An analysis of the phosphorylation and activation of extracellular-signal-regulated protein kinase 5 (ERK5) by mitogen-activated protein kinase kinase 5 (MKK5) *in vitro*

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MKK5 expressed as a glutathione S-transferase fusion protein in human embryonic kidney 293 cells activated full-length extracellular-signal-regulated protein kinase (ERK)5 (ERK5wt) as well as the isolated catalytic domain (ERK5cat) *in vitro*. Activation was accompanied by the phosphorylation of Thr²¹⁹ and Tyr²²¹, the former residue being phosphorylated preferentially. ERK5cat phosphorylated at Thr²¹⁹, but not Tyr²²¹, possessed 10 % of the activity of the doubly phosphorylated protein towards myelin basic protein, whereas ERK5cat phosphorylated at Tyr²²¹ alone was much less active. Activated ERK5 phosphorylated itself at a number of residues, including Thr²⁸, Ser⁴²¹, Ser⁴³³, Ser⁴⁹⁶, Ser⁷³¹ and Thr⁷³³. ERK5 phosphorylated at Thr²¹⁹, but not

Tyr²²¹, phosphorylated itself at a similar rate to ERK5 phosphorylated at both Thr²¹⁹ and Tyr²²¹. Activated ERK5 also phosphorylated mitogen-activated protein kinase kinase 5 (MKK5) extensively at Ser¹²⁹, Ser¹³⁷, Ser¹⁴² and Ser¹⁴⁹, which are located within the region in MKK5 that is thought to interact with ERK5.

Key words: extracellular-signal-regulated protein kinase 5 (ERK5), mitogen-activated protein (MAP) kinase kinase 5 (MKK5), big MAP kinase 1 (BMK1), MALDI-TOF MS, phosphopeptide mapping.

INTRODUCTION

Extracellular-signal-regulated protein kinase (ERK) 5 is a mitogen-activated protein (MAP) kinase family member that is switched on when cells are stimulated with epidermal growth factor (EGF) [1] or exposed to osmotic or oxidative stresses [2]. ERK5 was identified as a protein that interacts with MAP kinase kinase 5 (MKK5) in the yeast 'two-hybrid' system [3] and which became activated when co-transfected into cells with vectors expressing MKK5 [4]. The activation of ERK5 by extracellular signals, but not the activation of ERK1/ERK2, was prevented by the overexpression of a catalytically inactive mutant of MKK5, but not by a catalytically inactive mutant of MKK1 that prevented the activation of ERK1/ERK2 [4]. The ERK5 pathway was also activated when cells are transfected with the MAP kinase/ERK kinase (MEKK) family members MEKK2 or MEKK3 [5,6].

The information summarized above suggests that the protein kinases MEKK2/MEKK3, MKK5 and ERK5 may comprise a MAP kinase signalling module, distinct from the classical MAP kinase cascade, that results in the activation of ERK5 and the phosphorylation of its downstream substrates. The importance of this pathway has recently been highlighted by the finding that mice unable to express ERK5 die at an early stage of embryonic development [7,8]. However, to our knowledge, it has not yet been demonstrated that MKK5 can activate ERK5 *in vitro*. For example, one group [3] has reported that MKK5[ΔN11] (a MKK5 truncation mutant lacking the N-terminal 11 residues), but not full-length MKK5, was expressible in *Escherichia*

coli, but the bacterially expressed MKK5[ΔN11] was inactive. Another group expressed full-length MKK5 in *E. coli*, as well as mutants in which the putative activating phosphorylation sites (Ser³¹¹ and Thr³¹⁵) were changed to aspartic acid. These preparations were shown to phosphorylate myelin basic protein (MBP) weakly, but their ability to activate ERK5 was not reported [9]. A demonstration that MKK5 can activate ERK5 *in vitro* is clearly needed to validate the proposed pathway and to generate the large amounts of active ERK5 required for further work, including high-throughput screening. MKK5 is overexpressed in prostate cancer [10] and is constitutively active in breast cancer cells overexpressing ErbB2 [11]. Thus these protein kinases may be important targets for anti-cancer therapy. In the present study, we report the expression of MKK5 in a form that is capable of activating expressed ERK5 *in vitro*, and we use these preparations to make a detailed analysis of the reversible phosphorylation and activation of ERK5.

