kDa MAPKK band was excised, homogenised in five vol of 25 mM *N*-ethylmorpholine acetate pH 7.7 and SDS and 2-mercaptoethanol were added to final concentrations of 0.1% (w/v) and 5% (v/v), respectively. 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 $13,000 \times g$. The supernatants (containing 50–70% of the ^{32}P-radioactivity in the gel slice) were removed and dialysed for 8 h at 4°C against 25 mM *N*-ethylmorpholine acetate pH 7.7 with three changes of dialysis buffer. The samples were reduced to 0.3 ml in a vacuum concentrator and digested for 3 h at 42°C with 2.5 mg/ml thermolysin in 25 mM *N*-ethylmorpholine acetate containing 2 mM $CaCl_2$.

3. Results

3.1. Identification of the residues on MAPKK1 phosphorylated by MAP kinase in vitro

It has been reported that a MAPKK from *Xenopus* oocytes is a substrate for MAP kinase [11], and in the present study rabbit MAPKK1 was also found to be phosphorylated by MAP kinase. The initial rate of phosphorylation of MAPKK1 (4 μM) was 10% that of the standard substrate myelin basic protein (4 μM). The phosphorylation of MAPKK1 reached 1.3 and 1.8 mol/mol protein after incubation for 1 h with 5 U/ml and 20 U/ml MAP kinase, respectively, suggesting phosphorylation at more than one site. Phosphoamino acid analysis showed the presence of phosphothreonine, but no phosphotyrosine or phosphoserine was present (data not shown).

Digestion of the native ^{32}P-labelled enzyme (containing 1.3 mol phosphate/mol protein) with thermolysin released 90% of the ^{32}P-radioactivity as trichloroacetic acid soluble phosphopeptides and chromatography on a

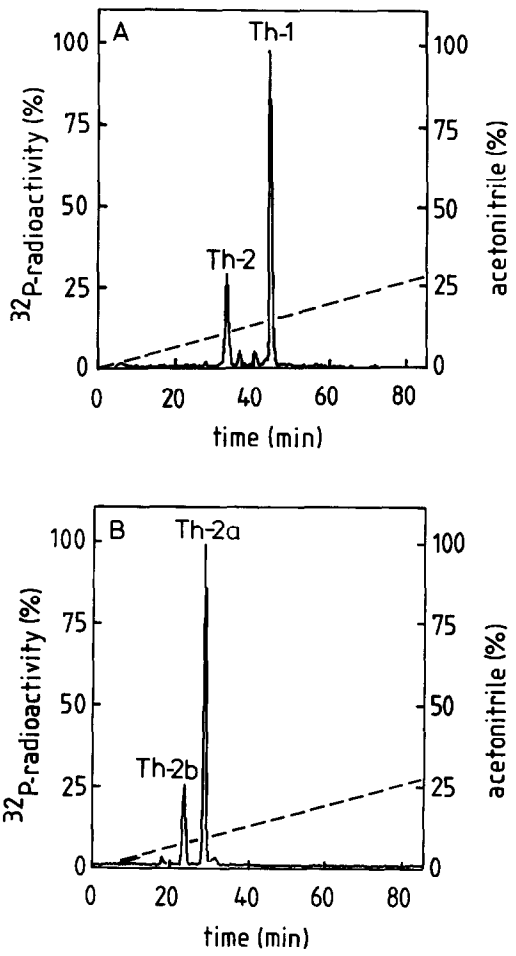

Fig. 1. Separation of ^{32}P-labelled thermolytic phosphopeptides from MAPKK1 phosphorylated by MAP kinase. MAPKK1 was phosphorylated to 1.3 mol/mol with MAP kinase, digested with thermolysin and in (A) chromatographed on a Vydac C_{18} column equilibrated in 0.1% (v/v) trifluoroacetic acid (pH 1.9) as described in section 2. The ^{32}P-radioactivity (full line) was recorded continuously with an on-line monitor and the acetonitrile gradient is shown by the broken line. The two major thermolytic peptides Th-1 and Th-2 (eluting at 14% and 10% acetonitrile, respectively) represented 66% and 23% of the ^{32}P-radioactivity applied to the C_{18} column, respectively. (B) Peptide Th-2 was rechromatographed as in (A) except that trifluoroacetic acid was replaced by 10 mM ammonium acetate pH 6.5.

