Synergistic activation of stress-activated protein kinase 1/c-Jun N-terminal kinase (SAPK1/JNK) isoforms by mitogen-activated protein kinase kinase 4 (MKK4) and MKK7

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Stress-activated protein kinase 1 (SAPK1), also called c-Jun N-terminal kinase (JNK), becomes activated *in vivo* in response to pro-inflammatory cytokines or cellular stresses. Its full activation requires the phosphorylation of a threonine and a tyrosine residue in a Thr-Pro-Tyr motif, which can be catalysed by the protein kinases mitogen-activated protein kinase kinase (MKK)4 and MKK7. Here we report that MKK4 shows a striking preference for the tyrosine residue (Tyr-185), and MKK7 a striking preference for the threonine residue (Thr-183) in three SAPK1/JNK1 isoforms tested (JNK1 α 1, JNK2 α 2 and JNK3 α 1). For this reason, MKK4 and MKK7 together produce a synergistic increase in the activity of each SAPK1/JNK isoform *in vitro*. The MKK7 β variant, which is several hundred-fold more efficient in activating all three SAPK1/JNK isoforms than is MKK7 α , is equally specific for Thr-183. MKK7 also phosphorylates JNK2 α 2 at Thr-404 and Ser-407 *in vitro*, Ser-407 being phosphorylated much more rapidly than Thr-183 *in vitro*. Thr-404/Ser-407 are phosphorylated in unstimulated human KB cells

and HEK-293 cells, and phosphorylation is increased in response to an osmotic stress (0.5 M sorbitol). However, in contrast with Thr-183 and Tyr-185, the phosphorylation of Thr-404 and Ser-407 is not increased in response to other agonists that activate MKK7 and SAPK1/JNK, suggesting that phosphorylation of these residues is catalysed by another protein kinase, such as CK2, which also phosphorylates Thr-404 and Ser-407 *in vitro*. MKK3, MKK4 and MKK6 all show a strong preference for phosphorylation of the tyrosine residue of the Thr-Gly-Tyr motifs in their known substrates SAPK2a/p38, SAPK3/p38 γ and SAPK4/p38 δ . MKK7 also phosphorylates SAPK2a/p38 at a low rate (but not SAPK3/p38 γ or SAPK4/p38 δ), and phosphorylation occurs exclusively at the tyrosine residue, demonstrating that MKK7 is intrinsically a 'dual-specific' protein kinase.

Key words: cellular stress, MAP kinase, proinflammatory cytokine; protein phosphorylation.

INTRODUCTION

Mitogen-activated protein kinase (MAPK) family members mediate many of the cellular effects of growth factors, cytokines and stress stimuli. Their activation requires the phosphorylation of a threonine and a tyrosine residue located in a Thr-Xaa-Tyr motif (where 'Xaa' can be Glu, Gly or Pro) in the 'activation loop' between subdomains VII and VIII of the kinase catalytic domain. The phosphorylation of the Thr-Xaa-Tyr motif is catalysed by MAPK kinases (MKKs) (reviewed in [1]). Originally, it was thought that each MKK catalysed the phosphorylation of both the threonine and the tyrosine residue of the Thr-Xaa-Tyr motif of its target MAPK. However, we recently reported that the MAPK family member known as stress-activated protein kinase-1 (SAPK1) or c-Jun N-terminal kinase (JNK) is activated synergistically *in vitro* by MKK4 [also called SAPK or extracellular-signal-regulated kinase (ERK) kinase (SEK), JNK kinase (JNKK)1 and SAPK kinase (SKK1)] and MKK7 (also called JNKK2 and SKK4) [2]. The basis for this unexpected phenomenon was revealed by the finding that MKK4 and MKK7 show marked preferences for the tyrosine and the threonine residue, respectively, of the Thr-Pro-Tyr motif in the SAPK1/JNK1

isoform [2]. Our results indicated that SAPK1/JNK phosphorylated at both Thr-183 and Tyr-185 was 5–10-fold more active than SAPK1/JNK phosphorylated at Thr-183 only, whereas SAPK1/JNK phosphorylated at Tyr-185 alone was inactive [2]. These observations raised two intriguing possibilities. First, the full activation of SAPK1 might require the concerted action of two different MKKs, providing a potential mechanism for synergizing the effects of different extracellular signals. Secondly, other MAPK family members might be activated by two or more MKKs, and some MKKs might have gone undetected if they phosphorylate only the tyrosine or the threonine residue, and therefore do not induce any activation until the other residue is phosphorylated by another MKK.

Our previous investigation of the regulation of SAPK1/JNK was performed using the JNK1 isoform that had been expressed in *Escherichia coli* [2]. This raises the possibility that the observed effects might be artefactual, resulting from incorrect folding of JNK1 in a prokaryotic expression system. It is also possible that the observed effects are unique to the JNK1 isoform. In the present study, we therefore extend our work to include all three isoforms, JNK1, JNK2 and JNK3, expressed from baculovirus vectors in insect cells.