MATERIALS AND METHODS

Materials

NuPAGE pre-cast gel electrophoresis products and MBP were from Invitrogen (Paisley, Renfrewshire, Scotland, U.K.), Complete protease inhibitor cocktail tablets were from Roche (Lewes, East Sussex, U.K.), Immobilon-P (PVDF) membranes were from Millipore Ltd (Norwich, Norfolk, U.K.), nickel-nitrilotriacetate agarose was from Qiagen Ltd (Crawley, West Sussex, U.K.) and glutathione-Sepharose 4B was from

Abbreviations used: a.m.u., atomic mass units; EGF, epidermal growth factor; ERK, extracellular-signal-regulated protein kinase; ERK5cat, ERK5 containing the catalytic domain; ERK5cat[T119F] etc., ERK5cat containing a Thr¹¹⁹ → Phe substitution etc.; ERK5kd, catalytically inactive ERK5 (Asp²⁰⁰ → Ala); ERK5wt, full-length ERK5; GST, glutathione S-transferase; HEK, human embryonic kidney; JNK, c-Jun N-terminal kinase; MALDI-TOF, matrix-assisted laser desorption-ionization time of flight; MAP, mitogen-activated protein; MBP, myelin basic protein; MEKK, MAP kinase/ERK kinase kinase; MKK, MAP kinase kinase; MKK5[ΔN11], MKK5 truncation mutant lacking the N-terminal 11 residues; PP2A, protein phosphatase 2A; PTP1B, protein tyrosine phosphatase 1B; TFA, trifluoroacetic acid.

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Amersham Biosciences (Little Chalfont, Bucks., U.K.). The catalytic subunit of protein phosphatase 2A (PP2A) purified from bovine heart was isolated by Miss Julie Diplexcito (MRC Protein Phosphorylation Unit, Dundee, Scotland, U.K.). Protein tyrosine phosphatase 1B (PTP1B) was a gift from Dr David Barford (Institute of Cancer Research, London, U.K.), and microcystin-LR was obtained from Dr Linda Lawton (Robert Gordon University, Aberdeen, Scotland, U.K.). Cellulose-coated plastic TLC plates for separation of phosphoamino acids were from Merck (Lutterworth, Leics., U.K.), and other chemicals were purchased from either Merck or Sigma (Poole, Dorset, U.K.).

Cell culture

Human embryonic kidney (HEK) 293 epithelial cells were maintained at 37 °C in 5 % CO₂ in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal calf serum, 2 mM L-glutamine and 1 % (v/v) antibiotic/antimycotic solution.

Expression and purification of MKK5[ΔN11]

A plasmid encoding human MKK5, obtained from Professor Jack Dixon (Department of Biological Chemistry and Chemistry, University of Michigan, Ann Arbor, MI, U.S.A.) [3], was found to lack a 3' alternatively spliced exon encoding a 10-amino-acid sequence (residues 349–358), corresponding to a region between subdomains IX and X that is conserved in other MKK isoforms. This splice variant is termed MKK5α-2 [9] (human MKK5 isoform B in the National Center for Biotechnology Information protein database, accession number NP_002748). Primers encoding the missing sequence were used to generate two separate PCR fragments of MKK5 that were subsequently fused together in another PCR reaction to generate full-length MKK5, termed MKK5α-1 [9] (human MKK5 isoform A in the National Center for Biotechnology Information protein database, accession number NP_660143). A further truncation mutant lacking the N-terminal 11 residues, termed MKK5[ΔN11], was also generated.

MKK5[ΔN11] was cloned into pEBG-2T, a glutathione S-transferase (GST) fusion vector [12], and transfected into HEK 293 cells using a modified calcium phosphate technique [13]. At 36 h post-transfection, the cells were lysed in ice-cold 50 mM Tris/HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1 % (w/v) Triton-X 100, 10 mM sodium glycerophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μM microcystin-LR, Complete protease inhibitor cocktail (one tablet per 50 ml) and 0.1 % (v/v) 2-mercaptoethanol. The lysates were clarified by centrifugation at 13 000 g for 15 min at 4 °C and GST–MKK5[ΔN11] was purified from the supernatant by affinity chromatography on glutathione–Sephadex. Between 0.5 and 1.0 mg of purified GST–MKK5[ΔN11] was obtained from 100 10-cm-diameter dishes of cells. The undegraded protein, molecular mass 75 kDa, accounted for 80 % of the material, as judged by densitometric analysis (results not shown). Aliquots of the preparation were snap frozen in liquid nitrogen and stored at –80 °C.

Expression and purification of ERK5cat

The cDNA encoding the N-terminal 491 residues of ERK5 including the catalytic domain (ERK5cat) was amplified by PCR from a vector encoding full-length human ERK5 (a generous gift from Professor Jack Dixon, University of Michigan), and cloned into pGEX-4T-1 for expression as a GST fusion protein