C_{18} column at pH 1.9 revealed two ^{32}P-labelled peptides, Th-1 and Th-2, eluting at 14% and 10% acetonitrile, respectively (Fig. 1A). Th-2 accounted for 23% and Th-1 for 66% of the ^{32}P-radioactivity applied to the column. Both peptides were purified by a further chromatography on the C_{18} column run at pH 6.5 instead of pH 1.9. Th-1 eluted as a single peak at 16% acetonitrile (data not shown), whereas Th-2 was resolved into two components, Th-2a and Th-2b (Fig. 1B).

The N-terminal sequence of peptide Th-1 (AAETPPRPRTPGRP) was found to correspond to residues 282–295 of the mature MAPKK1 protein (in which the initiating methionine is removed [7] and the site of phosphorylation was identified as Thr-291 (Fig. 2A).

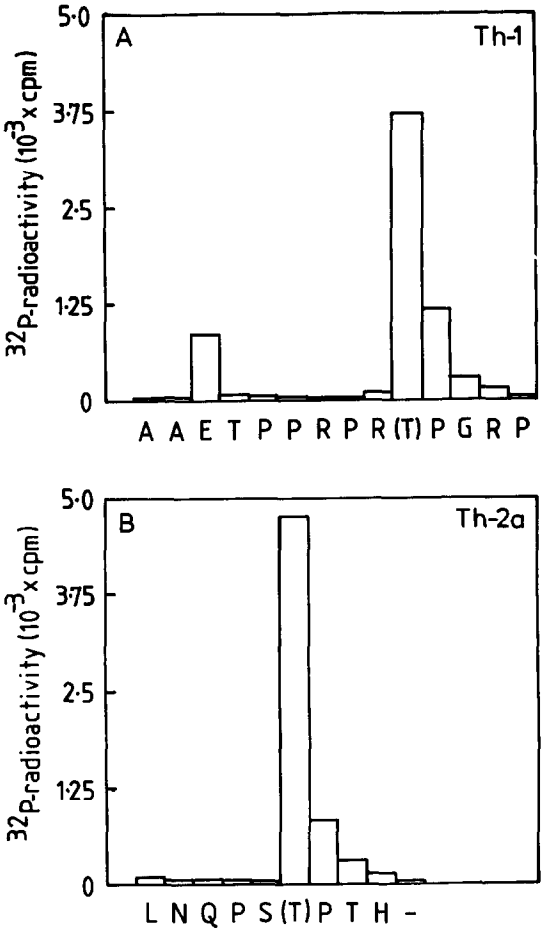

Fig. 2. Identification of the residues in MAPKK1 phosphorylated by MAP kinase in vitro. (A) Peptide Th-1 from Fig. 1A was further purified by chromatography on the C_{18} column in 10 mM ammonium acetate pH 6.5. The peptide, which eluted at 16% acetonitrile, was then sequenced twice on an Applied Biosystems 470A gas phase sequencer; firstly by conventional gas phase sequencing to identify amino acid residues; secondly by solid phase sequencing after coupling the peptide (15,000 cpm) covalently to a Sequelon arylamine membrane (Millipore, Watford, UK) via its carboxyl groups [13]. The figure shows ^{32}P-radioactivity released and amino acid residues (single letter code) identified after each cycle of Edman degradation. (B) Peptide Th-2a (9,600 cpm) from Fig. 1B was sequenced in an identical manner to Th-1. The phenylthiohydantoin derivative of threonine was not detected at the tenth cycle in A or at the sixth cycle in B and is therefore shown in parentheses. In A, the ^{32}P-radioactivity released after the third cycle of Edman degradation is caused by extraction of peptide which had been coupled to the membrane solely through the γ-carboxyl of glutamic acid and not through the α-carboxyl at the C terminus.

Amino acid analysis and fast atom bombardment mass spectrometry confirmed that the peptide terminated at residue 295. The N-terminal sequence of peptide Th-2a (the major peptide from Fig. 1B) corresponded to residues 380 to 388 of the mature MAPKK1 protein (LNQPSTPTH) and the site of phosphorylation was identified as Thr-385 (Fig. 2B). Amino acid analysis and fast atom bombardment mass spectrometry established that this peptide terminated at residue 388 (data not shown). The minor phosphothreonine-containing peptide Th-2b was not obtained in pure form, but amino acid sequencing also revealed a single burst of ^{32}P-radioactivity after the sixth cycle of Edman degradation as observed for Th-2a (data not shown). Th-2b may correspond to Th-2a in which the asparagine and/or glutamine residue is deamidated, which would account for its more acidic isoelectric point (see Fig. 4).

