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Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases

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A cDNA was cloned that encodes human stress-activated protein kinase-4 (SAPK4), a novel MAP kinase family member whose amino acid sequence is ~60% identical to that of the other three SAP kinases which contain a TGY motif in their activation domain. The mRNA encoding SAPK4 was found to be widely distributed in human tissues. When expressed in KB cells, SAPK4 was activated in response to cellular stresses and pro-inflammatory cytokines, in a manner similar to other SAPKs. SAPK4 was activated *in vitro* by SKK3 (also called MKK6) or when co-transfected with SKK3 into COS cells. SKK3 was the only activator of SAPK4 that was induced when KB cells were exposed to a cellular stress or stimulated with interleukin-1. These findings indicate that SKK3 mediates the activation of SAPK4. The substrate specificity of SAPK4 *in vitro* was similar to that of SAPK3. Both enzymes phosphorylated the transcription factors ATF2, Elk-1 and SAP-1 at similar rates, but were far less effective than SAPK2a (also called RK/p38) or SAPK2b (also called p38β) in activating MAPKAP kinase-2 and MAPKAP kinase-3. Unlike SAPK1 (also called JNK), SAPK3 and SAPK4 did not phosphorylate the activation domain of c-Jun. Unlike SAPK2a and SAPK2b, SAPK4 and SAPK3 were not inhibited by the drugs SB 203580 and SB 202190. Our results suggest that cellular functions previously attributed to SAPK1 and/or SAPK2 may be mediated by SAPK3 or SAPK4.

Keywords: cytokine/IL1/MAP kinase/MEK/stress/TNF

Introduction

Four mitogen-activated protein (MAP) kinase family members are activated by cellular stresses (chemical, heat and osmotic shock, UV radiation, inhibitors of protein synthesis), bacterial lipopolysaccharide (LPS), and the cytokines interleukin-1 (IL1) and tumour necrosis factor (TNF), and have therefore been termed stress-activated protein kinases or SAPKs (reviewed in Cohen, 1997). Isoforms of SAPK1 [also called c-Jun N-terminal kinases (JNKs)] phosphorylate Ser63 and Ser73 in the activation domain of c-Jun (Pulverer *et al.*, 1991), thereby increasing its transcriptional activity. The same sites in c-Jun also become phosphorylated when cells are exposed to the stresses and cytokines that activate SAPK1 (Pulverer *et al.*, 1991; Hibi *et al.*, 1993; Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994), suggesting that c-Jun is a physiological substrate for SAPK1.

SAPK2a [also termed p38 (Han *et al.*, 1994), p40 (Freshney *et al.*, 1994), RK (Rouse *et al.*, 1994), CSBP (Lee *et al.*, 1994) and Mxi2 (Zervos *et al.*, 1995)] is inhibited very specifically by the pyridinyl imidazoles SB 203580 and SB 202190 (Lee *et al.*, 1994; Cuenda *et al.*, 1995; reviewed in Cohen, 1997) which have been exploited to identify several physiological substrates. These include four protein kinases, namely MAP kinase-activated protein kinase-2 (MAPKAP-K2, Rouse *et al.*, 1994) and the closely related MAPKAP-K3 (Clifton *et al.*, 1996; McLaughlin *et al.*, 1996; Sithanandam *et al.*, 1996), as well as MAP kinase interacting protein kinases-1 and -2 (Mnk1 and Mnk2) (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997).

Physiological substrates of MAPKAP-K2/K3 include heat shock protein (HSP) 27 (Cuenda *et al.*, 1995; Huot *et al.*, 1995) and the transcription factor CREB (Tan *et al.*, 1996), whereas transcription factor eIF4E is a physiological substrate of Mnk1/2 (Waskiewicz *et al.*, 1997). SAPK2a also mediates the stress-induced phosphorylation and activation of the CEBPβ-related transcription factor CHOP (Wang and Ron, 1996) and the ternary complex factor Elk-1 (Price *et al.*, 1996).