in *E. coli*. A number of GST–ERK5cat variants were generated by site-directed mutagenesis as described in the Results section. The vectors encoding GST–ERK5cat and its mutants were used to transform *E. coli* strain BL21(DE3)-pLys-S, grown at 37 °C on a shaking platform in Luria–Bertani medium containing 100 μg/ml ampicillin until they had reached an *D*₆₀₀ of 0.4–0.6, and expression was induced with 10 μM isopropyl β-D-thiogalactoside for 16 h at 26 °C. The bacteria were harvested by centrifugation for 30 min at 4200 g and the pellets from each litre of bacterial culture were resuspended in 40 ml of ice-cold 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 % (w/v) Triton X-100, 0.1 % (v/v) 2-mercaptoethanol, 0.2 mM PMSF and 1 mM benzamide. The bacteria were lysed by sonication on ice and the suspension centrifuged at 27 000 g for 30 min at 4 °C. Fusion proteins were purified from the supernatants by affinity chromatography on glutathione–Sephadex. The fractions containing the bulk of the eluted protein were pooled, dialysed against 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1 mM EGTA, 0.03 % (w/v) Brij-35, 50 % (v/v) glycerol, 0.1 % (v/v) 2-mercaptoethanol, 1 mM benzamide and 0.2 mM PMSF, and stored unfrozen at –20 °C. Approx. 2.5 mg of GST–ERK5cat was obtained from each litre of bacterial culture. The undegraded protein, molecular mass 80 kDa, accounted for 32 % of the material, as judged by densitometric analysis (results not shown).

Expression and purification of ERK5wt and ERK5kd

The cDNA encoding full-length ERK5 (ERK5wt) preceded by six histidine residues was amplified by PCR, as described for ERK5cat, and cloned into pFASTBAC1. Site-directed mutagenesis was used to mutate Asp²⁰⁰ → Ala to create a catalytically inactive 'kinase dead' mutant (ERK5kd). These vectors were then used to express His₆-ERK5wt and His₆-ERK5kd in Sf21 cells using the Bac-to-Bac system (Invitrogen) as described previously [14]. The fusion proteins were purified from the extracts by affinity chromatography on nickel-nitrilotriacetate–agarose. Fractions containing the bulk of the protein were pooled, dialysed and stored as described for ERK5cat. Approx. 1.2 mg of ERK5wt and 1.7 mg of ERK5kd were obtained from each litre of Sf21 cell culture. Undegraded ERK5wt or ERK5kd, each of molecular mass 90 kDa, accounted for 65 % of the material, as judged by densitometric analysis (results not shown).

Assay of MKK5 and ERK5

MKK5 activity was measured by the direct phosphorylation of ERK5 using [γ -³²P]ATP or in a coupled assay in which the ERK5 activity generated upon phosphorylation by MKK5 was then measured by its ability to phosphorylate MBP. In the first assay, GST–MKK5[ΔN11] was incubated at 30 °C with GST–ERK5cat, His₆-ERK5wt or His₆-ERK5kd (2 μM) and 0.1 mM [γ -³²P]ATP in a 20 μl reaction containing 50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA, 10 mM magnesium acetate, 0.01 % (w/v) Brij-35 and 0.1 % (v/v) 2-mercaptoethanol. Reactions were terminated by the addition of SDS and analysed further as described in the Figure legends. In the second assay, unlabelled ATP replaced [γ -³²P]ATP during the phosphorylation by MKK5 and, at various times, aliquots (5 μl) were assayed for ERK5 activity at 30 °C in a 50 μl reaction containing 50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA, 10 mM magnesium acetate, 0.01 % (w/v) Brij-35, 0.1 % (v/v) 2-mercaptoethanol, 0.33 mg/ml

MBP and 0.1 mM [γ - 32 P]ATP (200–600 c.p.m./pmol). After incubation for 10 min, the incorporation of phosphate into MBP was determined by spotting on to phosphocellulose P81 paper, followed by washing to remove [γ - 32 P]ATP, drying and Cerenkov counting [15]. In this assay, ERK5 activity was linear with respect to time and ERK5 concentration. One unit of ERK5 was that amount which catalysed the phosphorylation of 1 nmol of MBP in the standard assay.

In order to assay 32 P-labelled ERK5, assays were terminated in SDS and subjected to PAGE. Gels were stained with Coomassie Blue, and the 32 P-band corresponding to MBP was excised and analysed by Cerenkov counting.

Mapping phosphorylation sites

The procedure for mapping phosphorylation sites is described in detail elsewhere [16]. Briefly, 32 P-labelled proteins were incubated with 0.5% (v/v) 4-vinylpyridine to alkylate cysteine residues, subjected to SDS/PAGE and the 32 P-labelled proteins digested with trypsin and chromatographed on a Vydac C₁₈ column equilibrated in 0.1% (v/v) trifluoroacetic acid (TFA). The column was developed with a gradient of acetonitrile in 0.1% (v/v) TFA from 0–30% acetonitrile (0–90 min), 30–50% (90–110 min) and 50–100% (110–120 min) at a flow rate of 0.8 ml/min.

Fractions of 0.4 ml were collected and the 32 P radioactivity determined by Cerenkov counting. The 32 P-labelled peptides were subjected to matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) MS on a Perseptive Biosystems Elite STR using a matrix of 10 mg/ml α -cyanocinnamic acid in 50% (v/v) acetonitrile/0.1% (v/v) TFA/2 mM diammonium citrate. Detection was performed in the reflector and linear modes. Sites of phosphorylation within peptides was determined by solid-phase sequencing after coupling the peptide covalently to a Sequelon-arylamine membrane, and 32 P radioactivity released after each cycle of Edman degradation was counted.