3.2. *Phosphorylation of MAPKK in PC12 cells*

^{32}P-labelled PC12 cells were stimulated with NGF for 40 min, lysed in the presence of protein phosphatase inhibitors and chromatographed on Mono Q to separate the two peaks of active MAPKK (eluting at 80–100 mM NaCl and 120–140 mM NaCl) from inactive MAPKK which does not bind to the column [10,15]. MAPKK was immunoprecipitated quantitatively from each fraction and SDS/polyacrylamide gel electrophoresis of the immunoprecipitates followed by autoradiography revealed a single ^{32}P-labelled band with the molecular mass of

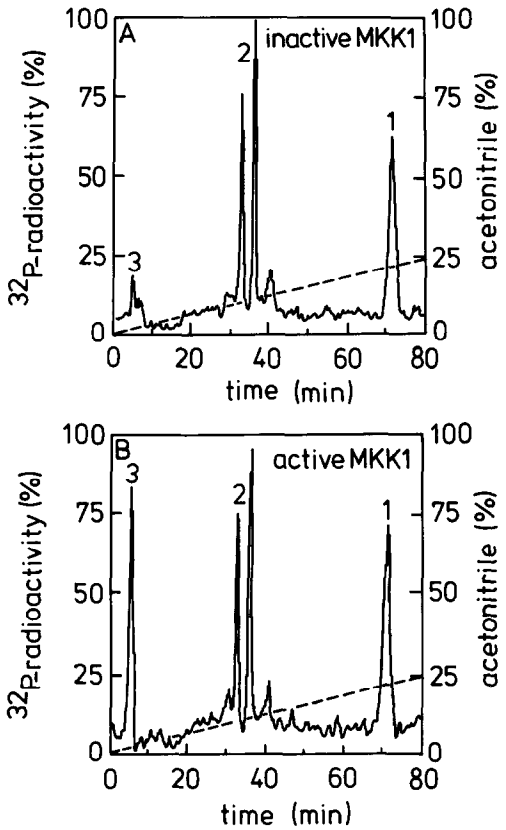

Fig. 3. Separation of thermolytic phosphopeptides generated from MAPKK1 after immunoprecipitation from extracts of ^{32}P-labelled PC12 cells. ^{32}P-labelled cells were stimulated for 40 min with NGF, lysed and chromatographed on Mono Q as in [10]. Inactive MAPKK1 (flowthrough fractions) and the first of the two peaks of active MAPKK1 were immunoprecipitated and digested with thermolysin as in section 2.4 and chromatographed on a Vydac C_{18} column at pH 1.9 as in Fig. 1A. The ^{32}P-radioactivity (full line) was recorded continuously with an on-line monitor and the acetonitrile gradient is shown by the broken line. A, inactive MAPKK1 (MKK1); B, active MAPKK1 (MKK1). The recovery of ^{32}P-radioactivity from each column was 90%.

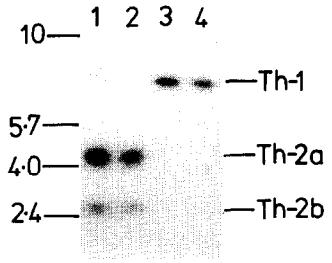

Fig. 4. Isoelectric focussing of thermolytic peptides from MAPKK1 phosphorylated by MAP kinase in vitro and in PC12 cells in vivo. The ^{32}P-labelled peptides were analysed by isoelectric focussing [28] and the figure shows an autoradiograph of the electrophoretogram. Lanes 1 and 3 show peptides 2 and 1 from Fig. 3 obtained by digestion of MAPKK1 immunoprecipitated from ^{32}P-labelled PC12 cells. Lanes 2 and 4 show peptides Th-2 and Th-1 from Fig. 1 obtained by thermolytic digestion of MAPKK1 phosphorylated by MAP kinase in vitro. The major ^{32}P-labelled band in lanes 1 and 2 corresponds to peptide Th-2a and the minor acidic ^{32}P-labelled band to peptide Th-2b in Fig. 1B. The isoelectric points of the markers Patent blue (2.4), acetylated cytochrome C (4.0) and azurin (5.7) are also shown.

