SAP kinase-3, a new member of the family of mammalian stress-activated protein kinases

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Received 8 February 1996

Abstract Stress-activated protein kinases are MAP kinase homologues that are activated by cellular stresses, bacterial endotoxin and inflammatory cytokines. They are activated by a dual threonine/tyrosine phosphorylation within a TPY sequence in the case of stress-activated protein kinase-1 and its isoforms (also called JNKs) or a TGY sequence in the case of stress-activated protein kinase-2 and its isoforms (also called p38, p40, RK, CSBPs, XMpk2 and Mxi2). Here we report the cloning and sequencing of a new protein kinase from rat with a TGY sequence in the activation domain. This stress-activated protein kinase-3 is 60% identical to mouse stress-activated protein kinase-2 and 45% identical to HOG1 from *Saccharomyces cerevisiae*. Transcripts encoding stress-activated protein kinase-3 are widely expressed, with high levels in skeletal muscle.

Key words: Mitogen-activated protein kinase; Stress-activated protein kinase; Stress-activated protein kinase-3; HOG1

1. Introduction

Mitogen-activated protein (MAP) kinases play important roles in signal transduction pathways in eukaryotic cells (reviewed in [1–3]). They are activated through phosphorylation by upstream cascades of protein kinases, resulting in a substantial amplification of the primary signal. The activation of the upstream kinase cascades is, in turn, triggered by a number of signalling molecules, chiefly growth factors. Once activated, MAP kinases phosphorylate a number of cytoplasmic and nuclear proteins, including some transcription factors, thereby effecting changes in gene expression. Activation of p42/p44 MAP kinases is sufficient to induce the proliferation or differentiation of several cell types. MAP kinases that participate in the growth factor-stimulated pathway are actived when a threonine and a tyrosine residue in a TEY sequence in kinase subdomain VIII just N-terminal to the conserved APE motif are phosphorylated. The activation is catalysed by the dual specificity enzymes MAP kinase kinase 1 (also called MEK1) or MAP kinase kinase 2 (also called MEK2) which phosphorylate both residues.

Two MAP kinase homologues have been identified in mammalian cells; they are activated by cellular stresses (chemical, heat and osmotic shock, ultraviolet radiation or inhibitors of protein synthesis), by bacterial endotoxin and by the inflammatory cytokines interleukin-1 and tumour necrosis factor-α and have consequently been named stress-activated protein (SAP) kinases [3]. Isoforms of SAP kinase-1 (also called JNKs) phosphorylate several transcription factors, such as c-Jun, ATF2, TCF/Elk-1 and p53 [4–9], and this has been shown to result in a stimulation of the transactivation functions of some of these proteins [4–8]. Isoforms of SAP kinase-2 (also called p38, p40, RK, CSBPs, XMpk2 and Mxi2) phosphorylate the transcription factor ATF2 and MAP kinase-activated protein (MAPKAP) kinase-2, one of whose intracellular targets is the small heat shock protein HSP27 [10–15]. Like MAP kinases, SAP kinases are activated by a dual threonine/tyrosine phosphorylation, with a TPY motif for SAP kinase-1 [4,5] and a TGY motif for SAP kinase-2 [10,11,13,15,16]. Although MAP kinase kinase 3 and MAP kinase kinase 4 (also known as SEK1 or JNKK) have been shown to activate SAP kinases [17–19], it appears likely that additional protein kinases are involved in their physiological activation [20,21].

At present, SAP kinase-2 and its alternatively spliced isoforms are the only known protein kinases from multicellular organisms with a TGY sequence in subdomain VIII. They are most similar to HOG1, a MAP kinase homologue from *S. cerevisiae* which lies in a signalling pathway that restores the osmotic gradient across the yeast cell membrane in response to high external osmolarity [22]. SAP kinase-2 and HOG1 both contain a TGY sequence in an equivalent position to the TEY sequence in MAP kinases and the TPY sequence in SAP kinase-1. We now report the cloning and sequencing of a novel protein kinase from rat with a TGY sequence in the activation domain. This protein kinase (which we call SAP kinase-3) is 60% identical to mouse SAP kinase-2 and 45% identical to HOG1. By Northern blotting we show that SAP kinase-3 mRNA is widely expressed, with high levels in skeletal muscle.