Based on the effects of SB 203580, the activation of SAPK2a is rate-limiting in the LPS-induced production of IL1 and TNF in monocytes (Lee *et al.*, 1994), in the TNF-stimulated transcription of IL6 and GM-CSF in fibroblasts (Beyaert *et al.*, 1996), in the IL1-induced stimulation of glucose uptake in epithelial cells (Gould *et al.*, 1995), in collagen-induced platelet aggregation (Saklatvala *et al.*, 1996) and in the stress-induced transcription of c-Jun and c-Fos in fibroblasts (Hazzalin *et al.*, 1996; Price *et al.*, 1996). The SAPK2a catalysed phosphorylation of Elk-1 (Price *et al.*, 1996) and the MAPKAP-K2 catalysed phosphorylation of CREB (Tan *et al.*, 1996) are both likely to contribute to the stress-induced transcription of c-Fos (Ginty *et al.*, 1994).

Recently, two additional SAP kinases were identified, called SAPK2b [or p38β (Jiang *et al.*, 1996)] and SAPK3 (Mertens *et al.*, 1996) [also called ERK6 (Lechner *et al.*, 1996) and p38γ (Li *et al.*, 1996)]. The amino acid sequence of SAPK2b is 73% identical to SAPK2a and it is inhibited by SB 202190 at similar concentrations to SAPK2a. In contrast, the amino acid sequence of SAPK3 is only 60% identical to SAPK2a and SAPK2b, and SAPK3 is not inhibited by SB 203580 (Cuenda *et al.*, 1997). SAPK2b and SAPK3 have been introduced into mammalian cells

by transient transfection and shown to be activated in response to pro-inflammatory cytokines and stressful stimuli in a manner similar to SAPK1 and SAPK2a. The physiological roles of SAPK2b and SAPK3 are unknown. The mRNAs encoding these enzymes are present in all mammalian tissues examined (Jiang *et al.*, 1996; Mertens *et al.*, 1996; Goedert *et al.*, 1997), with the mRNA encoding SAPK3 being most abundant in skeletal muscle. Expression of wild-type SAPK3 and an inactive mutant in the muscle cell line C2C12 respectively enhanced and inhibited differentiation into myotubes (Lechner *et al.*, 1996). *In vitro*, SAPK2b and SAPK3 phosphorylated several proteins that are also substrates for SAPK2a. SAPK2b was reported to phosphorylate the transcription factor ATF2 more efficiently than SAPK2a (Jiang *et al.*, 1996) but, since the stress- and cytokine- induced phosphorylation of ATF2 in fibroblasts is unaffected by SB 203580 (Beyaert *et al.*, 1996; Hazzalin *et al.*, 1996), neither SAPK2a nor SAPK2b appears to be rate-limiting for ATF2 phosphorylation *in vivo*, in contrast to earlier studies using transfection-based approaches (Gupta *et al.*, 1995). The substrate specificity of SAPK3 *in vitro* was similar to that of SAPK2a, except that it was much less effective in activating MAPKAP-K2/K3 and (like SAPK1, but unlike SAPK2a) phosphorylated ATF2 at Ser90, as well as at Thr69 and Thr71 (Cuenda *et al.*, 1997). However, whether SAPK1 and/or SAPK3 are rate-limiting for ATF2 phosphorylation *in vivo* is unknown.

