SKK4, a novel activator of stress-activated protein kinase-1 (SAPK1/JNK)

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Abstract A cDNA was cloned and expressed that encodes human stress-activated protein kinase kinase-4 (SKK4), a novel MAP kinase kinase family member whose mRNA is widely expressed in human tissues. SKK4 activated SAPK1/JNK in vitro, but not SAPK2a/p38, SAPK2b/p38β, SAPK3/ERK6 or SAPK4. It appears to be the mammalian homologue of HEP, an activator of SAPK1/JNK in *Drosophila*. In human epithelial KB cells SKK4 and SKK1/MKK4 (another activator of SAPK1/JNK) were both activated by stressful stimuli, but only SKK4 was activated by proinflammatory cytokines. The identification of SKK4 explains why the major SAPK1/JNK activator detected in many mammalian cell extracts is chromatographically separable from SKK1/MKK4.

Key words: SAPK; JNK; p38; MAP kinase; Cytokine; Stress

1. Introduction

Seven mitogen-activated protein kinase (MAPK) family members have been identified that are activated strongly by adverse stimuli (e.g. chemical, heat and osmotic shock, ultraviolet radiation and the protein synthesis inhibitor anisomycin) or by signals produced/released during infection (e.g. lipopolysaccharide and the proinflammatory cytokines interleukin-1 (IL-1) and tumour necrosis factor (TNF)), but only weakly (in most cell contexts) by polypeptide growth factors or phorbol esters (reviewed in [1]). For these reasons, they are termed stress-activated protein kinases (SAPKs).

The three isoforms of SAPK1/JNK bind tightly to the activation domain of c-Jun and are the only SAPKs that phosphorylate (at significant rates) the residues in this transcription factor that become phosphorylated in vivo in response to adverse stimuli and proinflammatory cytokines [2–4]. The two isoforms of SAPK2/p38 (SAPK2a/p38 and SAPK2b/p38β) are inhibited specifically by the pyridinyl imidazoles SB 203580 and SB 202190 at submicromolar concentrations [4–6] and their physiological substrates include the transcription factors Elk1 [7], SAP1 [7], CHOP [8] and MEF2C [9], as well as MAPK-activated protein kinase-2 (MAPKAP-K2) [10], MAPKAP-K3 [11,12], MAPK-interacting protein kinase-1 (Mnk1) and Mnk2 [13,14]. Intracellular targets for MAPKAP-K2/MAPKAP-K3 include heat shock protein 27 [5,15], tyrosine hydroxylase [16] and the transcription factor CREB [17], while one of the substrates for Mnk1/Mnk2 is eukaryotic initiation factor eIF4E [13]. SAPK3/ERK6 [18–20] and SAPK4 [4,6] have only been identified recently and their physiological substrates are unknown. SAPK3 and SAPK4 are 60% identical to each other or to SAPK2a and SAPK2b, but they are not inhibited by SB 203580 and SB 202190 [4,6,18].

The SAPKs are activated by MAPK kinase (MKK) family members, termed here SAPK kinases (SKKs). The major activator of SAPK2a/p38, SAPK2b/p38β, SAPK3 and SAPK4 that has been detected biochemically is SKK3/MKK6 [4,18,21–23], although SAPK2/p38 can also be activated by SKK2/MKK3 in vitro [24]. Neither SKK2/MKK3 nor SKK3/MKK6 are capable of activating SAPK1/JNK, which is activated by a distinct enzyme termed SKK1/MKK4 [24,25]. SKK1/MKK4 is the only activator of SAPK1/JNK that can be detected biochemically in extracts prepared from PC12 cells that have been exposed to adverse stimuli [21], and in 293 cells the activation of transfected SAPK1/JNK by anisomycin can be prevented by overexpressing a catalytically inactive form of SKK1/MKK4 [25]. In addition, heat shock or anisomycin failed to activate SAPK1/JNK in murine embryonic stem cells lacking SKK1/MKK4 [26,27].

SKK1/MKK4 can activate SAPK2/p38 in vitro [24,28] but, in contrast to SAPK1/JNK which is activated strongly by cotransfection with MEK kinase (MEKK) [29] (an upstream activator of SKK1/MKK4), SAPK2/p38 is not activated [24] or activated much more weakly [30]. Moreover the activation of SAPK2/p38 by osmotic shock and anisomycin is unimpaired in murine embryonic stem cells lacking SKK1/MKK4 [26,27]. For these reasons, SKK1/MKK4 does not seem to be rate-limiting for the activation of SAPK2/p38 in vivo.