2. Materials and methods

2.1. PCR, cDNA cloning and sequencing

A number of degenerate oligonucleotides were designed based on conserved kinase subdomains and used as polymerase chain reaction (PCR) primers to isolate and clone fragments of protein kinase cDNAs from adult rat brain cDNA. One set of primers from kinase subdomains I [5′-AA(A/G)AT(A/C/T)GG(T/C/A/G)GA(A/G)GG(T/C/A/G)AC(T/C/A/G)TA(T/C)GG-3′, sense, for KIGEGTYG] and VII [5′-CC(A/G)AA(A/G)TC(T/C/A/G)A(A/G)(T/G/A)AT(T/C)TT-3′, anti-sense, for KILDFG] produced a clone that exhibited a high degree of sequence similarity with SAP kinase-2 (PCR conditions: 1 min 94°C, 1 min 48°C, 1 min 72°C, for 33 cycles, with a 10 min elongation in the last cycle). This PCR fragment was then used as a probe to screen a rat brain cDNA library (Clontech) at high-stringency. One partial clone of 600 bp (clone rSAPK31) was obtained after screening 10^6 phage. Following sequencing the insert was gel-purified, labelled with [^{32}P]dCTP by random priming and used as a probe to screen a rat skeletal muscle cDNA library (Clontech) under high-stringency conditions. 24 hybridisation-positive clones were ob-

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The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank and Nucleotide Sequence Databases under Accession number X96488.

SSDI S0014-5793(96)00255-4

tained after screening 100000 plaques; 12 clones were isolated, subcloned into M13mp18 and partially sequenced. Sequencing was performed both manually using synthetic oligonucleotides as primers and on an Applied Biosystems 377 DNA sequencer with fluorescent primers. Full-length sequence was compiled from both strands of cDNA clone rSAPK37. The EMBL, Swissprot, GSDB and dBEST sequence databases were searched using the FastA algorithm via the GenQuest integrated sequence comparison server (GRAILMAIL@ornl.gov) and the EBI network file server (Fasta@ebi.ac.uk). A multiple alignment of SAP kinase-3, SAP kinase-2 and HOG1 was built up by eye, based on the optimal alignments of each pair of sequences produced by the Align program [23].

2.2. RNA blot analysis

RNA blots were performed using the rat multiple tissue Northern blot from Clontech, with 2 μg poly(A)$^+$ RNA per lane. Probes were labelled with [^{32}P]dCTP by random priming and hybridised under high-stringency conditions. The SAP kinase-3 probe was prepared from the gel-purified insert of the partial brain cDNA clone rSAPK31. The human β-actin cDNA probe was purchased from Clontech.

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GTTGGCGGCTATACAGAGTTCAGGGTGGCGACCCCGAGAGAAGAGCCCGCAAAGGAAAGC          60

                                                        *
CCCAGAGGCGGGCAGGCGGGTGGCCGGCGCGGAGCCGGCCCTGCCACGCAGTGACCCGGG         120

                *                   *
GCGCACGGGCGGAGCCCCTGATCCCAAGTCCGGTCCTAGAGCGCGGTGCTCCGGCCGGGG         180

 M  S  S  P  P  P  A  R  K  G  F  Y  R  Q  E  V  T  K  T  A      20
ATGAGCTCCCCGCCACCCGCCCGCAAGGGCTTTTACCGCCAGGAGGTGACCAAAACGGCC         240

 W  E  V  R  A  V  Y  Q  D  L  Q  P  V  G  S  G  A  Y  G  A      40
TGGGAGGTGCGCGCCGTGTACCAGGACCTGCAGCCCGTTGGCTCTGGTGCCTATGGTGCA         300

 V  C  S  A  V  D  S  R  T  G  N  K  V  A  I  K  K  L  Y  R      60
GTGTGCTCTGCAGTAGACAGCCGCACTGGCAACAAGGTGGCCATCAAGAAGTTGTACCGG         360

 P  F  Q  S  E  L  F  A  K  R  A  Y  R  E  L  R  L  L  K  H      80
CCCTTCCAGTCGGAGCTGTTTGCCAAGCGCGCCTACAGAGAGTTGCGCCTCCTCAAACAC         420

 M  R  H  E  N  V  I  G  L  L  D  V  F  T  P  D  E  T  L  D     100
ATGCGCCACGAGAACGTCATTGGGCTGCTGGATGTGTTCACTCCCGATGAGACTCTGGAT         480

 D  F  T  D  F  Y  L  V  M  P  F  M  G  T  D  L  G  K  L  M     120
GACTTCACAGACTTCTACCTGGTGATGCCATTCATGGGCACTGACCTGGGCAAGCTCATG         540

 K  H  E  T  L  S  E  D  R  I  Q  F  L  V  Y  Q  M  L  K  G     140
AAGCACGAGACTCTGAGTGAAGACAGAATCCAGTTTCTTGTGTATCAGATGCTGAAGGGG         600