Five chromatographically distinct SAP kinase kinases (SKKs) have been identified in mammalian cells (Meier *et al.*, 1996; Cuenda *et al.*, 1996). *In vitro*, SKK1 [also termed MKK4 (Dérijard *et al.*, 1995), SEK1 (Sanchez *et al.*, 1994) and XMEK2 (Yashar *et al.*, 1993)] activates all four SAPKs (Sanchez *et al.*, 1994; Dérijard *et al.*, 1995; Doza *et al.*, 1995; Jiang *et al.*, 1996; Cuenda *et al.*, 1997), although SAPK2b and SAPK3 are phosphorylated less efficiently. SKK2 [also termed MKK3 (Dérijard *et al.*, 1995)] and SKK3 (Cuenda *et al.*, 1996) [also called MKK6 (Han *et al.*, 1996; Moriguchi *et al.*, 1996; Raingeaud *et al.*, 1996) and MEK6 (Stein *et al.*, 1996)] activate SAPK2a but not SAPK1, while SKK3 was the only detectable activator of SAPK3 induced by pro-inflammatory cytokines and stressful stimuli in human epithelial KB cells or human embryonic kidney 293 cells (Cuenda *et al.*, 1997). SKK3 was also the most efficient activator of SAPK2b in co-transfection experiments (Jiang *et al.*, 1996). SKK4 and SKK5 activate SAPK1 but not SAPK2a (Meier *et al.*, 1996) or SAPK3 (Cuenda *et al.*, 1997). SKK4/SKK5 have not been purified or cloned and their amino acid sequences are thus still unknown.

In this paper, we report the cloning and characterization of a novel MAP kinase family member that we call SAPK4. This enzyme, which also contains a TGY sequence in the activation domain, shows ~60% identity to SAPK2a, SAPK2b and SAPK3, and its mRNA is widely expressed in human tissues. We show that SAPK4 is activated by the same stimuli that activate other SAP kinases and that SKK3 is likely to be the major upstream activator of SAPK4 *in vivo*. The substrate specificity of SAPK4 *in vitro* is similar to that of SAPK3 and, like SAPK3, SAPK4 is not inhibited by SB 203580 or SB 202190.