Although SKK1/MKK4 is clearly essential for the activation of SAPK1/JNK in some cells, the dominant activator of SAPK1/JNK detected biochemically in extracts from human epithelial KB cells [21] or Rat 3Y1 fibroblasts [31] is a chromatographically distinct enzyme(s). Moreover, the activation of SAPK1/JNK by UV radiation and osmotic shock in murine embryonic stem cells lacking SKK1/MKK4 was either unimpaired [26] or only inhibited partially [27]. These observations indicate that mammalian cells contain at least one further activator of SAPK1/JNK distinct from SKK1/MKK4. In this paper we have cloned a novel MKK family member, termed here SKK4, that is activated by stressful stimuli and proinflammatory cytokines and which activates SAPK1/JNK, but not other SAPKs.

2. Materials and methods

2.1. Materials

An *E. coli* plasmid encoding a fusion protein comprising glutathione S-transferase (GST) linked to residues 19–96 of activating transcription factor-2 (GST-ATF2[19–96]) was provided by Dr. N. Jones (ICRF, London) and a plasmid encoding GST-SKK1/MKK4 by Dr. J. Woodgett (Ontario Cancer Institute, Toronto). A plasmid encoding

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the maltose-binding protein (MalE) linked to the *Xenopus* homologue of SAPK2a/p38 (MalE-Mpk2) was a gift from Dr. A.R. Nebreda (EMBL, Heidelberg, Germany), while a plasmid encoding MEKK preceded by six histidine residues (6-His-MEKK) was provided by Dr. G. Johnson (National Jewish Centre for Immunology and Respiratory Medicine, Denver, USA). All plasmids were transformed into *E. coli* strain BL21 (DE3), and expressed and purified as described [4,18,21]. 6-His SAPK1/JNK1γ [32] was expressed and purified as reported previously [33]. PKI, the specific peptide inhibitor of cAMP-dependent protein kinase (TTYADFIASGRTGRRNAIHD) was synthesised by Mr. F.B. Caudwell in the MRC Protein Phosphorylation Unit in Dundee and other peptides by Dr. Graham Bloomberg, University of Bristol, UK.

2.2. cDNA cloning and sequencing

Two oligonucleotides corresponding to part of the EST encoded by GenBank ID H85962, (forward, 5′-GACGGATCCGACCCCAC-CAAGCCGGACTTT; reverse, 5′-GGCCAAGCTTGTCTTTGAC-GAAGGACTGGAA) were used to amplify a 209-bp fragment from a λgt10 human skeletal muscle cDNA library (Clontech). The nucleotide sequence of this product was identical to that in the database. The PCR fragment was ^{32}P-labelled by random priming and used to probe the same cDNA library at high stringency. One strongly hybridising plaque was identified after screening 500 000 plaques. This clone was isolated, the phage DNA purified, and the insert cloned into pBluescript (Stratagene) and sequenced using an Applied Biosystems model 373A DNA sequencer.

2.3. Expression of GST-SKK4 in E. coli

The open reading frame of SKK4 was amplified by PCR and subcloned as a *Bam*HI/*Eco*RI fragment into the expression vector pGEX4T-3 (Pharmacia) followed by transformation into *E. coli* strain BL21 (DE3). Expression and purification of GST-SKK4 were carried out as described in [34]. The purified protein was dialysed against 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.03% (m/v) Brij35, 0.1% (v/v) 2-mercaptoethanol and 50% (v/v) glycerol, and stored unfrozen at −20°C at 2 mg/ml.

2.4. Preparation of anti-SKK4 and anti-SKK1/MKK4 antibodies

Peptides were synthesised corresponding to the C-terminal 12 residues of human SKK 4 (GVLSQPHLPFFR) and the last 14 residues of SKK1/MKK4 (DQMPATPSSPMYVD) [24], coupled to both bovine serum albumin and keyhole limpet haemocyanin using glutaraldehyde and injected into sheep at the Scottish Antibody Production Unit (Carluke, Ayrshire, UK). The anti-SKK4 and anti-SKK1/MKK4 antibodies were purified on peptide antigen-CH-Sepharose columns [22].