 L  K  Y  I  H  A  A  G  V  I  H  R  D  L  K  P  G  N  L  A     160
CTGAAGTATATCCACGCTGCCGGCGTCATCCACAGGGACTTGAAACCTGGAAACCTGGCT         660

 V  N  E  D  C  E  L  K  I  L  D  F  G  L  A  R  Q  A  D  S     180
GTGAACGAGGACTGTGAGCTGAAGATCCTAGATTTTGGCCTTGCCAGGCAGGCGGACAGT         720

 E  M  T  G  Y  V  V  T  R  W  Y  R  A  P  E  V  I  L  N  W     200
GAGATGACAGGATATGTGGTAACCCGGTGGTATCGGGCACCAGAGGTCATCTTGAATTGG         780

 M  R  Y  T  Q  T  V  D  I  W  S  V  G  C  I  M  A  E  M  I     220
ATGCGTTACACACAGACAGTGGACATTTGGTCTGTTGGCTGCATCATGGCAGAGATGATT         840

 T  G  K  I  L  F  K  G  N  D  H  L  D  Q  L  K  E  I  M  K     240
ACTGGAAAGATCCTGTTCAAAGGCAATGACCACCTGGACCAGCTGAAGGAGATCATGAAA         900

 V  T  G  T  P  P  P  E  F  V  Q  K  L  Q  S  A  E  A  K  N     260
GTCACAGGGACACCCCCTCCTGAGTTTGTACAGAAGCTACAGAGTGCTGAGGCCAAGAAC         960

 Y  M  E  G  L  P  E  L  E  K  K  D  F  A  S  V  L  T  N  A     280
TACATGGAAGGCCTCCCTGAGTTGGAAAAGAAGGATTTTGCTTCTGTCCTGACCAATGCA        1020

 S  P  Q  A  V  N  L  L  E  K  M  L  V  L  D  A  E  Q  R  V     300
AGCCCTCAGGCCGTGAATCTCCTGGAAAAGATGCTGGTGTTGGATGCGGAACAGCGGGTG        1080

 T  A  A  E  A  L  A  H  P  Y  F  E  S  L  R  D  T  E  D  E     320
ACAGCAGCTGAGGCATTAGCCCACCCATACTTTGAGTCCCTTCGGGACACTGAGGATGAG        1140

 P  K  A  Q  K  Y  D  D  S  F  D  D  V  D  R  T  L  E  E  W     340
CCCAAGGCCCAGAAATATGATGACTCCTTTGATGACGTAGACCGCACCCTTGAGGAATGG        1200

 K  R  V  T  Y  K  E  V  L  S  F  K  P  P  R  Q  L  G  A  R     360
AAGCGTGTTACGTATAAGGAAGTGCTCAGCTTCAAGCCTCCCAGGCAGCTAGGAGCCAGA        1260

 V  P  K  E  T  A  L  *                                        367
GTTCCAAAGGAGACAGCTCTGTGAAGACCTCCGGGTGGTTTGGGGGGTATCCTAAGGAGG        1320

CTGTCTGGGAGCTTCGCAGACACCTTGGCTTCCCTTCTCCGGAAGAGGAATCCTGGTTGG        1380

CACCAGTGCCTGGTGCTTTTATCCCAAGTCATCCACCTGGAAAGGCTGTGTAGACCCCTT        1440

GAATCACGAACCCTCCATCTCCAAGCCAGTTCTTCAGATTTTGAGCGCCCGAGATGACCC        1500

TGGCAGAACATCTAAGCTTTTTTTTTTTCTTTTTTCTTTTTTTCGGAGCTGGGGACCGAA        1560

CCCAGGGCCTTGCGCTTGCTAGGCAAGCGCTCTACTACTGAGCTAAATCCCCAACCCCAC        1620

ATCTAAGCTTTCTGTCCAAGACCCCTACCCAACATGGGACTAGCC                        1665
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Fig. 1. Nucleotide and predicted amino acid sequence of rat SAP kinase-3. Nucleotides are numbered in the 5′ to 3′ direction and the amino acids are shown in single-letter code above the nucleotide sequence. In-frame termination codons are marked by an asterisk.