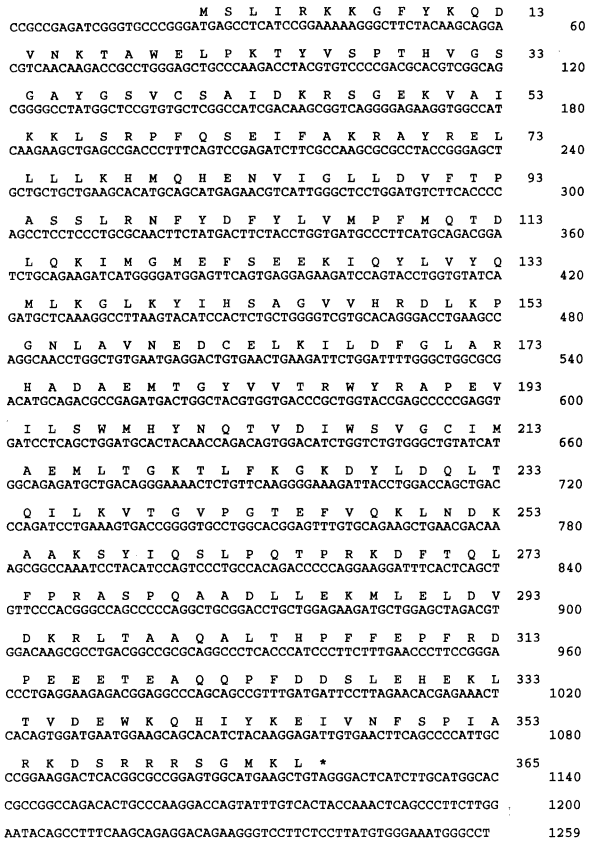


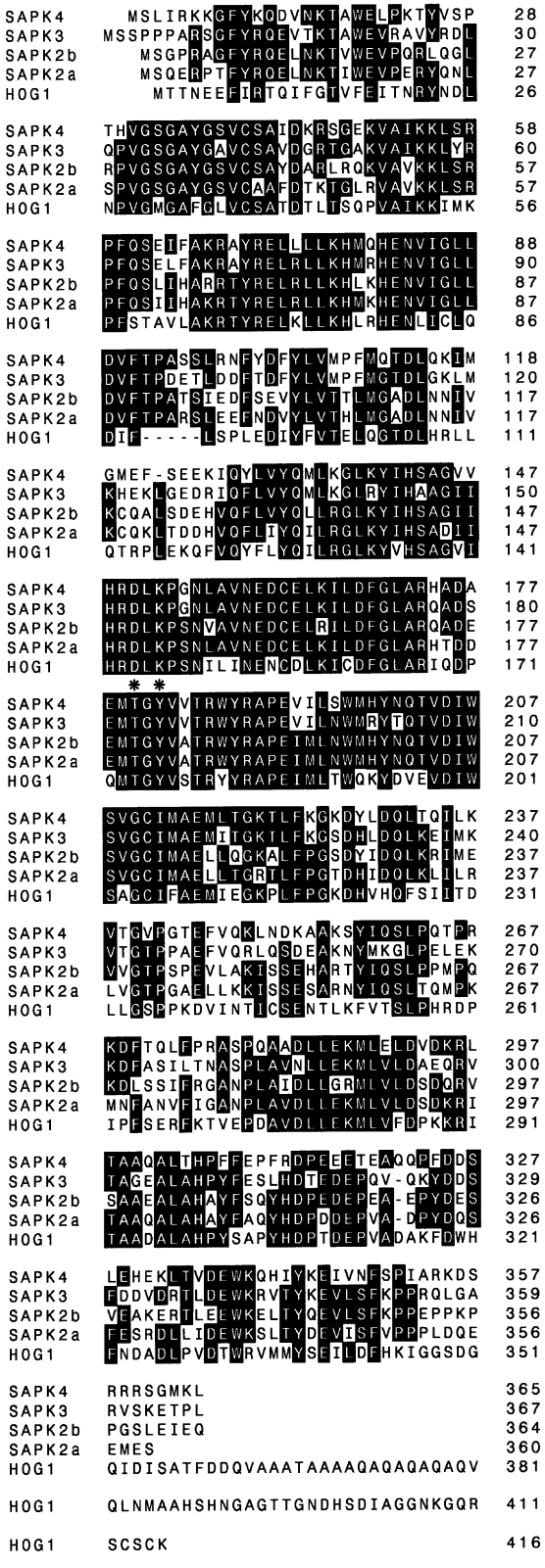
Fig. 1. Nucleotide and predicted amino acid sequence of human SAP kinase-4 (GenBank Accession Number Y10488). Nucleotides are numbered in the 5′ to 3′ direction and amino acids are shown in single-letter code above the nucleotide sequence. The in-frame termination codon is marked by an asterisk.

Results

Molecular cloning of human SAPK4

To identify novel members of the SAPK family, we used EST clone 156272 (GenBank accession number R72662), which encodes a portion of SAPK2b (p38β), as the probe to screen a human pituitary gland cDNA library. Sequencing of positive clones showed three distinct sequences. The first set of clones encoded portions of ERK5 (Zhou *et al.*, 1995) [also called BMK1 (Lee *et al.*, 1995)], whereas the second set encoded a novel form of p38β (see Discussion). The third set of clones encoded a protein whose sequence was similar to, but differed from that of known SAPKs and which was consequently named stress-activated protein kinase-4 (SAPK4). The nucleotide and deduced amino acid sequence of human SAPK4 is shown in Figure 1. An open reading frame encodes a protein of 365 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 64% sequence identity with SAPK3, 59% identity with SAPK2a, 58% identity with SAPK2b, 42% identity with HOG1 from *Saccharomyces cerevisiae* (Figure 2), 45% identity with SAPK1 and 41% identity with p42 MAP kinase. Residues Thr180 and Tyr182 in subdomain VIII are in an equivalent position to the TEY, TPY and TGY sequences in known MAP kinases and SAP kinases, phosphorylation of which is required for enzymatic

activity. SAPK4 shares a TGY sequence with SAPK2a, SAPK2b, SAPK3 and HOG1 (Figure 2). Moreover, as in SAPK2a, SAPK2b, SAPK3 and HOG1, subdomain VII is separated by only six amino acids from the activation region in subdomain VIII (Figure 2), whereas this gap is eight residues in SAPK1 and over 12 residues in MAP kinases. The tissue distribution of SAPK4 mRNA in human tissues was assessed by RNA blotting (Figure 3). Hybridization of ^{32}P-labelled SAPK4 cDNA to multiple tissue Northern blots showed a transcript of ~2.3 kb which was present in most of the 16 tissues examined, albeit at variable levels (Figure 3). Highest levels were detected in pancreas, testis, small intestine and prostate gland. Hybridization of the blots with a probe for β-actin showed approximately equal loading of RNA (data not shown).