MAPKK1 (44 kDa) [10]. This band was present in the flowthrough fractions containing inactive MAPKK, as well as both peaks of active MAPKK [10]. ^{32}P-radioactivity associated with the inactive MAPKK in the flowthrough fractions was much greater than that associated with the two peaks of active MAPKK, and in two experiments was 2.3 ± 0.1-fold higher than both peaks of active MAPKK combined.

The immunoprecipitated MAPKK protein was digested with thermolysin and chromatographed on a C_{18} column. The digests of inactive MAPKK (Fig. 3A), the first peak of active MAPKK (Fig. 3B) and the second peak of active MAPKK (data not shown) each contained three major ^{32}P-labelled peptides eluting at 33, 36 and 70 min, while both peaks of active MAPKK contained an additional component eluting after 5 min. The latter was a phosphoserine-containing peptide(s) which was also generated by thermolytic digestion of MAPKK1 that had been phosphorylated by p74$^{raf\text{-}1}$ in vitro (data not shown) and therefore contains the peptides comprising residues 217 and 221 (see section 1). The peptides eluting at 33, 36 and 70 min only contained phosphothreonine (data not shown).

The component (peptide 1) eluting after 70 min in Fig. 3A comigrated in isoelectric focussing experiments with peptide Th-1 from Fig. 1A (Fig. 4, lanes 3 and 4). Furthermore, when re-chromatographed on the C_{18} column this phosphopeptide now eluted at 43 min, the same time as peptide Th-1 (Fig. 1A). Also, the addition of SDS (1%) to peptide Th-1 from Fig. 1A greatly retarded its elution from the C_{18} column (data not shown). The abnormal elution of this peptide during the first C_{18} chromatography (Fig. 3A) can therefore be explained by the presence of SDS in the thermolytic digest (see section 2) which binds to the peptide (presumably to the three arginine residues) retarding its elution from the C_{18} column. This was confirmed by phosphorylating MAPKK1 in vitro with MAP kinase followed by SDS/polyacrylamide gel electrophoresis and thermolytic digestion as described above. Chromatography of this digest on a C_{18} column yielded the same pattern of phosphopeptides to that observed in Fig. 3A (data not shown). SDS clearly dissociates from the peptide during either isoelectric focussing or re-chromatography. The peptide eluting at 70 min (Fig. 3) was subjected to solid phase sequencing and, consistent with its identity as Th- 1, ^{32}P-radioactivity was released at the tenth cycle of Edman degradation (Fig. 5A). This demonstrates that Thr-291 of MAPKK1 is phosphorylated in PC12 cells after stimulation by NGF.

The peptide eluting after 33 min in Fig. 3 comigrated with peptide Th-2 from Fig. 1A during both C_{18} chrom-