Abbreviations used: ATF2, activating transcription factor 2; CK2, (formerly known as) casein kinase II; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; IL-1, interleukin-1; JNK, c-Jun N-terminal kinase; JNKK, JNK kinase; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MEKK, MAPK or ERK kinase kinase; SAPK, stress-activated protein kinase; SKK, SAPK kinase; SEK, SAPK or ERK kinase; TNF, tumour necrosis factor.

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While our studies were in progress it was reported that two splice variants of MKK7 (MKK7 β and MKK7 γ) that possess long N-terminal extensions interact more strongly with JNK, and are more effective activators of JNK than the shorter MKK7 α variant [3]. MKK7 α is itself five residues longer than the form of MKK7 that was cloned in our laboratory [4], which is therefore termed MKK7 α' in this paper. These findings raised the possibility that the more active MKK7 variants might be more effective JNK activators by virtue of their ability to phosphorylate the tyrosine, as well as the threonine, residue of Thr-Pro-Tyr motif. We therefore extended the study to include the most active MKK7 β variant. We also examined the site-specific phosphorylation of three other MAPK family members, namely SAPK2a/p38, SAPK3 (also called ERK6 and p38 γ) and SAPK4 (also called p38 δ) by MKK3, MKK4, MKK6 and MKK7 in order to see whether other MAPK family members can be activated synergistically by two different MKK family members.

EXPERIMENTAL

Materials

Glutathione S-transferase (GST)–MKK4, GST–MKK7 α' , GST–activating transcription factor 2 (ATF2) (19–96) and His₆–MAPK or ERK kinase kinase (MEKK)1 were expressed in *E. coli* and purified as described previously [4]. GST–MKK4, GST–MKK7 α' and GST–MKK7 β were activated by treatment with His₆–MEKK1 [4]. Constitutively active mutants of GST–MKK3 (made by Dr A. Cuenda in this Unit) and GST–MKK6 [5] were expressed in *E. coli* and purified by affinity chromatography on glutathione–Sepharose. In these mutants, the two serine/threonine residues equivalent to those in MKK1 that are phosphorylated by c-Raf (Ser-217 and Ser-221 [6]) were both mutated to aspartate. GST–SAPK2a, GST–SAPK3 and GST–SAPK4 were expressed and purified as described previously [7–9]. The CK2 (protein kinase formerly known as casein kinase II) holoenzyme was expressed in Sf9 cells and purified by successive chromatography on phosphocellulose and Mono Q.

Expression and purification of SAPK1/JNK

There are three genes encoding isoforms of SAPK1/JNK that can be alternatively spliced to generate a total of ten different variants [10]. cDNAs encoding the JNK1 α 1, JNK2 α 2 and JNK3 α 1 isoforms of SAPK1/JNK were amplified by PCR from the vectors pRSET/JNK1 α 1 [2], pRSET/JNK2 α 2 and pCal-n/JNK3 α 1 using the oligonucleotide pairings listed below: 5'-AGGATCCACCATGCACCATCACCATCACCATATGAGCAGAGCAAGCGTGAC-3' and 5'-GCGGTCGACTCAGTGCTGCACCTGTGCTAAAG-3' (for JNK1 α 1); 5'-AGGATCCACCATGCACCATCACCATCACCATATGAGCGACAGTAAATGTGACAG-3' and 5'-CTCGAGTCATCGACAGCCTTCAAGGG-3' (for JNK2 α 2); and 5'-AGGATCCACCATGCACCATCACCATCACCATATGAGCAAAAGCAAA-GTTGACAACC-3' and 5'-CTCGAGTCACTGCTGCACCTGTGCTGAAG-3' (JNK3 α 1). The JNK1 α 1 and JNK2 α 2 clones were full length, whereas the JNK3 α 1 vector (generously provided by Miss Linda Trotter and Dr Ross Kinloch, Pfizer Inc., Sandwich, U.K.) encoded a protein that lacked 38 residues at the N-terminus and was preceded by a calmodulin tag. This 38-residue truncation was found to be critical to obtain a reasonable level of expression of JNK3 α 1. The PCR products were cloned into either the *Bam*HI/*Sal*I sites of the pFASTBAC1 vector

expressing JNK1 α 1, or the *Bam*HI/*Xho*I sites of the same vector expressing JNK2 α 2 and JNK3 α 1(Δ 1–38). These vectors were then used to generate recombinant baculovirus using the Bac-to-Bac system (Life Technologies, Paisley, Scotland, U.K.). The resulting viruses, encoding the SAPK1 isoforms with an N-terminal hexahistidine sequence, were used to infect Sf21 insect cells (1.5×10^6 /ml) at a multiplicity of infection of 5. The infected cells were harvested 72 h post-infection, and the histidine-tagged SAPK1 proteins were purified by Ni²⁺-nitrilotriacetate agarose chromatography [11]. The purified proteins were then dialysed against 50 mM Tris/HCl (pH 7.5), 50 % (v/v) glycerol, 150 mM NaCl, 0.1 mM EGTA, 0.1 % (v/v) 2-mercaptoethanol, 0.03 % (w/v) Brij-35, 1 mM benzamidinium and 0.2 mM PMSF, and stored at -20°C . JNK1 α 1, JNK2 α 2 and JNK3 α 1 were recovered with yields of 19, 16 and 13 mg/l of infected Sf21 cells respectively and were > 90 % homogeneous, as judged from SDS/PAGE followed by staining with Coomassie Brilliant Blue.