2.5. Cell culture and cell lysis

KB cells were cultured [18] and exposed for 15 min to osmotic stress (0.5 M sorbitol) or proinflammatory cytokines (20 ng/ml IL-1α or 100 ng/ml TNFα), or for 30 min to anisomycin (10 μg/ml), or for 10 min to 100 ng/ml epidermal growth factor (EGF) or 300 ng/ml phorbol myristate acetate (PMA). UV-C irradiation was carried out at 60 J/m^2 and the cells then incubated for a further 30 min at 37°C. Cells were lysed as described [21].

2.6. Immunoprecipitation of SKK4 and SKK1/MKK4

KB cell lysates (250 μg) were incubated for 60 min at 4°C with 10 μg of affinity purified antibody coupled to 5 μl protein G-Sepharose. The protein G-Sepharose immunoprecipitates were washed twice with 1 ml lysis buffer containing 0.5 M NaCl, twice with lysis buffer without NaCl and assayed as described below. In control experiments, antibodies bound to protein G-Sepharose were incubated for 30 min at 4°C with the peptide immunogen (1 mg/ml) prior to the addition of cell lysate.

2.7. Assay of SKK1/MKK4 and SKK4 immunoprecipitates

Immunoprecipitates (~6.5 μl) were incubated on a shaking platform for 30 min at 30°C with 1 μl of 20 μM 6-His-SAPK1/JNK in 50 mM Tris/HCl (pH 7.4), 0.1 mM EGTA, 0.03% (m/v) Brij-35, 0.1% (v/v) 2-mercaptoethanol and 5% (v/v) glycerol, and the reactions initiated with 2.5 μl of 40 mM magnesium acetate-0.4 mM unlabelled ATP. After 30 min, the active SAPK1/JNK generated was assayed by adding 40 μl of a solution containing 31.25 mM Tris/HCl (pH 7.4), 0.125 mM EGTA, 1.25 mM sodium orthovanadate, 3.1 μM PKI, 0.1% (by vol.) 2-mercaptoethanol and 0.25 mg/ml GST-ATF2[19–96], 12.5 mM magnesium acetate and 0.125 mM [γ-^{32}P]ATP. After 30 min at 30°C reactions were stopped by adding 5 μl of 6% (m/v) SDS, 400 mM Tris/HCl (pH 6.8), 50% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.2% (m/v) bromophenol blue. A 40 μl aliquot was withdrawn, electrophoresed on a 10% SDS/polyacrylamide gel and autoradiographed. The ^{32}P-labelled band corresponding to GST-ATF2[19–96] was excised and counted. One unit of SKK1/MKK4 or SKK4 activity was that amount which increased the activity of SAPK1/JNK by 1 U/min. One Unit of SAPK1/JNK was that amount which incorporated 1 nmol of phosphate into GST-ATF2[19–96] in one min.

SKK1/MKK4 and SKK4 were also assayed for their ability to activate *Xenopus* SAPK2a. The assay was identical except that SAPK2a (2 μM) replaced SAPK1/JNK.

2.8. Activation and assay of expressed GST-SKK4 and GST-SKK1/MKK4

Each GST fusion protein (4 μM) was activated by incubation for 60 min at 30°C with MEKK (1 μM) in 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 10 mM magnesium acetate and 0.1 mM unlabelled ATP. Activated GST-SKK4 and GST-SKK1/MKK4 were then measured by their ability to activate SAPK1/JNK or SAPK2a/p38, which were assayed by the phosphorylation of ATF2. The assays (50 μl) were carried out at 30°C and comprised 0.1 μM GST-SKK4 or GST-SKK1/MKK4, 0.2 μM 6-His SAPK1/JNK, 0.2 mg/ml GST-ATF2[19–96], 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 10 mM magnesium ace-

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TGCTCCTGCCCCGTCCCAACGAGCAGCCCTGCAGCTCCCGCTGGCCAACGATGGGGGCAG      60

CCGCCCGCCATCCTCAGAGAGCTCCCCGCAGCACCCCACGCCCCCCGCCCGGCCCCGCCA     120

                                                M  E  S  I  E  T  D    7
CATGCTGGGGCTCCCGTCAACCCTGTTCACACCCCGCAGCATGGAGAGCATTGAGATTGA     180

 Q  K  L  Q  E  I  M  K  Q  T  G  Y  L  T  I  G  G  Q  R  Y    27
CCAGAAGCTGCAGGAGATCATGAAGCAGACGGGCTACCTGACCATCGGGGGCCAGCGCTA     240