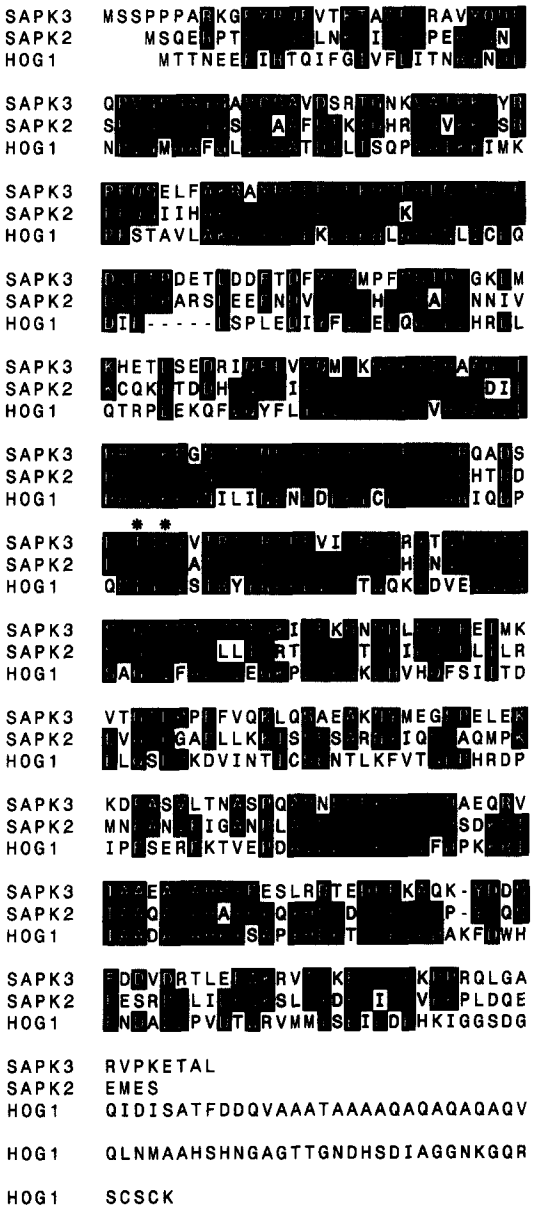

Fig. 2. Sequence comparison of rat SAP kinase-3, mouse SAP kinase-2 [10] (also called p38, p40, RK, CSBP2 and XMpk2) and HOG1 from *S. cerevisiae* [22]. Amino acids were aligned and two gaps were introduced to maximise the homology. Amino acid identities between at least two of the three sequences are indicated by black bars. Asterisks denote phosphorylation sites in the TGY sequence of the activation domain.

3. Results and discussion

To identify novel members of the MAP kinase and SAP kinase group, we employed a PCR strategy using degenerate primers to amplify sequences from rat brain cDNA. Sequence analysis of PCR products led to the identification of one cDNA fragment that exhibited homology to known SAP kinases. This fragment was used as a probe to screen an adult rat brain cDNA library at high-stringency and partial cDNA clone rSAPK31 was in turn used to screen a cDNA library from rat skeletal muscle. Screening of 100000 plaques gave 24 positives, several of which contained the entire coding region. The nucleotide and deduced amino acid sequence of cDNA

clone rSAPK37 is shown in Fig. 1. In-frame termination codons in the 5′ and 3′ regions of the DNA sequence indicate that it contains the entire coding region. A single open reading frame encodes a protein of 367 amino acids, with a predicted molecular mass of 42 kDa. It possesses the conserved amino acid domains (I–XI) characteristic of protein kinases and shows 60% sequence identity with mouse SAP kinase-2, 45% identity with HOG1 from *S. cerevisiae* (Fig. 2), 47% identity with human SAP kinase-1 (JNK1) and 42% identity with p42 MAP kinase (ERK2) from rat. The Thr^{183} and Tyr^{185} residues in subdomain VIII are in an equivalent position to the TEY, TPY or TGY sequences in known MAP kinases and SAP kinases, phosphorylation of which is required for enzymatic activity. The sequence shares a TGY sequence with SAP kinase-2 and HOG1. Moreover, as in SAP kinase-2 and HOG1, kinase subdomain VII is separated by only 6 amino acids from the activation region in subdomain VIII, whereas the gap is 8 residues in SAP kinase-1 and over 12 amino acids in known MAP kinases. The predicted amino acid sequence and overall structure identify this protein as a novel member of the SAP kinase-2 family that we designate SAP kinase-3. SAP kinase-2 has been shown to exist as different isoforms resulting from alternative mRNA splicing [13,14]. It remains to be seen whether the same is true of SAP kinase-3. It also remains to be seen whether additional mammalian protein kinases exist with a TGY sequence in the activation domain. A recent study [24] has reported the sequence of a trapped exon from a human chromosome 22 cosmid library (accession number H55067) that is 88% identical to nucleotides 438–496 from rat SAP kinase-3 and completely identical to the corresponding nucleotide sequence of human SAP kinase-3 (our unpublished observation). The human SAP kinase-3 gene therefore probably maps to chromosome 22.