Antibodies

A phosphopeptide corresponding to residues 399–412 of human JNK2 α 2 and phosphorylated at Thr-404 and Ser-407 (MSTEQT*LAS*DTDSS, where the phosphorylated residues are marked with asterisks) was coupled separately with BSA and keyhole-limpet haemocyanin, mixed together and injected into a sheep at the Scottish Antibody Production Unit (Carlisle, U.K.). The antisera were affinity-purified on phosphopeptide–agarose, followed by chromatography on a dephosphopeptide–agarose column to remove and purify antibodies that recognize dephosphorylated and phosphorylated JNK2 α 2 equally well. Polyclonal anti-(SAPK1/JNK) antibodies were also raised in sheep against the JNK1 α 1 and JNK2 α 2 proteins and affinity-purified by chromatography on JNK1 α 1–agarose and JNK2 α 2–agarose respectively. Aliquots of these antibodies were mixed together before immunoblotting cell extracts. Further phospho-specific antibodies that recognize all JNK isoforms after they are phosphorylated at Thr-183 and/or Tyr-185 were purchased from New England Biolabs (Beverly, MA, U.S.A.), and were used according to the manufacturer's instructions. All antibodies were used at a concentration of 1 $\mu\text{g}/\text{ml}$. Affinity-purified antisera raised against rabbit and sheep IgG were purchased from Pierce (Rockford, IL, U.S.A.) and used at dilutions of 1:2000. Immunoreactive proteins were detected by the enhanced chemiluminescence (ECL[®]) technique (Amersham Pharmacia Biotech, Amersham, U.K.).

Cloning of MKK7 β

A search of the National Center for Biotechnology Information expressed sequence tag database identified a human cDNA clone (accession no. AA194047) corresponding to the N-terminal 269 residues of human MKK7 β . Sequencing of this clone revealed that it overlapped with our human MKK7 α' sequence [4]. An overlapping *Not*I restriction site was therefore used to splice these two clones together, generating the full-length MKK7 β sequence. MKK7 β was subcloned into pGEX-4T3 for expression in *E. coli* as a GST-fusion protein [4].

Other methods

All the His-tagged SAPK1/JNK isoforms purified from Sf9 cells were completely inactive. The phosphorylation and activation of SAPK1/JNK isoforms by MKK4 and MKK7, tryptic digestion of phosphoproteins, C₁₈ chromatography, phospho amino acid

Figure 1 MKK4 and MKK7α' activate JNK1α1 synergistically

JNK1α1 (2 μM) was incubated for 20 min with MgATP²⁻ and 0.01 μM MKK4, 0.08 μM MKK7α' or 0.01 μM MKK4 plus 0.08 μM MKK7α'. JNK1α1 activity was assayed as described in [2]. JNK activity was undetectable before phosphorylation by MKK4 or MKK7. The results are shown ± S.E.M for three separate experiments, each performed in triplicate.

analysis and amino acid sequencing was performed as described previously [2]. SAPK1/JNK isoforms were assayed using GST-ATF2(19–96) as substrate [2]. The phosphorylation and activation of SAPK2a/p38, SAPK3/p38γ and SAPK4/p38δ by MKK3, MKK4, MKK6 and MKK7 was performed in an assay identical with that used to study the activation of SAPK1/JNK. SAPK2a/p38, SAPK3/p38γ and SAPK4/p38δ activities were measured as for SAPK1/JNK, except that myelin basic protein (0.33 mg/ml) was used as substrate instead of ATF2. All protein kinase assays were performed in the presence of 10 mM magnesium acetate and 0.1 mM ATP. One unit of SAPK activity was that amount which catalysed the phosphorylation of 1 nmol of myelin basic protein or ATF2 in 1 min. CK2 was assayed using the peptide RRRDDSDDDD (165 μM) as substrate, and one unit of activity was that amount which catalysed the phosphorylation of 1 nmol of substrate in 1 min. DNA sequencing was carried out on an Applied Biosystems 373A automatic DNA sequencer using the *Taq* dye-terminator cycle-sequencing kit.

Figure 2 Tryptic phosphopeptide mapping of JNK1α1

JNK1α1 was phosphorylated with [γ -³²P]ATP and MKK4 or MKK7α', as in Figure 1. Following SDS/PAGE, the bands corresponding to JNK1α1 were excised, digested with trypsin and chromatographed on a Vydac C₁₈ column equilibrated in 0.1% (v/v) trifluoroacetic acid and developed with a linear water/acetonitrile gradient (diagonal line) [17]. MKK4 was found to phosphorylate a single major peptide eluting in fraction 149 (A), whereas the major peptide phosphorylated by MKK7α' eluted slightly later in fraction 152 (B). Fractions 149 (C) and 152 (D) were subjected to solid-phase sequencing to identify the sites of phosphorylation. Matrix-assisted laser-desorption/ionization–time-of-flight (MALDI–TOF) MS showed that both peptides had an identical mass of 1773.8, corresponding to that predicted for the tryptic peptide comprising residues 175–189 of JNK1α1 plus one phosphate group. Peptide sequences are shown using the single-letter code for amino acids.