 Q  A  E  I  N  D  L  E  N  L  G  E  M  G  S  G  T  C  G  Q    47
CCAGGCAGAAATCAACGACCTGGAGAACTTGGGCGAGATGGGCAGCGGCACCTGCGGCCA     300

 V  W  K  M  R  F  R  K  T  G  H  V  I  A  V  K  Q  M  R  R    67
GGTGTGGAAGATGCGCTTCCGGAAGACCGGCCACGTCATTGCCGTTAAGCAAATGCGCCG     360

 S  G  N  K  E  E  N  K  R  I  L  M  D  L  D  V  V  L  K  S    87
CTCCGGGAACAAGGAGGAGAACAAGCGCATCCTCATGGACCTGGATGTGGTGCTGAAGAG     420

 H  D  C  P  Y  I  V  Q  C  F  G  T  F  I  T  N  T  D  V  F   107
CCACGACTGCCCCTACATCGTGCAGTGCTTTGGGACGTTCATCACCAACACGGACGTCTT     480

 I  A  M  E  L  M  G  T  C  A  E  K  L  K  K  R  M  Q  G  P   127
CATCGCCATGGAGCTCATGGGCACCTGCGCTGAGAAGCTCAAGAAGCGGATGCAGGCCCC     540

 I  P  E  R  I  L  G  K  M  T  V  A  I  V  K  A  L  Y  Y  L   147
CATCCCCGAGCGCATTCTGGGCAAGATGACAGTGGCGATTGTGAAGGCGCTGTACTACCT     600

 K  E  K  H  G  V  I  H  R  D  V  K  P  S  N  I  L  L  D  E   167
GAAGGAGAAGCACGGTGTCATCCACCGCGACGTCAAGCCCTCCAACATCCTGCTGGACGA     660

 R  G  Q  I  K  L  C  D  F  G  I  S  G  R  L  V  D  S  K  A   187
GCGGGGCCAGATCAAGCTCTGCGACTTCGGCATCAGCGGCCGCCTGGTGGACTCCAAAGC     720

 K  T  R  S  A  G  C  A  A  Y  M  A  P  E  R  I  D  P  P  D   207
CAAGACGCGGAGCGCCGGCTGTGCCGCCTACATGGCACCCGAGCGCATTGACCCCCAGA     780

 P  T  K  P  D  Y  D  I  R  A  D  V  W  S  L  G  I  S  L  V   227
CCCCACCAAGCCGGACTATGACATCCGGGCCGACGTATGGAGCCTGGGCATCTCGTTGGT     840

 E  L  A  T  G  Q  F  P  Y  K  N  C  K  T  D  F  E  V  L  T   247
GGAGCTGGCAACAGGACAGTTTCCCTACAAGAACTGCAAGACGGACTTTGAGGTCCTCAC     900

 K  V  L  Q  E  E  P  P  L  L  P  G  H  M  G  F  S  G  D  F   267
CAAAGTCCTACAGGAAGAGCCCCCGCTTCTGCCCGGACACATGGGCTTCTCGGGGGACTT     960

 Q  S  F  V  K  D  C  L  T  K  D  H  R  K  R  P  K  Y  N  K   287
CCAGTCCTTCGTCAAAGACTGCCTTACTAAAGATCACAGGAAGAGACCAAAGTATAATAA    1020

 L  L  E  H  S  F  I  K  R  Y  E  T  L  E  V  D  V  A  S  W   307
GCTACTTGAACACAGCTTCATCAAGCGCTACGAGACGCTGGAGGTGGACGTGGCGTCCTG    1080

 F  K  D  V  M  A  K  T  E  S  P  R  T  S  G  V  L  S  Q  P   327
GTTCAAGGATGTCATGGCGAAGACTGAGTCACCGCGGACTAGCGGCGTCCTGAGCCAGCC    1140

 H  L  P  F  F  R  *                                         333
CCACCTGCCCTTCTTCAGGTAGCTGCTTGGCGGCGGCCAGCCCCACAGGGGGCCAGGGGC    1200

CGG                                                           1203
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Fig. 1. Nucleotide and predicted amino acid sequence of human SKK4. Nucleotides are numbered in the 5′ to 3′ direction and amino acids are shown in single-letter code above the nucleotide sequence. The in-frame termination codon is marked with an asterisk.

tate and 0.1 mM [γ^{32}P]ATP. The reactions were terminated by spotting 40 μl on to 2×2 cm squares of phosphocellulose p81 paper followed by immersion into 75 mM phosphoric acid. After washing the papers several times in phosphoric acid, followed by immersion in acetone, the papers were dried and counted.