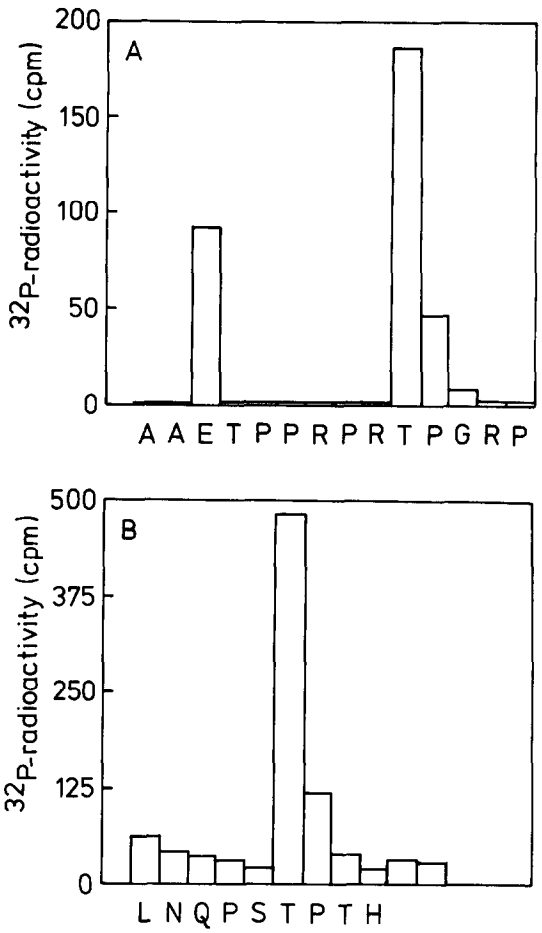

Fig. 5. Thr-291 and Thr-385 of MAPKK1 are phosphorylated in NGF-stimulated PC12 cells. (A) Peptide 1 from Fig. 3 (1200 cpm) was analysed by solid phase sequencing as in Fig. 2. The figure shows ^{32}P-radioactivity released after each cycle of Edman degradation. Peptide 1 comigrated in isoelectric focussing experiments (Fig. 4) with the peptide comprising residues 282–295 of MAPKK1, the sequence of which is shown on the abscissa. The reason why some ^{32}P-radioactivity is released after the third cycle of Edman degradation is explained in the legend to Fig. 2. (B) Peptide-2 from Fig. 3 (2,100 cpm) was analysed by solid phase sequencing as in A. This peptide comigrated in isoelectric focussing experiments (Fig. 4) with the peptide comprising residues 380–388 of MAPKK1, the sequence of which is shown on the abscissa.

atography and isoelectric focussing (Fig. 4, lanes 1 and 2). Both in vitro and in vivo-labelled peptides exhibited a major and a minor band upon isoelectric focussing, which correspond to peptides Th-2a and Th-2b respectively (Fig. 4). The peptide eluting at 33 min in Fig. 3 was subjected to solid phase sequencing and, consistent with its identity as Th-2, ^{32}P-radioactivity was only released after the 6th cycle of Edman degradation (Fig. 5B). This demonstrates that Thr-385 is phosphorylated in vivo in response to NGF. The proportions of the two components eluting at 33 min and 36 min varied from preparation to preparation and the latter also showed two ^{32}P-labelled bands in isoelectric focussing experiments which comigrated with peptides Th-2a and Th-2b (data not shown). The peptide eluting at 36 min therefore comprises peptides Th-2a and Th-2b plus bound SDS.

3.3. *Effect of phosphorylation by MAP kinase on the activity of MAPKK1*

The finding that the inactive MAPKK in the flowthrough fractions from Mono Q was phosphorylated at both Thr-291 and Thr-385 in PC12 cells implied that phosphorylation of these residues by MAP kinase does not activate MAPKK. This was confirmed by phosphorylating MAPKK1 with MAP kinase in vitro. Bacterially expressed MAPKK1 was virtually (but not completely) inactive [10] and phosphorylation to 1.8 mol/mol by MAP kinase did not increase this very low basal level of activity. In contrast, maximal phosphorylation of MAPKK1 by p74^{raf-1} resulted in a 7000-fold increase in activity [10].

4. Discussion

The sequence surrounding the residue (Thr-291) in MAPKK1 phosphorylated most rapidly by MAP kinase (Pro-Arg-Thr-Pro) is identical to that surrounding the major phosphorylation site in myelin basic protein [16], the substrate normally used to assay MAP kinase. A similar sequence (Pro-Arg-Ser-Pro) surrounds the residue in the TH-3 and TH-4 forms of human tyrosine hydroxylase which are the alternatively spliced variants of this enzyme phosphorylated most rapidly by MAP kinase [17]. MAP kinase substrates possessing an arginine residue immediately N-terminal to the phosphorylation site may therefore be phosphorylated preferentially. Pro-Xaa-Ser/Thr-Pro sequences are not only found at the two MAP kinase phosphorylation sites in MAPKK1, but also in other proteins thought to be physiological substrates for MAP kinase, such as MAPKAP kinase-2 [13].

However, although the C-terminal proline is essential for phosphorylation by MAP kinase, the N-terminal proline is not since Leu/Ile-Xaa-Ser/Thr-Pro sequences are found in other physiological substrates, such as MAPKAP kinase-1 [18], c-jun [19] and stathmin [20]. In the case of stathmin, the major site phosphorylated by MAP kinase in vitro lies in an Ile-Xaa-Ser-Pro sequence and the minor site in a Pro-Xaa-Ser-Pro sequence [20].