RESULTS

Phosphorylation and activation of ERK5 by MKK5

GST–MKK5[Δ N11], but not the full-length protein, could be expressed in HEK 293 cells. The purified GST–MKK5[Δ N11] was found to activate both full-length ERK5 (ERK5wt; Figure 1A) and the isolated catalytic domain (ERK5cat; Figure 1C), with activation reaching a plateau after 1 h. Activation was accompanied by phosphorylation which, in the case of ERK5wt, exceeded 1 mol of phosphate/mol of protein, indicating that at least two residues were undergoing phosphorylation. However, in

Figure 1 GST–MKK5[Δ N11] expressed in HEK 293 cells phosphorylates and activates ERK5 *in vitro*

His₆-ERK5wt, His₆-ERK5kd or GST–ERK5cat, each at 2 μ M, were phosphorylated at 30 °C with 2 μ M GST–MKK5[Δ N11] and MgATP (see Materials and methods section). At the times indicated, two aliquots were removed. One aliquot was assayed for ERK5 activity [(●) ERK5wt and (■) ERK5kd in (A), and (●) ERK5cat in (C)], and the second aliquot was denatured in SDS, subjected to PAGE, stained with Coomassie Blue, followed by autoradiography (B, D). The bands were excised and 32 P incorporated into ERK5 was analysed by Cerenkov counting. Stoichiometries of phosphorylation were determined using the molecular mass of ERK5 and the amount of undegraded ERK5 protein in the assays [(○) ERK5wt, and (□) ERK5kd in (A), and (○) ERK5cat in (C)]. The results are shown as means $\pm$ S.D. for two separate assays performed in duplicate. Similar results were obtained in two other experiments.

Figure 2 Separation of tryptic phosphopeptides from ERK5cat

The ^{32}P -labelled ERK5cat from the 15 min (A) and 60 min (B) time points in Figure 1(D) was excised from the gel, digested with trypsin and the resulting peptides separated on a Vydac C₁₈ column equilibrated in 0.1% (v/v) TFA and developed with an acetonitrile gradient (broken lines). The major ^{32}P -labelled peptides (T1, T2, T3 and T4) were eluted in fractions 75, 140, 155 and 179 respectively. Peptides T1, T2 and T3 (C, D and E respectively) were subjected to solid-phase sequencing and the ^{32}P radioactivity released after each cycle of Edman degradation was measured (filled bars). The identity of each peptide was determined by MALDI-TOF MS as described in the text. Single-letter amino-acid notation is used for the amino acid sequence.

order to achieve this level of activation and phosphorylation, high concentrations of GST-MKK5[ΔN11] were required, similar to the amount of ERK5 in the assays. Although the GST-MKK5[ΔN11] that we expressed in HEK 293 cells is active, the activity was low and not increased by prior stimulation of the cells with EGF (results not shown). This suggests that the activity of the expressed MKK5 may represent the inherent basal activity of the unphosphorylated enzyme or a slightly elevated level caused by the small N-terminal truncation.

Following phosphorylation, a very small proportion of the ERK5wt protein, not detectable by protein staining, was converted into a slower-migrating ^{32}P -labelled form (Figure 1B), which was not observed with ERK5cat (Figure 1D). This slower migrating band was later confirmed to be ERK5 by protein sequencing (see below). The full-length catalytically inactive mutant of ERK5 (ERK5kd) was phosphorylated to a lesser extent than ERK5wt (or ERK5cat) and no conversion into a form of lower electrophoretic mobility took place. As expected, the phosphorylation of ERK5kd was not accompanied by any activation (Figures 1A and 1B).

Interestingly, during the activation of ERK5wt and ERK5cat, MKK5 also became phosphorylated and to a similar extent as ERK5 (Figures 1B and 1D). This did not appear to result from autophosphorylation of MKK5, because it did not occur in the absence of ERK5 (results not shown) or when ERK5kd was the substrate (Figure 1B). These observations indicate that MKK5 is phosphorylated by ERK5 once the latter has been activated. However, it cannot be formally excluded that MKK5

can autophosphorylate when it is bound to, or phosphorylated by, active ERK5.

Identification of the phosphorylation sites on ERK5cat

^{32}P -Labelled ERK5cat was digested with trypsin and subsequent chromatography on a C₁₈ column revealed four major ^{32}P -labelled peptides, termed T1, T2, T3 and T4 (Figures 2A and 2B). Analysis by MALDI-TOF MS showed that peptide T1 had a molecular mass of 1303.6 atomic mass units (a.m.u.), corresponding to that predicted for the tryptic peptide encompassing residues 23–35 plus one phosphate. ^{32}P Radioactivity was released after the sixth cycle of Edman degradation, identifying Thr²⁸ as the site of phosphorylation (Figure 2C). Peptide T2 had a molecular mass of 2586.9 a.m.u., corresponding to that predicted for the tryptic peptide encompassing residues 206–225 plus two phosphate groups and with Met²¹⁸ oxidized to methionine sulphone. T3 had a molecular mass of 2506.7 a.m.u., corresponding to the same tryptic peptide with only one phosphate group. ^{32}P Radioactivity was released from T2 after the 14th and 16th cycles of Edman degradation, indicating that the phosphorylated residues were Thr²¹⁹ and Tyr²²¹ (Figure 2D). In contrast, ^{32}P radioactivity was released from T3 only after the 14th cycle, indicating that this peptide was phosphorylated at Thr²¹⁹, but not Tyr²²¹ (Figure 2E). The amount of T2 relative to T3 was higher after 60 min than 15 min (Figures 2A and 2B).