SAPK4 is activated by cellular stresses and cytokines

SAPK4 is most closely related to SAPK3, SAPK2a and SAPK2b, enzymes that are activated by cellular stresses and the cytokines IL1 and TNF (see Introduction). We therefore investigated whether the same stimuli would activate SAPK4. Human epithelial KB cells were transiently transfected with a myc epitope-tagged SAPK4 and, after exposure to cellular stresses or cytokines, the enzyme was immunoprecipitated and assayed. These experiments showed that the stimuli which trigger the activation of SAPK2a, SAPK2b or SAPK3 also activate SAPK4 (Figure 4), while stimuli that do not activate SAPK2a, SAPK2b or SAPK3 [such as insulin-like growth factor-1 (IGF-1) and phorbol esters] also failed to activate SAPK4 (Figure 4).

Identification of SKK3 as the major activator of SAPK4 in epithelial cells

We have shown previously that SKK3 accounts for 95% and SKK2 for 5% of the SAPK2a activator detected after Mono S chromatography of lysates from KB cells that have been stressed in several ways or stimulated with IL1 (Cuenda *et al.*, 1996; Meier *et al.*, 1996). As shown in Figure 5, the single peak of SAPK4 activator detected after subjecting KB cells to IL1 or the protein synthesis inhibitor anisomycin co-migrated with SKK3 on Mono S and was immunoprecipitated quantitatively and specifically by anti-MKK6 antibodies. This experiment also demonstrated that SKK4 and SKK5, which are activated in KB cells by these stimuli, and which elute from Mono S at a higher NaCl concentration than SKK3 (Meier *et al.*, 1996), do not activate SAPK4. SKK3 was also the only activator of SAPK4 after subjecting KB cells to osmotic stress using sorbitol (data not shown). Further evidence that SKK3 can activate SAPK4 *in vivo* was obtained by co-transfection into COS cells. SAPK4 activity was elevated 12.5 ± 0.63-fold by co-expression with SKK3 ($n = 3$). In contrast, SAPK4 was not activated significantly by co-transfection with MEK kinase, under conditions where MEK kinase activated the endogenous SKK1 in COS cells (data not shown).

Fig. 2. Sequence comparison of human SAPK4, human SAPK3 (Goedert *et al.*, 1997) (also called ERK6 and p38γ), human SAPK2a (Lee *et al.*, 1994) (also called p38, p40, RK, CSBP2, Mxi2 and XMpk2), human SAPK2b (also called p38β) and HOG1 from *S.cerevisiae* (Brewster *et al.*, 1993). Amino acids were aligned and gaps were introduced to maximize the homology. Amino acid identities between at least three of the five sequences are indicated by black bars. Asterisks denote the dual phosphorylation sites in the TGY motif of the activation domain. The SAPK2b sequence shown here differs from the published p38β sequence (Jiang *et al.*, 1996) (see Discussion). The amino acid sequence of SAPK3 (Goedert *et al.*, 1997) differs in 13 positions from the ERK6 sequence of Lechner *et al.* (1996) and in 1 amino acid from the p38γ sequence of Li *et al.* (1996).