RESULTS

Phosphorylation and activation of SAPK1/JNK isoforms expressed in insect cells by MKK4 and MKK7 α'

As reported previously for JNK1 α 1 expressed in *E. coli* [2], the combination of MKK4 and MKK7 α' produced a synergistic activation of Sf21-cell-expressed JNK1 α 1 (Figure 1). Low levels of MKK4 or MKK7 α' produced only a slight activation of JNK1 α 1, but the effect was greatly amplified when both activators were added together. Phospho amino acid analysis confirmed that, under these conditions, MKK4 was highly specific for the phosphorylation of tyrosine, while MKK7 α' was highly specific for threonine. Tyrosine and threonine were both phosphorylated when MKK4 and MKK7 α' were added together (results not shown). The phosphorylated JNK1 α 1 was resolved from MKK7 α' by SDS/PAGE. The gel slice was excised, digested with trypsin, and the resulting phosphopeptides were chromatographed on a C₁₈ column (see the Experimental section). These experiments showed that MKK4 phosphorylated one major peptide that was eluted in fraction 149 (Figure 2A), whereas MKK7 α' phosphorylated one major peptide that was eluted in fraction 152 (Figure 2B). Amino acid sequencing confirmed that both peptides corresponded to residues 175–190, the former

being phosphorylated at Tyr-185 (Figure 2C) and the latter at Thr-183 (Figure 2D).

The same experiments were performed using JNK2 α 2 and JNK3 α 1(Δ 1–38) instead of JNK1 α 1. MKK4 and MKK7 α' also activated JNK2 α 2 (Figure 3A) and JNK3 α 1(Δ 1–38) (Figure 3B) synergistically, although the synergism was not quite as striking as for JNK1 α 1. MKK4 was again highly specific for tyrosine, whereas MKK7 α' phosphorylated JNK3 α 1(Δ 1–38) on threonine specifically, and JNK2 α 2 on threonine and serine residues (Figures 3C and 3D). Interestingly, the phosphoserine residue(s) in JNK2 α 2 were phosphorylated much more rapidly than the threonine residue(s) (Figure 3C). JNK3 α 1(Δ 1–38) was subjected to tryptic digestion, and the resulting phosphopeptides were chromatographed on a C₁₈ column and sequenced. The results (Figure 4) were identical with those of JNK1 α 1, consistent with JNK3 α 1(Δ 1–38) having an amino acid sequence identical with that of JNK1 α 1 in the immediate vicinity of the two phosphorylation sites.

JNK2 α 2 phosphorylated by MKK4 was digested with trypsin and chromatographed on the C₁₈ column. One major phosphopeptide was eluted at fraction 147 (Figure 5A), which was shown to correspond to the expected 'activation loop' peptide containing the Thr-Pro-Tyr motif phosphorylated specifically on the

Figure 3 Synergistic activation of JNK2 α 2 and JNK3 α 1 by MKK4 and MKK7 α'

(A) JNK2 α 2 (2 μ M) was incubated for 20 min with MgATP²⁻ and 0.01 μ M MKK4, 0.08 μ M MKK7 α' or 0.01 μ M MKK4 plus 0.08 μ M MKK7 α' . The results shown represent two separate experiments (each performed in triplicate) $\pm$ S.E.M. (B) Same as in (A), except that JNK3 α 1(Δ N1–38) replaced JNK2 α 2 and the concentration of MKK4 was increased to 0.2 μ M, and the concentration of MKK7 α' was reduced to 0.01 μ M. (C) As in (A), except that [γ -³²P]ATP replaced unlabelled ATP and, after SDS/PAGE and transfer to nitrocellulose membranes, the JNK2 α 2 band was excised, subjected to partial acid hydrolysis and electrophoresed on thin-layer cellulose to separate the different phospho amino acids [2]. Equal amounts of ³²P radioactivity were applied to each lane before electrophoresis on the thin-layer cellulose. This is the reason why amounts of each phospho amino acid are lower in the samples phosphorylated by MKK4 and MKK7. (D) As in (C), except that JNK3 α 1(Δ N1–38) was activated as in (B). JNK2 α 2 and JNK3 α 1 were completely inactive before phosphorylation with MKK4 and/or MKK7. pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine.

Figure 4 Tryptic phosphopeptide mapping of JNK3 α 1

JNK3 α 1(Δ N1–38) was phosphorylated with [γ -³²P]ATP and MKK4 or MKK7 α ' as in Figure 3(B). Following SDS/PAGE, the bands corresponding to JNK3 α 1(Δ N1–38) were excised, digested with trypsin and chromatographed on a Vydac C₁₈ column, as in Figure 2. As for JNK1 α 1 (Figure 2), a single major peptide was found to be phosphorylated by MKK4, which was eluted in fraction 149 (A), whereas the major peptide phosphorylated by MKK7 α ' was eluted later in fraction 152 (B). Fractions 149 (C) and 152 (D) were subjected to solid-phase sequencing to identify the sites of phosphorylation. MALDI-TOF MS showed that both peptides had an identical mass of 1773.79, corresponding to that predicted for the tryptic peptide comprising residues 175–189 of JNK1 α 1 plus one phosphate group with both methionines oxidized to methionine sulphone. The amino acid sequence of the peptide is shown using the single-letter code for amino acids.