Thr-291 and Thr-385 represent two of the sites in MAPKK1 that are phosphorylated in PC12 cells and in several experiments the ^{32}P-labelling of these residues increased by twofold to threefold after stimulation for 40 min with NGF (data not shown). This indicates that MAPKK1 is phosphorylated by MAP kinase in vivo, but the physiological role is unclear because MAP kinase does not activate MAPKK1. We have also been unable to detect any significant effect of Thr-291 and Thr-385 phosphorylation on the activity of activated MAPKK1, or on its rate of activation by p74^{raf-1} or rate of inactivation by the catalytic subunit of PP2A. Other proteins that lie upstream of MAP kinase in this growth factor-stimulated pathway also appear to be phosphorylated by MAP kinase in vivo, such as p74^{raf-1} [21] and the EGF receptor [22]. The effects of MAP kinase phosphorylation on these two proteins is also unknown. Perhaps the phosphorylation by MAP kinase of 'upstream' components in this signalling pathways does not alter their activity directly, but their subcellular location or ability to interact with other proteins.

MAPKK is inactive in unstimulated rat PC12 cells. Activation is maximal five min after stimulation with NGF and maintained at a similar level for at least 90 min [15]. When the cells are lysed in the presence of phosphatase inhibitors and chromatographed on Mono Q, most of the inactive MAPKK does not bind to the column whereas activated MAPKK is retained and eluted at two peak. The second peak accounts for < 20% of the MAPKK activity after stimulation with NGF for 5 min, but its proportion increases to 40–50% after 40 min [10,15]. The two active forms of MAPKK comigrate on SDS-polyacrylamide gels in immunoprecipitation [10] or immunoblotting experiments and are inactivated similarly by the MAPKK1-antisera (data not shown). Furthermore, both peaks of active MAPKK are phosphorylated at Ser-217 and Ser-221 [10] as well as at Thr-291 and Thr-385. Thus neither species corresponds to the MAPKK2 isoform which lacks Thr-291 [23–25]. The two peaks of MAPKK activity may either represent very closely related isoforms, or the second peak may be derived from the first by an as yet unidentified post-translational modification. Thr-291 is also absent in the MAPKKs from *Xenopus* eggs [26] and *Drosophila* [27] that have been identified. However, the Pro-Ser-Thr-Pro sequence surrounding Thr-385 is totally conserved in all MAPKKs cloned to date from vertebrates and invertebrates.

If it is assumed that Thr-291 and Thr-385 are phosphorylated to the same extent in the inactive and active forms of MAPKK, then it can be calculated from the total amount of ^{32}P-radioactivity associated with in-

active MAPKK in the Mono Q flowthrough fractions (which is phosphorylated at two residues-Thr-291 and Thr-385) and the two active MAPKK peaks retained by the column (which are phosphorylated at four residues-Ser-217, Ser-221, Thr-291 and Thr-385) that only 20% of the MAPKK is converted to the active form after stimulation with NGF. In fact, the extent of activation may be even lower, because some inactive MAPKK appears to bind to Mono Q and elute with the first peak of active MAPKK (Y. Saito unpublished experiments). This explains why MAPKK immunoprecipitated from ^{32}P-labelled cells contain far less phosphoserine than phosphothreonine [11,12]. Nevertheless, this low degree of activation is sufficient to fully activate both $p42^{mapk}$ and $p44^{mapk}$, as shown by gel-mobility shift experiments (D. Cross and P. Cohen, unpublished observations). This emphasizes the high degree of amplification inherent in this protein phosphorylation cascade and suggests that only trace activation of MAPKKKs, such as $p74^{raf-1}$, may be sufficient to generate the MAPKK activity needed for the full activation of MAP kinase.

The activation of $p74^{raf-1}$ is normally assayed after its immunoprecipitation from extracts of cells that have been stimulated with a growth factor or transformed with an oncogene. The finding that MAPKK is a substrate for at least two protein kinases in vivo and in vitro indicates that it is potentially dangerous to assay $p74^{raf-1}$ solely by the phosphorylation of MAPKK, because of the risk of contamination of the immunoprecipitates with MAP kinase and/or other protein kinases that phosphorylate MAPKK at residues other than those involved in activation. When assaying $p74^{raf-1}$, it would seem essential to measure the activation of MAPKK, rather than its phosphorylation, or at least to ensure that the MAPKK substrate has been phosphorylated on serine and not on threonine residues.

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