Figure 3 Separation of tryptic phosphopeptides from ERK5wt and ERK5kd

The upper ^{32}P -labelled ERK5wt (A) and the ERK5kd (B) from the 60 min time points in Figure 1(B) were excised from the gel, digested with trypsin and the resulting peptides separated on a Vydac C_{18} column equilibrated in 0.1% (v/v) TFA and developed with an acetonitrile gradient (broken line). The major ^{32}P -labelled peptides T1–T14 (full line) were then analysed as described in the text.

Peptide T4 could not be identified by MS, but ^{32}P radioactivity was released after the 21st cycle of Edman degradation, with a hint of further release after the 33rd cycle (results not shown). This peptide contained phosphoserine, but not phosphothreonine or phosphotyrosine, and inspection of the sequence of ERK5 revealed that only one theoretical tryptic peptide (residues 401–437) contained serine at positions 21 (Ser^{421}) and 33 (Ser^{433}). Moreover, both serines are followed by proline residues, as is usually the case for residues phosphorylated by MAP kinase family members (see Discussion). Peptide T4 was sub-digested with Asp-N protease and re-subjected to solid-phase sequencing. ^{32}P radioactivity was now released after the sixth cycle of Edman degradation (results not shown). This is consistent with phosphorylation of Ser^{421} and Ser^{433} , since residues 416 and 428 are both aspartic acid.

Identification of phosphorylation sites on ERK5wt and ERK5kd

Following phosphorylation of ERK5wt by MKK5, the slower-migrating more highly phosphorylated band, corresponding to ^{32}P -labelled ERK5, was excised, digested with trypsin and chromatographed on the C_{18} column (Figure 3A). Peptides T1–T4 were all present (in the case of T2 and T3 confirmed by MS analysis), together with many other peptides (T5–T14).

Peptide T5 contained phosphothreonine and phosphotyrosine. It had the same molecular mass as peptide T2 and, as expected, ^{32}P radioactivity was released after the 14th and 16th cycles of Edman degradation (results not shown). These results demonstrate that peptide T5 comprises residues 206–225 phosphorylated at Thr^{219} and Tyr^{221} and with Met^{218} oxidized to methionine sulphone. This indicates that peptide T2 is the non-oxidized version of the peptide in which the methionine only became oxidized between its elution from the C_{18} column and its analysis by MS.

Figure 4 Activation of ERK5cat and mutants derived from ERK5cat

ERK5cat (●), ERK5cat[T219A] (○), ERK5cat[Y221F] (□) and ERK5cat[T219A,Y221F] (■), each at $2\ \mu\text{M}$, were phosphorylated at $30\ ^\circ\text{C}$ with MKK5[ΔN11] ($2\ \mu\text{M}$) as in Figure 1(C). Aliquots were removed at the times indicated and ERK5 assayed. The results are shown as means $\pm$ S.D. from two different reactions performed in parallel and each assayed in duplicate. Similar results were obtained in two other experiments.

Peptide T6 had a molecular mass of 1857.8 a.m.u., corresponding to residues 720–735 phosphorylated at two sites. It contained phosphoserine and phosphothreonine and ^{32}P radioactivity was released after the 12th and 14th cycles of Edman degradation (results not shown), identifying Ser^{731} and Thr^{733} as the sites of phosphorylation. Peptide T7 had a molecular mass of 1777.8 a.m.u., corresponding to that predicted for the tryptic peptide encompassing residues 720–735 plus one phosphate group, and ^{32}P radioactivity was released after the 12th cycle of Edman degradation identifying Ser^{731} as the site of phosphorylation (results not shown).

Peptide T8 had a molecular mass of 1684.8 a.m.u., corresponding to residues 491–505 plus one phosphate group. The peptide contained phosphoserine only and ^{32}P -radioactivity was released after the sixth cycle of Edman degradation, identifying Ser^{496} as the site of phosphorylation.

The minor peptides T9 and T10 and the peptides eluting as broad peaks at high acetonitrile concentrations (T11–T14) could not be identified.

ERK5kd was subjected to the same analysis. Only three tryptic phosphopeptides were identified, T2, T3 and T5 (Figure 3B), which are all phosphorylated at Thr^{219} and/or Tyr^{221} . Thus all the additional ^{32}P -labelled peptides derived from ERK5wt arise from autophosphorylation.