tyrosine residue (results not shown). JNK2 α 2 phosphorylated by MKK7 α ' was also digested with trypsin and chromatographed on the C₁₈ column. Two major phosphopeptides were obtained that were eluted in fractions 150 and 152 (Figure 5B). The phosphopeptide eluting in fraction 150 was the expected 'activation loop' peptide containing the Thr-Pro-Tyr motif phosphorylated specifically on the threonine residue (results not shown). The 'activation loop' peptides from JNK2 α 2 differ at two positions from the sequences of the corresponding peptides from JNK1 α 1 and JNK3 α 1(Δ 1–38), explaining their slightly earlier elution from the C₁₈ column.

The peptide eluting in fraction 152 (Figure 5B) contained phosphoserine and some phosphothreonine. This fraction contained three peptides, two of which corresponded to the long C-terminal tryptic peptide (residues 374–424) and a slightly longer peptide (residues 369–424) that resulted from the failure of trypsin to cleave the Lys–Asp bond between residues 373 and 374. The third peptide corresponded to residues 141–150. Since no ³²P radioactivity was released even after 23 cycles of Edman degradation, this suggested that the phosphorylation sites were

contained within the long C-terminal tryptic peptide beyond residue 397. The C-terminal peptide contained a methionine at residue 399. JNK2 α 2 phosphorylated by MKK7 α ' was therefore cleaved with CNBr, and the resulting peptides were again chromatographed on the C₁₈ column. One [³²P]peptide that peaked at fraction 135 (results not shown) was phosphorylated on serine and threonine, the majority of the ³²P radioactivity being associated with phosphoserine (inset to Figure 5C). MS and Edman sequencing revealed that this peptide was a mixture of the dephosphorylated, monophosphorylated and diphosphorylated derivatives of the C-terminal CNBr peptide comprising residues 400–424. ³²P radioactivity was released after the fifth and eighth cycles of Edman degradation (Figure 5C), identifying Thr-404 and Ser-407 as the sites of phosphorylation.

Phosphorylation of SAPK1/JNK isoforms by MKK7 β and MKK7 α '

The experiments described above were performed with MKK7 α ', which is five amino acid residues shorter than MKK7 α at its N-terminus [4]. MKK7 β , which has a 73-residue extension at its

Figure 5 Identification of the sites on JNK2 α 2 phosphorylated by MKK4 and MKK7

JNK2 α 2 was phosphorylated with [γ - 32 P]ATP and MKK4 (**A**) or MKK7 α' (**B**, **C**) as in Figure 3(A). Following SDS/PAGE, the bands corresponding to JNK2 α 2 were excised, digested with trypsin (**A**, **B**) or cleaved with CNBr (**C**) and chromatographed on a Vydac C₁₈ column, as in Figure 2. (**C**) Fraction 135 from the CNBr digest was analysed by Edman degradation and solid-phase sequencing to identify the peptide sequence and sites of phosphorylation, and also subjected to phospho amino acid analysis (inset to Figure). The sequence of the peptide is shown using the single-letter code for amino acids.

N-terminus compared with MKK7 α , is reported to interact with and activate SAPK1/JNK more effectively than MKK7 α . We therefore compared the site-specific phosphorylation of MKK7 β

Figure 6 Relative rates of phosphorylation of JNK1 α 1 by MKK7 α' and MKK7 β

JNK1 α 1 (2 μ M) was incubated for the times indicated with MgATP $^{2-}$ and 200 nM MKK7 α' ($\circ$) or 2 nM MKK7 β ($\bullet$). JNK1 α 1 activity was then assayed using ATF2 as substrate. The results are shown $\pm$ S.E.M for three separate experiments, each performed in triplicate.

and MKK7 α' with all three SAPK1/JNK isoforms. MKK7 β activated JNK1 α 1 approximately 500-fold faster than did MKK7 α' (Figure 6), and similar results were obtained with the other SAPK1/JNK isoforms (results not shown). Phospho amino acid analysis revealed that, like MKK7 α' , MKK7 β was highly specific for threonine, although traces of phosphotyrosine could also be detected (Figure 7). Like MKK7 α' , MKK7 β phosphorylated JNK2 α 2 on serine, as well as threonine, residue(s) (Figure 7). Tryptic digestion and chromatography on the C₁₈ column confirmed that the phosphoserine residue was located in the C-terminal tryptic peptide comprising residues 374–424, as expected.

JNK2 α 2 is phosphorylated at Thr-404 and Ser-407 in human epithelial KB cells and human HEK-293 cells

A phosphospecific antibody was raised in sheep that recognized JNK2 α 2 phosphorylated at Thr-404 and Ser-407. The specificity of this antibody was established by the finding that it did not recognize dephosphorylated JNK2 α 2, JNK2 α 2 phosphorylated by MKK4, or JNK1 α 1 phosphorylated by either MKK4 or MKK7 (Figure 8A). Moreover, recognition by the antibody was prevented if it was preincubated with the phosphopeptide immunogen (Figure 8A), but not if it was mixed with the corresponding dephosphopeptide (results not shown).