3. Results

3.1. Molecular cloning of SKK4

To identify novel members of the SKK family, we used the DNA sequence encoding SKK1/MKK4 to interrogate a number of EST databases. This search identified a 250-bp sequence (GenBank ID H85962) that was distinct from any previously identified SKK homologue, but was much more closely related to members of this family than to any other protein kinase. A suitable PCR probe (see the Methods in Section 2) was therefore used to screen a human skeletal muscle cDNA library. This resulted in the isolation of a 1.2-kbp clone that encoded a novel MKK family member, hereafter termed SKK4. The nucleotide and deduced amino acid sequence of human SKK4 is shown in Fig. 1. The open reading frame encodes a protein of 333 residues, with a predicted molecular mass of 37.9 kDa. It possesses all the conserved amino acid domains (I–XI) characteristic of protein kinases and shows 62% identity with the *Drosophila* MKK homologue HEP (see Section 4), 48% identity with human SKK1/MKK4, 39% identity with human SKK2/MKK3, and 41% identity with human SKK3/MKK6 (Fig. 2). The sequence identity with MKK1 and MKK2 is 36% and 35%, respectively. Ser[185] and Thr[189] in kinase subdomain VIII are in an equivalent position to the Ser/Thr residues in other MKK family members, whose phosphorylation is required to generate enzymatic activity.Hybridisation of ^{32}P-labelled SKK4 cDNA to multiple tissue Northern blots (Clontech) showed an RNA transcript of ~4 kbp that was present in heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (data not shown). The presence of ESTs in databases revealed that SKK4 is also expressed in retina and germinal centre B-cells. Thus SKK4 mRNA is widely expressed in mammalian cells.

3.2. SKK4 is activated by proinflammatory cytokines and stressful stimuli

An antibody was raised against the unique C-terminal sequence of SKK4 (Section 2.4) and used to immunoprecipitate SKK4 from KB cell extracts after stimulation with a variety of agonists (Fig. 3). These studies revealed that SKK4 was activated rapidly in KB cells in response to the same stimuli that trigger the activation of SAPK1/JNK [21], namely proinflammatory cytokines and stressful stimuli (UV radiation, osmotic shock and anisomycin). SKK4 was activated weakly by EGF, but not by PMA. SKK4 was also activated by stressful stimuli in COS cells (data not shown). SKK4 was activated to a greater extent by interleukin-1 (IL-1) and tumour necrosis factor (TNFα) than by UV irradiation, osmotic shock or anisomycin.

SKK1/MKK4, another activator of SAPK1/JNK (see Introduction) is also present in KB cells and, like SKK4, was activated by UV irradiation, osmotic shock and anisomycin. However, in contrast to SKK4, SKK1/MKK4 was hardly activated by interleukin-1, TNFα or EGF (Fig. 3A).

The specificities of the antibodies used in these experiments were established by the finding that immunoprecipitation of