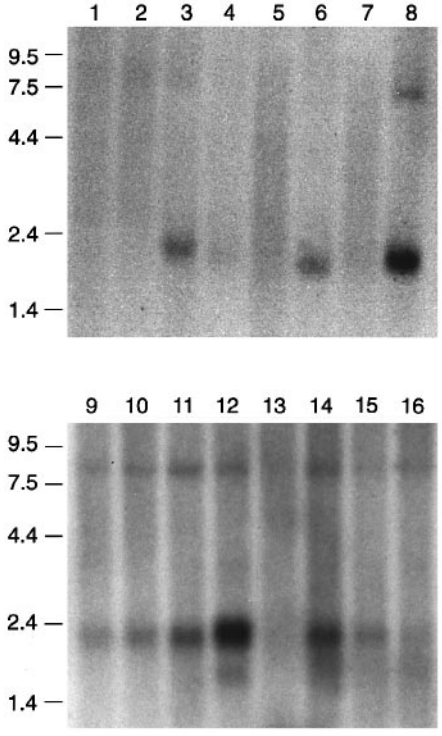


Fig. 3. RNA blot analysis of poly(A)$^+$ RNA from adult human tissues, with ^{32}P-labelled SAPK4 DNA as the probe. Size markers (in kb) are marked to the left. Lanes: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, thymus; 11, prostate gland; 12, testis; 13, ovary; 14, small intestine; 15, colon; 16, leucocytes.

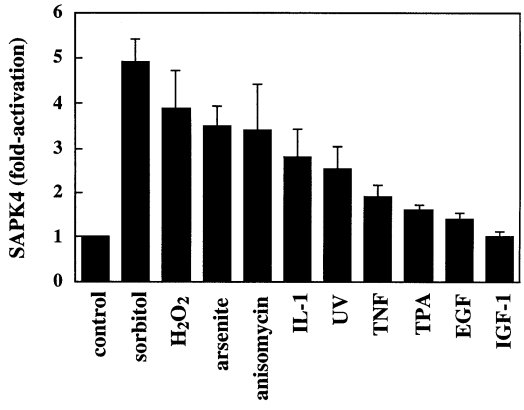


Fig. 4. SAPK4 is activated by cytokines and cellular stresses in KB cells. KB cells were transiently transfected with a DNA construct encoding myc epitope-tagged SAPK4, as indicated in Materials and methods. Cells were stimulated in DMEM for 15 min with 0.5 M sorbitol, 1 mM H_2O_2, 20 ng/ml IL-1α, 0.5 mM sodium arsenite, 100 ng/ml TNFα, 100 ng/ml EGF or 300 ng/ml TPA; for 30 min with 10 μg/ml anisomycin or for 10 min with 100 ng/ml IGF-1. UV irradiation was at 60 J/m^2 and the cells were lysed 30 min later. Following stimulation, the myc epitope-tagged SAPK4 was immunoprecipitated from cell lysates using monoclonal antibody 9E10 and the kinase activity measured using MBP as the substrate, as indicated in Materials and methods. The results are shown as the average ± SEM of four experiments.

Activation of SAPK4 by SKK3 in vitro

SAPK4 was activated *in vitro* by a highly purified preparation of SKK3 from skeletal muscle (Figure 6), but could not be activated by MKK1 under conditions where p42 MAP kinase was activated maximally (data not shown).