Dual phosphorylation of Thr^{219} and Tyr^{221} is required for the full activation of ERK5

In order to investigate the relative roles of phosphorylation of Thr^{219} and Tyr^{221} in the activation of ERK5, mutant ERK5cat proteins were generated in which Thr^{219} or Tyr^{221} were changed to alanine (ERK5cat[T219A]) and phenylalanine (ERK5cat[Y221F]) respectively. A further construct was also made in which both Thr^{219} and Tyr^{221} were mutated (ERK5cat[T219A,Y221F]). Following phosphorylation by MKK5, ERK5cat[Y221F] attained approx. 10% of the activity of activated ERKcat, whereas ERK5cat[T219A] attained only 1–2% of the activity of ERK5cat. ERK5cat[T219A,Y221F] was not activated (Figure 4).

Figure 5 Phosphorylation of ERK5cat and mutants derived from ERK5cat

(A) The 32 P-labelled ERK5cat (WT), ERK5cat[T219A] (T219A), ERK5cat[Y221F] (Y221F) and ERK5cat[T219A,Y221F] (T219A,Y221F), obtained by phosphorylation for 60 min with MKK5 and SDS/PAGE as in Figure 1(D), were transferred on to PVDF membrane, excised and hydrolysed for 90 min at 110 °C in 6 M HCl and subjected to phosphoamino acid analysis as described previously [26]. An autoradiograph of the separation on thin-layer cellulose is shown. pSer, pThr and pTyr show the positions of phosphoserine, phosphothreonine and phosphotyrosine respectively. Other spots are partially hydrolysed peptides. (B) The different ERK5cat preparations used in (A) were phosphorylated with MKK5 and stoichiometries of phosphorylation were determined as in Figure 1(C). The symbols are as in the legend to Figure 4.

Following phosphorylation for 60 min by MKK5, ERK5cat-[T219A] was largely phosphorylated on tyrosine (presumably Tyr²²¹) with only small amounts of phosphoserine and phosphothreonine, presumably arising from trace autophosphorylation (Figure 5A). As expected, ERK5cat[Y221F] was not phosphorylated on tyrosine (Figure 5A) but, interestingly, became as strongly phosphorylated on serine and threonine residues as ERK5cat (Figures 5A and 5B). ERK5cat[T219A,Y221F] was not phosphorylated by MKK5 (Figures 5A and 5B). These results, which suggest that the rates of autophosphorylation of ERK5cat[Y221F] and ERK5cat are similar, are considered further in the Discussion.

We also mutated ERK5cat to change Thr²⁸, Ser⁴²¹ and Ser⁴³³ individually to alanine and generated a further mutant in which both Ser⁴²¹ and Ser⁴³³ were changed to alanine. All four mutants were activated by MKK5 to the same level as ERK5cat. Moreover, MKK5 became phosphorylated to the same extent under these conditions (results not shown).

Figure 6 Effect of site-specific dephosphorylation of ERK5cat on activity

ERK5cat (2 μ M) was phosphorylated for 60 min at 30 °C using [γ - 32 P]ATP (10 000 c.p.m./pmol) as in Figure 1(D). The reaction was desalted on a PD-10 column equilibrated in 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1 mM EGTA, 0.01 % (v/v) Brij-35, 5 % (v/v) glycerol and 0.1 % (v/v) 2-mercaptoethanol and incubated with PP2A (10 units/ml) and/or PTP1B (30 μ g/ml) for 60 min at 30 °C. Aliquots of the reactions were then analysed by SDS/PAGE and autoradiography (A), subjected to phosphoamino acid analysis as in Figure 5(A) (B), or assayed for activity towards MBP (C). The results in (C) are the means $\pm$ S.D. from two independent incubations each assayed in duplicate. Similar results were obtained in two other experiments.

In order to investigate the relative roles of phosphorylation of Thr²¹⁹ and Tyr²²¹ by a second method, ERK5cat, which had been maximally phosphorylated by MKK5, was incubated with PTP1B to dephosphorylate Tyr²²¹ or with PP2A, a serine/threonine-specific phosphatase (Figure 6A). Phosphoamino acid analysis confirmed that PTP1B dephosphorylated Tyr²²¹ selectively. In contrast, PP2A dephosphorylated the threonine residues (Thr²¹⁹ and Thr²⁸) without dephosphorylating Tyr²²¹, Ser⁴²¹ and Ser⁴³³ (Figure 6B). The resistance of serine residues to dephosphorylation that lie in Ser-Pro sequences has been noted previously [17].

Dephosphorylation of Tyr²²¹ decreased ERK5cat activity towards MBP by 90 %, similar to the activity of ERK5cat[Y221F] phosphorylated at Thr²¹⁹. Dephosphorylation of Thr²¹⁹ (and Thr²⁸) consistently decreased ERK5cat activity slightly more (95 %). Treatment with both PTP1B and PP2A almost abolished activity (Figure 6C).