In order to examine whether Thr-404 and Ser-407 were phosphorylated in cells, lysates from KB cells (Figure 8B) and HEK-293 cells (Figure 8C) were electrophoresed on SDS/polyacrylamide gels, and immunoblotted with phospho-specific antibodies. These experiments revealed that Thr-404/Ser-407 were phosphorylated, even in unstimulated cells. Phosphorylation was not increased by stimulation with anisomycin or interleukin (IL)-1 α , or by exposure to UV radiation, these agonists actually causing a small decrease in the basal level of phosphorylation, especially in KB cells. In contrast, the phosphorylation of Thr-404/Ser-407 increased in both cells in response to an osmotic shock (0.5 M sorbitol) (uppermost panels in Figures 8B and 8C). Increased phosphorylation of Thr-404/Ser-407 was detectable after 5 min, and maximal after 30–60 min (results not shown). All the stimuli increased the

Figure 7 Phospho amino acid analysis of SAPK1/JNK isoforms after phosphorylation by MKK7 α' and MKK7 β

JNK1 α 1, JNK2 α 2 or JNK3 α 1(Δ N1–38) (each at 2 μ M) were incubated for 60 min with 0.8 μ M MKK7 α' or MKK7 β in the presence of Mg[γ - 32 P]ATP. Following SDS/PAGE and transfer to nitrocellulose membranes, the bands corresponding to each SAPK1/JNK isoform were excised and subjected to phospho amino acid analysis. pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine.

phosphorylation of the Thr-Pro-Tyr motif, as judged from immunoblotting with a different phospho-specific antibody that recognizes the 'activation loop' phosphorylation sites specifically (middle panels in Figures 8B and 8C). This antibody, and a further antibody raised against the JNK1 α 1 and JNK2 α 2 proteins (bottom panels in Figures 8B and 8C), detect all the SAPK1/JNK variants.

As observed previously in many other cell types, the major forms of SAPK1/JNK migrate with apparent molecular masses of 46 kDa and 54 kDa (Figure 8B, bottom two panels). The antibody that recognizes JNK2 α 2 phosphorylated at Thr-404/Ser-407 consistently detected a single 55 kDa band that migrates just above the major 54 kDa band of SAPK1/JNK.

Phosphorylation of Thr-404/Ser-407 by CK2

The finding that Thr-404/Ser-407 was already phosphorylated in unstimulated cells, and that phosphorylation did not increase (but actually decreased) in response to several agonists [anisomycin, UV light, IL-1 α and tumour necrosis factor (TNF) α] that activate MKK7, suggested that these residues might be phosphorylated *in vivo* by another protein kinase. We therefore examined whether CK2 was able to phosphorylate Thr-404/Ser-407, because this protein kinase phosphorylates serine and threonine residues that lie three residues N-terminal to an acidic amino acid. Ser-407 lies three residues N-terminal to Asp-410, while the phosphorylation of Ser-407 would permit the CK2-catalysed phosphorylation of Thr-404. As shown in Figure 8(D), CK2 was indeed able to phosphorylate Thr-404/Ser-407 *in vitro*. This was confirmed by phosphopeptide mapping and phospho amino acid analysis (results not shown).

Site-specific phosphorylation of other SAPKs by MKK family members

The site-specific phosphorylation of SAPK1/JNK isoforms by MKK4 and MKK7 raised the question of whether other MAPK family members are phosphorylated at distinct sites by different MKKs. We therefore examined the phosphorylation of SAPK2 α /p38, SAPK3/p38 γ and SAPK4/p38 δ by MKK3, MKK4 and MKK6, which are all known to be capable of activating these enzymes *in vitro*. Phosphorylation experiments were performed at low levels of each MKK, alone or in

combination, under conditions where each kinase individually phosphorylated each MAPK family member to only about 0.1 mol/mol protein. These experiments failed to reveal any synergistic activation, similar to observations with MKK4 and MKK7 acting on SAPK1/JNK, the effects being purely additive (Figure 9A). The reason for lack of synergism was revealed by phospho amino acid analysis, which showed that all three MKKs phosphorylated SAPK2 α /p38, SAPK3/p38 γ and SAPK4/p38 δ preferentially on a tyrosine residue(s) (Figure 9B). Interestingly, MKK7 β also phosphorylated SAPK2 α /p38, although at a rate that was more than 100-fold lower than MKK3, MKK4 and MKK7. Similar to the phosphorylation of SAPK1/JNK isoforms, MKK7 β was much more effective than MKK7 α' in phosphorylating SAPK2 α /p38 at Tyr-182 (results not shown). The weak effect of MKK7 β on SAPK2 α activity was not synergistic with MKK3, MKK4 or MKK6 (Figure 9A) because, in contrast with the phosphorylation of SAPK1/JNK at Thr-183, MKK7 β phosphorylated SAPK2 α /p38 almost exclusively on a tyrosine residue (Figure 9B). Tryptic digestion, followed by chromatography of the resulting phosphopeptides on a C₁₈ column, demonstrated that MKK7 β phosphorylated the same peptide on SAPK2 α /p38 as MKK6 (results not shown). MKK7 did not phosphorylate SAPK3/p38 γ and SAPK4/p38 δ at detectable rates.