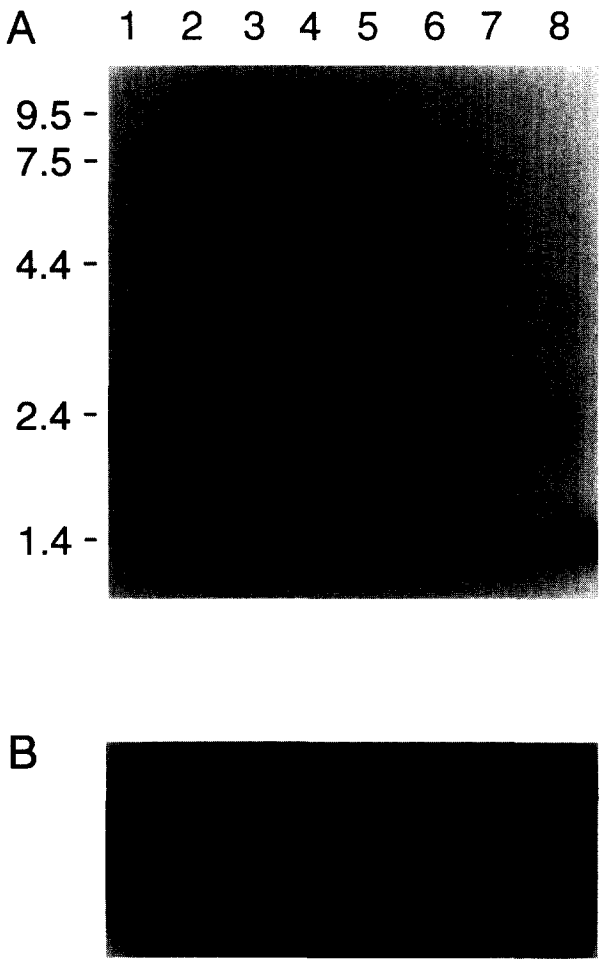

Fig. 3. RNA blot analysis of poly(A)$^+$ RNA from adult rat tissues, with ^{32}P-labelled SAP kinase-3 (A) or β-actin DNA (B) used as the probe. The blot was re-hybridised with the actin probe following elution of the SAP kinase-3 signal. Size markers (in kb) are marked to the left in (A). Lanes: 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, testis.

To examine the tissue distribution of SAP kinase-3, we used RNA blot analysis to investigate the levels of SAP kinase-3 mRNA (Fig. 3). A cDNA probe specific for SAP kinase-3 recognised a major transcript of approx. 2.2 kb (Fig. 3A). This size is larger than the DNA sequence shown in Fig. 1, suggesting that it lacks untranslated sequence; this is also indicated by the absence of a poly(A) tail at the 3′ ends of the cDNA clones. SAP kinase-3 mRNA is widely expressed, with high levels in skeletal muscle (Fig. 3A). In some tissues a transcript of approx. 3.6 kb was observed in addition to the 2.2 kb band. It is at present unclear whether this transcript results from alternative splicing, alternative polyadenylation, or whether it indicates the existence of a closely related gene. Re-hybridisation of the blot with a human β-actin probe showed that comparable amounts of poly(A)$^+$ RNA had been loaded in each lane (Fig. 3B). The tissue distribution of rat SAP kinase-3 mRNA is similar to that of human SAP kinase-2 mRNA [13] which is also expressed at high levels in skeletal muscle.

SAP kinase-2 is activated by various cellular stresses, by bacterial endotoxin and by inflammatory cytokines [10,13–15]. It will be interesting to determine whether the same is true of SAP kinase-3. Similarly, it will be important to identify the SAP kinase kinases that activate SAP kinase-3, as well as its downstream targets. This will show whether SAP kinase-2 and SAP kinase-3 are activated by a common pathway or whether they form part of parallel signal transduction pathways, each with its own specific downstream targets. SAP kinase-2 is the only known protein kinase that is inhibited by a novel class of pyridinyl imidazoles called CSAIDs, which were developed as inhibitors of the lipopolysaccharide-induced synthesis of interleukin-1 and tumour necrosis factor in monocytes, suggesting that activation of SAP kinase-2 results in protein phosphorylation events which trigger cytokine gene transcription [13]. Moreover, CSAIDs inhibit the activation of MAPKAP kinase-2 and the phosphorylation of HSP27 in vivo in response to cellular stresses, bacterial endotoxin and inflammatory cytokines [25]; they also inhibit the stimulation of 2-deoxyglucose uptake in response to interleukin-1 and anisomycin [26]. It will be interesting to determine whether CSAIDs inhibit SAP kinase-3.

Acknowledgements: We thank Philip Cohen for helpful comments on the manuscript. S.M. was supported by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft.

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