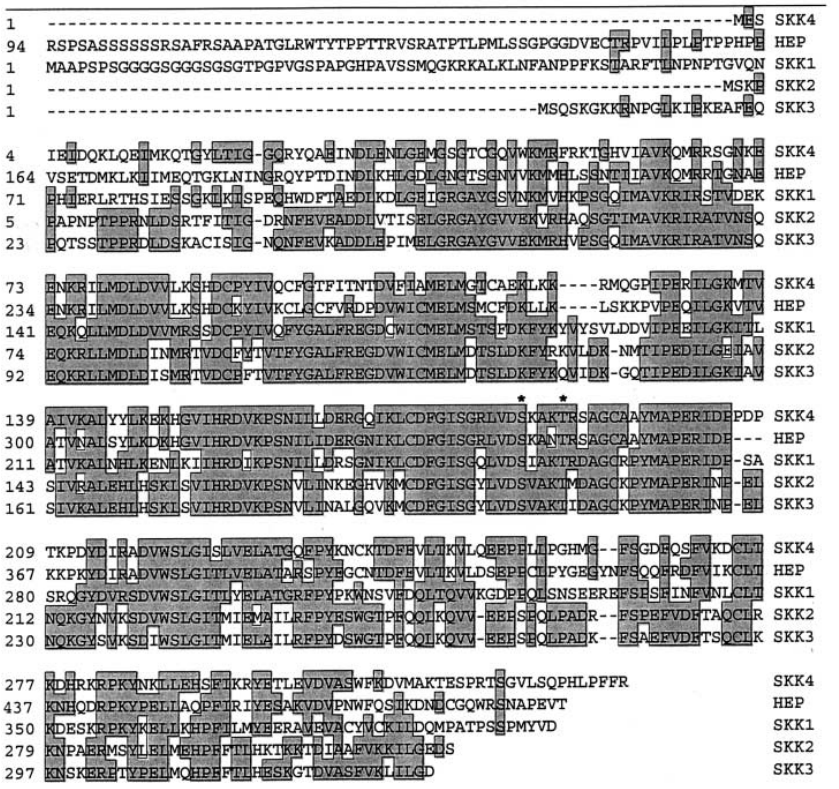

Fig. 2. Sequence comparison of human SKK4, *Drosophila* JNKK (HEP), human SKK1/MKK4, human SKK2/MKK3 and human SKK3/MKK6 [24,39]. Amino acids were aligned using the CLUSTAL program. Amino acid identities are shown by filled bars and asterisks denote the putative phosphorylation sites in the activation domain. Residues 94–487 of HEP are shown.

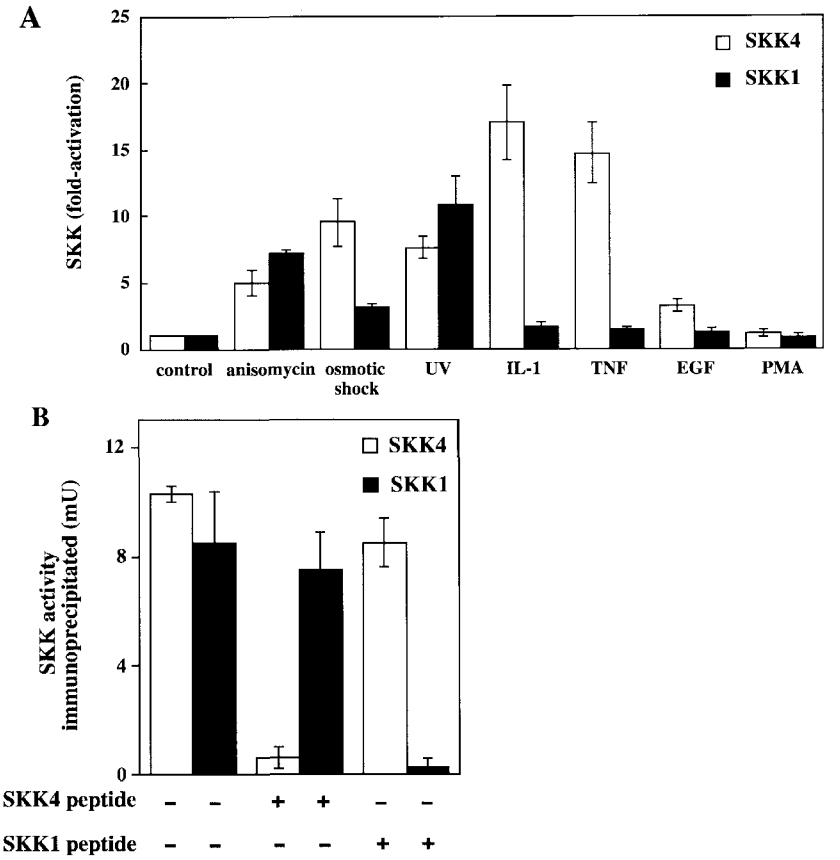

Fig. 3. Activation of SKK4 and SKK1/MKK4 in KB cells. (A) KB cells were incubated in the absence (control) or presence of the indicated agonists as described in Section 2.7. and SKK4 (open bars) and SKK1/MKK4 (filled bars) immunoprecipitated from the lysates and assayed for their ability to activate SAPK1/JNK1. The results are shown as means ± S.E.M. for six dishes of cells with each agonist (two separate experiments). The basal activity of SKK4 and SKK1/MKK4 in unstimulated cells was 5.0 ± 0.4 mU/mg and 2.0 ± 0.2 mU/mg, respectively. Abbreviations: IL-1, interleukin-1; TNFα, tumour necrosis factor α; EGF, epidermal growth factor; PMA, phorbol myristate acetate. (B) SKK4 and SKK1/MKK4 were immunoprecipitated from extracts prepared from UV-irradiated KB cells. Where indicated, the antibodies were incubated with the peptides used to raise the anti-SKK4 or anti-SKK1/MKK4 antibodies. The results are presented as means ± S.E.M. for at least three experiments.