DISCUSSION

The results presented in this paper confirm and considerably extend our earlier study in which the synergistic activation of SAPK1/JNK by MKK4 and MKK7 was first noted [2]. In particular, the present work establishes that the original result is not an artefact resulting from the incorrect folding of SAPK1/JNK1 in *E. coli*, and demonstrates that it is a phenomenon that extends to all three isoforms. Importantly, even the MKK7 β splice variant, which is 500-fold more efficient than the MKK7 α' variant used earlier in activating each SAPK1/JNK isoform (Figure 6), still displays a remarkable selectivity for the threonine residue of the Thr-Pro-Tyr motif (Figure 7).

JNK3 α 1(Δ N1–38) was an extremely poor substrate for MKK4 compared with JNK1 α 1 or JNK2 α 2, the rate of activation being much lower even at a 20-fold higher concentration of MKK4 (see the legend to Figure 3). In contrast, JNK3 α 1(Δ N1–38) was activated by MKK7 at a similar rate to the other SAPK1/JNK

Figure 8 The JNK2 α 2 variant is phosphorylated at Thr-404 and Ser-407 in human cells

(A) JNK1 α 1 and JNK2 α 2 were phosphorylated by MKK4, MKK7 α' or both activators (MKK4/MKK7 α') as in Figure 1, or left unphosphorylated. Each phosphorylation reaction (100 ng of JNK) was then spotted on to nitrocellulose, and blotted with an antibody raised against a synthetic peptide corresponding to residues 399–412 of JNK2 α 2 (top panel) and an antibody raised against the same peptide phosphorylated at Thr-404 and Ser-407 (middle and bottom panels). In the experiment shown in the bottom panel, the phospho-specific antibody was first preincubated with the phosphopeptide immunogen (20 μ g/ml). (B) Human epithelial KB cells were cultured as described in [16] and either left unstimulated or stimulated for 30 min with

isoforms. Similar results were reported by others [12] while this manuscript was in preparation. These investigators reported that MKK4 was unable to phosphorylate JNK3 α 1(Δ N1–38) significantly at the concentrations used in their experiments, but became able to phosphorylate Tyr-185 once MKK7 had phosphorylated Thr-183. It will be important to see whether the same results are obtained with full-length JNK3 α 1 in order to assess whether this difference is likely to be physiologically significant. The extremely poor expression of full-length JNK3 α 1 in insect cells has so far prevented us from performing this experiment.

Interestingly, JNK2 α 2 became highly phosphorylated at two further residues, Ser-407 and Thr-404, after incubation with MgATP $^{2-}$ and MKK7 *in vitro* (Figure 5). One possible explanation for this result was that, after phosphorylation at Thr-183 by MKK7, JNK2 α 2 then phosphorylated itself at Ser-407 and Thr-404. However, several lines of evidence would appear to exclude this possibility and strongly indicate that both residues are phosphorylated directly by MKK7. First, neither Ser-407 nor Thr-404 is followed by a proline, which is a prerequisite for the phosphorylation of other proteins by SAPK1/JNK. Secondly, the phosphorylation of Ser-407 occurred more rapidly than the phosphorylation of Thr-183 (Figure 3C), whereas the reverse situation would be expected if these residues were phosphorylated by JNK2 α 2 itself, after it had been activated. Thirdly, no phosphorylation of Ser-407 occurred if JNK2 α 2 was activated to a similar level with MKK4 (Figure 3C).

We found that Ser-407/Thr-404 were phosphorylated in unstimulated KB cells and HEK-293 cells, and that phosphorylation did not increase (and even decreased slightly) in response to several signals (anisomycin, UV light, IL-1 α , TNF α) that activate MKK7 and SAPK1/JNK (Figure 8). These observations suggested that these residues are phosphorylated *in vivo* by another protein kinase, and led us to demonstrate that both these residues are phosphorylated by CK2 *in vitro* (Figure 8D). The known requirements of CK2 for an acidic residue three amino acids C-terminal to the phosphorylation site suggests that Ser-407 would be phosphorylated initially, the phosphorylation of this residue then acting as the recognition site for the phosphorylation of Thr-404 [13]. However, the phosphorylation of Thr-404/Ser-407 was strongly stimulated by exposure to an osmotic shock (0.5 M sorbitol) (Figures 8B and 8C), which is not known to be an activator of CK2. If CK2 is not activated by osmotic stress, then Thr-404/Ser-407 might be phosphorylated *in vivo* by a protein kinase distinct from either MKK7 or CK2. Alternatively, osmotic shock may induce the inhibition of a Thr-404/Ser-407 phosphatase.