Figure 7 Separation of tryptic phosphopeptides from MKK5

(A) The ^{32}P -labelled MKK5 from the 60 min time point in Figure 1(B) was excised from the gel, digested with trypsin and the resulting peptides separated on a Vydac C_{18} column equilibrated in 0.1% (v/v) TFA and developed with an acetonitrile gradient (broken line). The major ^{32}P -labelled peptides (T1, T2, T3 and T4) were eluted in fractions 48, 51, 108 and 112 respectively. Peptides T1 (B), T3 (C) and T4 (D) were subjected to solid-phase sequencing and the ^{32}P radioactivity released after each cycle of Edman degradation was measured (solid bars). Peptide T2 gave the same result as peptide T1. The identity of peptides T1, T3 and T4 was determined by MALDI-TOF MS as described in the text.

Identification of sites on MKK5 phosphorylated by ERK5

After incubating MKK5 and ERK5 for 60 min with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, the ^{32}P -labelled MKK5 band was excised, digested with trypsin and chromatographed on the C_{18} column. Four ^{32}P -labelled peptides were detected, termed T1–T4 (Figure 7A).

T1 had a molecular mass of 842.5 a.m.u., corresponding to residues 147–153 of MKK5 plus one phosphate group. ^{32}P Radioactivity was released from T1 after the third cycle of Edman degradation identifying Ser¹⁴⁹ as the site of phosphorylation (Figure 7B). The minor peptide T2 was not identified, but ^{32}P radioactivity was also released after the third cycle of Edman degradation. T2 is likely to be the same as T1 plus an additional lysine residue (residue 154) at the C-terminus.

T4 had a mass of 2146.1 a.m.u., corresponding to the tryptic peptide encompassing residues 126–146 plus one phosphate group. However, ^{32}P radioactivity was released after the 4th,

12th and 17th cycles of Edman degradation, indicating that T4 is singly phosphorylated at Ser¹²⁹, Ser¹³⁷ or Ser¹⁴² (Figure 7D). T3 contained two peptides with molecular masses of 2274.1 a.m.u. and 2225.9 a.m.u. (corresponding to residues 126–147 plus one phosphate group and residues 126–146 plus two phosphate groups respectively). Thus T3 was the same as T4, except for an additional lysine residue at the C-terminus or an additional phosphate group. ^{32}P radioactivity was released after the 12th and 17th cycles of Edman degradation (Figure 7C), indicating phosphorylation at Ser¹³⁷ and/or Ser¹⁴².

DISCUSSION

In the present study, we show for the first time that a purified preparation of MKK5 is capable of phosphorylating and activating ERK5 *in vitro* (Figure 1), activation being accompanied by the phosphorylation of Thr²¹⁹ and Tyr²²¹. These residues lie in a Thr-Glu-Tyr sequence equivalent to the Thr-Xaa-Tyr motifs of other MAP kinases, whose phosphorylation is required for activation. However, in contrast with MKK1, MKK3, MKK4 and MKK6, which phosphorylate the tyrosine residue of the Thr-Xaa-Tyr motif preferentially [14,18], MKK5 resembles MKK7 in phosphorylating the threonine residue of the Thr-Xaa-Tyr motif preferentially [19]. Thus ERK5 phosphorylated at Thr²¹⁹ was the only monophosphorylated species detected during the activation reaction (Figures 2 and 3). These results suggest that the phosphorylation of Thr²¹⁹ induces a conformational change in ERK5 that allows MKK5 to phosphorylate Tyr²²¹ *in vitro*. The same observation was made when ERK5wt was replaced by the catalytically inactive mutant ERK5kd (Figure 3). This argues against the possibility that the MKK5-catalysed phosphorylation of Thr²¹⁹ induces a conformational change that then allows ERK5 to phosphorylate itself at Tyr²²¹.

MKK7 catalyses the phosphorylation of Thr¹⁸³ in c-Jun N-terminal kinase (JNK), stimulating the subsequent phosphorylation of Tyr¹⁸⁵ by MKK4 [20]. By analogy with JNK, it therefore cannot be excluded that a protein kinase distinct from, or in addition to, MKK5 phosphorylates ERK5 at Tyr²²¹ *in vivo*. The phosphorylation of ERK5 by MKK5 also resembles the phosphorylation of JNK by MKK7 [19] in that phosphorylation of the threonine residue of the Thr-Xaa-Tyr motif is sufficient to generate approx. 10% of maximal activity towards the standard substrate MBP (Figure 4). However, ERK5 phosphorylated at Thr²¹⁹ alone was as efficient at phosphorylating itself as ERK5 phosphorylated at both Thr²¹⁹ and Tyr²²¹ (Figure 5). This raises the possibility that the activity of Thr²¹⁹-phosphorylated ERK5, relative to that of the doubly phosphorylated enzyme, may vary from substrate to substrate.