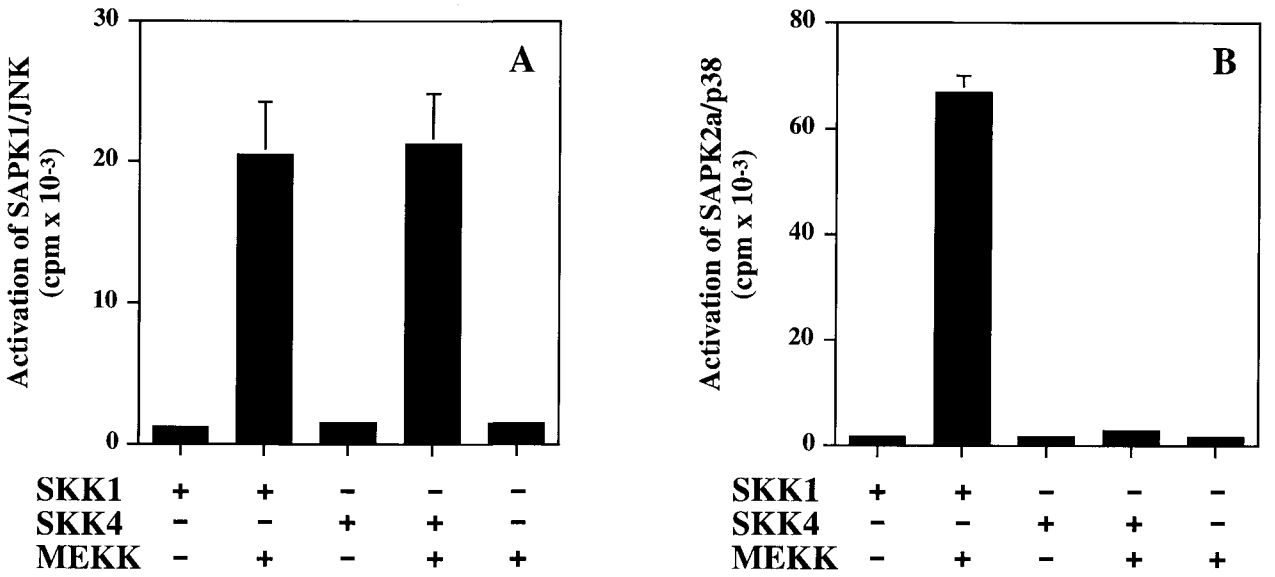

Fig. 4. GST-SKK4 is activated by MEKK and activates SAPK1/JNK, but not SAPK2a/p38. GST-SKK4 (0.25 μM) and GST-SKK1/MKK4 (0.1 μM) were incubated with MgATP in the presence or absence of MEKK and then assayed for their ability to activate SAPK1/JNK (A) or SAPK2a/p38 (B) (Section 2.8). The activation of SAPK1/JNK and SAPK2a/p38 were monitored by the phosphorylation of GST-ATF2[19–96]. The results are presented as means ± S.E.M. for at least three experiments.

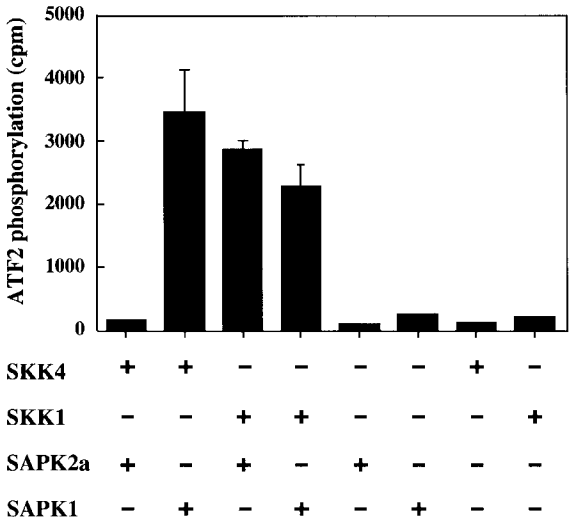

Fig. 5. SKK4 immunoprecipitated from extracts prepared from UV-irradiated KB cells activates SAPK1/JNK, but not SAPK2a/p38. SKK4 and SKK1/MKK4 immunoprecipitates were incubated with MgATP and SAPK2a/p38 or SAPK1/JNK. At the end of the reaction SAPK2a/p38 and SAPK1/JNK activity was then measured using ATF2 as substrate. The results are expressed as means ± S.E.M. for three experiments.