The role of Ser-407/Thr-404 phosphorylation remains to be determined. It seems to have little or no direct effect on activity because, upon incubation with MgATP $^{2-}$ and MKK7, the level of activation of JNK2 α 2 correlates with the phosphorylation of

anisomycin (10 μ g/ml) or sorbitol (0.5 M), or for 15 min with IL-1 α (20 ng/ml) or exposed to UV-C radiation (60 J/m 2 , and left for 30 min). The cells were lysed as described in [17], and 0.1 mg of lysate protein was electrophoresed on SDS/PAGE (10% gels), transferred to nitrocellulose and immunoblotted with antibodies raised against JNK2 α 2(399–412) phosphorylated at Thr-404 and Ser-407 (top panel), a phospho-specific antibody that recognizes all SAPK1/JNK isoforms phosphorylated at Thr-183 and Tyr-185 (middle panel), or antibodies that recognize phosphorylated and dephosphorylated JNK1 and JNK2 equally well (anti-JNK; bottom panel). The Figure shows the result of a single experiment in which two different dishes of cells were stimulated with each agonist. The experiment was performed twice with similar results. (C) HEK-293 cells were cultured as described [17], and then treated exactly as in (B), except that TNF α (100 ng/ml) replaced IL-1 α . (D) JNK2 α 2 was phosphorylated as in (A), except that CK2 was also used at 50 units/ml. Aliquots of the reaction were electrophoresed on SDS/PAGE (10% gel) and, after transfer to nitrocellulose, immunoblotted as in (B) and (C).

Thr-183, and not that of Ser-407/Thr-404. Moreover, JNK2 α 2 is not activated after phosphorylation by CK2. Furthermore, the mutation of Thr-404 and Ser-407 to alanine or aspartate did not affect the activation of JNK2 α 2 in response to anisomycin, TNF, sorbitol or UV radiation (Y. Fleming and P. Cohen, unpublished work). Nevertheless, the phosphorylation of these two residues could play other roles; for example, in regulating the stability of JNK2 α 2, its interaction with other proteins, the rate of dephosphorylation of Thr-183 or Tyr-185, or the translocation of JNK2 α 2 from one cellular compartment to another.

The synergistic activation of SAPK1/JNK isoforms by MKK4 and MKK7 raised the question of whether other MAPK family members might be regulated in a similar manner. However, experiments with SAPK2a/p38, SAPK3/p38 γ and SAPK4/p38 δ using their known activators MKK3, MKK4 and MKK6 failed to provide any evidence in support of this idea. These three SAPKs were phosphorylated preferentially on tyrosine by each MKK family member (Figure 9). Nevertheless, it remains possible that the threonine residues of the Thr-Gly-Tyr motifs of these SAPKs are phosphorylated by one or more protein kinases that have not yet been identified. Such enzymes may have escaped detection previously if the phosphorylation of the threonine residue alone does not induce any activation.

We and others have reported that stimulation of several mammalian cells with pro-inflammatory cytokines induces the activation of MKK7, but not that of MKK4 [4,14,15]. However, preliminary experiments have indicated that IL-1 induces the phosphorylation of JNK1 α 1 and JNK2 α 2 at Tyr-185, as well as at Thr-183, in KB cells (Y. Fleming and P. Cohen, unpublished work). This raises the question of how Tyr-185 becomes phosphorylated under these conditions. In an earlier paper [2], we reported that MKK7 was intrinsically a 'dual-specificity' protein kinase, because it not only phosphorylated bacterially expressed JNK1 α 1 at Thr-183, but also at a tyrosine residue located in the enterokinase-sensitive 'linker' region that connected the protein kinase to the six-histidine tag attached to its N-terminus. The tyrosine residue was phosphorylated at a similar rate to that of Thr-183. In the present study, we found that MKK7 phosphorylates SAPK2a/p38 exclusively at Tyr-182, albeit at a low rate. Therefore one possibility is that the interaction of MKK7 and/or SAPK1/JNK with another cellular protein alters the conformation of one of these enzymes in such a way as to facilitate phosphorylation of Tyr-185 by MKK7 *in vivo*. Alternatively, Tyr-185 may be phosphorylated by another IL-1-activated protein kinase that has yet to be identified. It will clearly be critical to investigate whether pro-inflammatory cytokines induce the phosphorylation of SAPK1/JNK at Tyr-185 in cells that lack MKK7.

Figure 9 Different MKKs do not activate SAPK2a/p38, SAPK3/p38 γ and SAPK4/p38 δ synergistically because they all phosphorylate tyrosine preferentially

(A) SAPK2a/p38, SAPK3/p38 γ and SAPK4/p38 δ (2 μ M) were phosphorylated in the presence of MgATP $^{2-}$ to about 0.1 mol of phosphate per mol of protein by incubation for 10 min with 0.4 μ M MKK3, for 3 min with 0.04 μ M MKK6 or 0.02 μ M MKK4, or for 30 min with 0.2 μ M MKK7 β , and assayed as described in [2]. SAPK2a, SAPK3/p38 γ and SAPK4/p38 δ activities were undetectable before phosphorylation by MKKs. The results are shown $\pm$ S.E.M. for two separate experiments, each performed in triplicate. (B) Same as (A), except that [γ - 32 P]ATP replaced unlabelled ATP. After phosphorylation, the samples were denatured in SDS and subjected to SDS/PAGE. After transfer to nitrocellulose membranes, the bands corresponding to each SAPK were excised and subjected to phospho amino acid analysis. SAPK3/p38 γ and SAPK4/p38 δ were not phosphorylated by MKK7, and this result is therefore omitted. Abbreviations are: pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine.

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