In the present study, we have also identified some of autophosphorylation sites on ERK5, which include residues near the N-terminus (Thr²⁸) and C-terminus (Ser⁷³¹ and Ser⁷³³), as well as in the catalytic domain (Ser⁴²¹, Ser⁴³³ and Ser⁴⁹⁶). However, there are likely to be other sites of autophosphorylation near the C-terminus, because several additional tryptic phosphopeptides were observed with ERK5wt compared with ERK5cat (Figure 3 versus Figure 2). These sites proved difficult to identify because the C-terminus of ERK5 has an unusual amino acid sequence that is virtually devoid of sites for cleavage by trypsin or other proteinases, giving rise to very large peptides in which phosphorylation site identification has, so far, proved intractable.

The generation and exploitation of phospho-specific antibodies that recognize the sites of autophosphorylation will be needed to investigate whether the autophosphorylation of ERK5 occurs *in vivo*. However, this may well be the case, because both the

activation of ERK5 in cells [1,4,22] and the autophosphorylation of ERK5 *in vitro* (Figure 1B) are accompanied by a decrease in electrophoretic mobility on SDS/polyacrylamide gels. In contrast, the MKK5-catalysed phosphorylation of Thr²¹⁹ and Tyr²²¹ alone is insufficient to decrease the electrophoretic mobility of ERK5, as shown by experiments with ERK5kd (Figure 1B). The isolated C-terminus of ERK5 has been reported to stimulate gene transcription in cell transfection experiments, raising the possibility that it is a transcription factor or a transcriptional co-activator [21]. This raises the possibility that one of the important cellular substrates of ERK5 is ERK5 itself and that the transcriptional activity of the C-terminus may be stimulated by autophosphorylation. Consistent with this notion, a catalytically inactive mutant of ERK5 was reported to be transcriptionally inactive [21].

Although the GST-MKK5[ΔN11] that we expressed in HEK 293 cells is active, the activity is very low. Equimolar molar concentrations of MKK5 were needed to fully activate ERK5 (Figure 1). Moreover, the expressed GST-MKK5[ΔN11] did not become activated when the cells were exposed to EGF (results not shown). Thus the activity of the expressed GST-MKK5[ΔN11] may represent the inherent basal activity of the unphosphorylated enzyme. We have reported previously [22] that MKK5 becomes phosphorylated at Ser³¹¹ and Thr³¹⁵ in response to EGF, just prior to the time at which ERK5 is activated, as judged by experiments with a phospho-specific antibody raised against the amino acid sequence surrounding these sites. Ser³¹¹ and Thr³¹⁵ are equivalent to Ser²¹⁷ and Ser²²¹ of MKK1, the residues whose phosphorylation is required for activation by the protein kinase Raf [23]. We therefore changed both of these residues to aspartic acid to try to generate a constitutively active enzyme, but found that the mutated GST-MKK5[ΔN11] activated ERK5 at a similar rate to the unmutated GST-MKK5[ΔN11]. Other investigators [9] have also reported that the weak MBP kinase activity of an MKK5 variant (truncated by 89 residues at the N-terminus) was not increased by the mutation of Ser³¹¹ and Thr³¹⁵ to aspartic acid. Therefore changing these amino acids to acidic residues either does not induce the active conformation or the activation of MKK5 requires the phosphorylation of an additional residue(s) that remains to be identified. Nevertheless, the amounts of active ERK5 that we can generate using GST-MKK5[ΔN11] expressed from 100 10-cm-diameter dishes of HEK 293 cells (see the Materials and methods section) is enough for 100 000 assays, sufficient to initiate high-throughput screening studies aimed at developing specific inhibitors of this enzyme.

During the present study, we found that MKK5 was phosphorylated extensively by ERK5 at residues 129, 137, 142 and 149. These residues lie in the region of MKK5 thought to interact with ERK5, because the analogous region in MKK1 is known to span the docking site for ERK1 and ERK2 [24]. The generation of phospho-specific antibodies that recognize these phosphorylation sites will be needed to investigate whether they are phosphorylated by ERK5 *in vivo*, but this finding raises the possibility that phosphorylation may affect the interaction of MKK5 with ERK5 and represent a mechanism for feedback control of the pathway.

Interestingly, none of the residues on MKK5 phosphorylated by ERK5, as well as three of the sites of autophosphorylation on ERK5 (Thr²⁸, Ser⁴⁹⁶ and Ser⁷³¹), are followed by proline residues, whereas nearly all other MAP kinase family members are highly specific for serine and threonine residues that precede a proline residue. These observations suggest that the specificity of ERK5 may differ markedly from other MAP kinase family members. Consistent with this possibility, ERK5 is inhibited

by submicromolar concentrations of the bis-indolymaleimide Ro 318220 (N. Mody and P. Cohen, unpublished work), a compound that interacts with the ATP-binding sites of protein kinases. In contrast, other MAP kinases are resistant to inhibition by Ro 318220 [25].

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