SKK4 was prevented by incubating the anti-SKK4 antibody with the SKK4 peptide immunogen used to generate it, but not with the SKK1/MKK4 peptide immunogen. Conversely, immunoprecipitation of SKK1/MKK4 was prevented by incubating the anti-SKK1/MKK4 antibody with the SKK1/MKK4 peptide immunogen, but not the SKK4 peptide immunogen (Fig. 3B).

3.3. Activation and substrate specificity of SKK4

GST-SKK4 was expressed in *E. coli* (Section 2.3.) and 10 mg of purified enzyme could be isolated from 500 ml of bacterial culture. The preparation showed a single protein-staining band with the predicted molecular mass of 64 kDa (data not shown). GST-SKK4 was inactive, but could be activated by incubation with MgATP and MEKK (Fig. 4), an enzyme that also activates SKK1/MKK4 [29]. The activated GST-SKK4 was able to activate SAPK1/JNK in vitro, but not SAPK2a/p38 (Fig. 4), SAPK2b/p38β, SAPK3/ERK6 or SAPK4 (data not shown). In contrast, activated GST-SKK1/MKK4 activated SAPK2a/p38 as well as SAPK1/JNK (Fig. 4).

SKK4 and SKK1/MKK4 immunoprecipitated from the lysates of UV-stimulated KB cells had the same specificity as the bacterially expressed enzymes. Thus SKK4 activated SAPK1/JNK but not SAPK2a/p38, while SKK1/MKK4 activated both enzymes (Fig. 5).

4. Discussion

In this paper we have cloned a novel MKK homologue that is activated strongly by proinflammatory cytokines and stressful stimuli, and has therefore been termed SKK4. SKK4 expressed in *E. coli* and activated in vitro, or immunoprecipitated from KB cell extracts activated SAPK1/JNK, but was unable to activate other SAPKs, suggesting that SAPK1/JNK may be a physiological substrate of SKK4. This view is reinforced by the striking similarity between SKK4 and the *Drosophila* MKK homologue, termed HEP (Fig. 2). HEP was identified by genetic dissection of a signalling pathway that is required for dorsal closure during early embryonic development and shown to be situated 'upstream' of *Drosophila* JNK [35–37]. Thus HEP is almost certainly the enzyme responsible for activating JNK in *Drosophila*. The generation of mice lacking SKK4 will be needed to find out if SKK4 also plays an essential role in mammalian embryonic development.

SKK4 is activated in vitro by MEKK (Fig. 4), one of many protein kinases [38] that have been shown to activate SKK1/MKK4 in vitro and to trigger the activation of SAPK1/JNK in cotransfection experiments. These observations, and the finding that overexpression of inactive mutants of SKK1/MKK4 prevent the activation of SAPK1/JNK by MEKK, other upstream activators and stressful stimuli [25,29,38], suggested that SKK1/MKK4 mediates the activation of SAPK1/JNK in vivo. However, since the inactive mutant of SKK1/MKK4 probably exerts its dominant negative effect by binding to SAPK1/JNK, it remained possible that MEKK and other upstream activators trigger the activation of SAPK1/JNK via another MKK homologue. This view was confirmed by biochemical experiments which show that the major activator(s) of SAPK1/JNK in several cell extracts is chromatographically separable from SKK1/MKK4 [21,31], and by the finding that UV radiation and osmotic shock can still trigger the activation of SAPK1/JNK in stem cells from mice that do not express SKK1/MKK4 [26,27]. The present work indicates that one of the additional SAPK1/JNK activators is likely to be SKK4. Whether mammalian cells express additional MKK homologues that activate SAPK1/JNK remains to be established.

The present work (Fig. 3) and our earlier biochemical studies [21] showed that SKK4 is activated strongly by proinflammatory cytokines as well as stressful stimuli in KB cells, whereas SKK1/MKK4 is activated by stress stimuli only. These observations suggest that SKK4 may be a more attractive target for an anti-inflammatory drug than SAPK1/JNK because inhibitors of SKK4 may prevent the activation of SAPK1/JNK by proinflammatory cytokines, without affecting the activation of SAPK1/JNK by other agents.

Note added in proof. Cloning of murine SKK4 was recently reported by Tournier et al. [40] and MKK7 by these investigators.

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