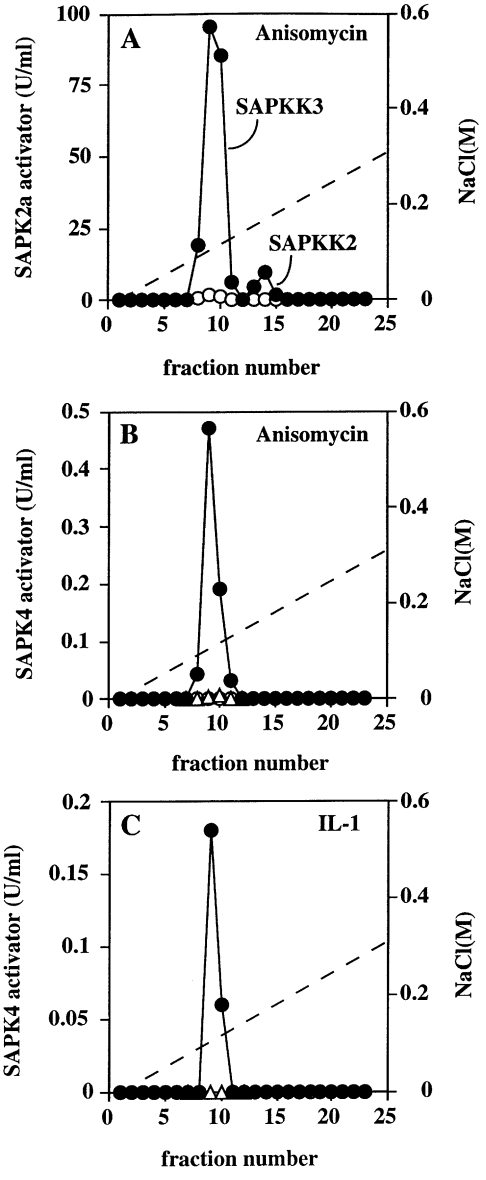


Fig. 5. SKK3 is the major SAPK4 activator in KB cells. Cell lysates (0.6 mg protein) from quiescent KB cells (open circles in A) or cells stimulated for 30 min with 10 μg/ml anisomycin (closed circles in A and B) or for 15 min with 20 ng/ml IL-1α (closed circles in C) were chromatographed on Mono S, and activators of SAPK2a (**A**) or activation of SAPK4 (B and C) assayed as described in Materials and methods. Aliquots from the peak of SAPK4 activator seen in (**B**) and (**C**) were immunoprecipitated, as described previously (Cuenda *et al.*, 1996) using anti-MKK6 antibodies and the SAPK4 activator remaining in the supernatant was assayed (open triangles in B and C). Similar results were obtained in three different experiments.

The activation of SAPK4 by SKK3 occurred 2- to 3-fold more slowly than that of SAPK2a, although both enzymes attained the same specific activity towards myelin basic protein (MBP) after 2 h (Figure 6A). The activity of SAPK2a and SAPK4 towards MBP is 25- to 50- fold lower than the activity of p42 MAP kinase towards this substrate (Stokoe *et al.*, 1992). The activation of SAPK4, like that of SAPK3 (Cuenda *et al.*, 1997), reached a plateau at ~1.6 mol/mol subunit (data not shown) and was accompanied by the appearance of phosphotyrosine and phosphothreonine in similar amounts (Figure 6B). Interestingly, SAPK4, like SAPK3 but unlike SAPK2a, also

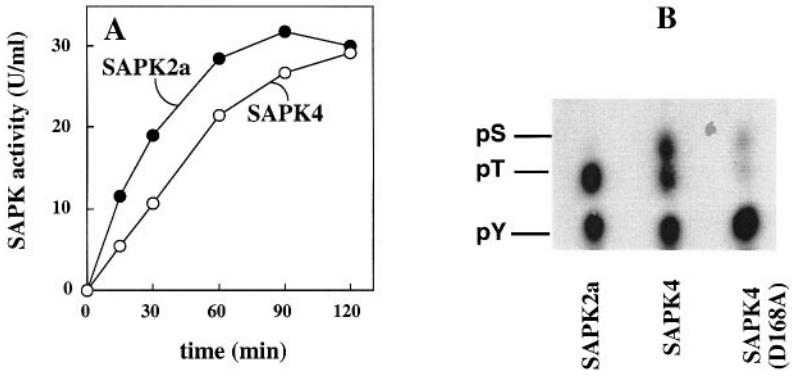


Fig. 6. Phosphorylation and activation of SAPK4 and SAPK2a by purified SKK3. (**A**) GST–SAPK4 (0.5 μM, open circles) or MalE–Mpk2, the *Xenopus* homologue of SAPK2a (0.5 μM, closed circles), were phosphorylated using 100 U/ml SKK3 and unlabelled ATP and SAPK activities were measured at the times indicated using MBP as substrate. (**B**) GST–SAPK4, an inactive mutant GST–SAPK4(D168A) and *Xenopus* SAPK2a were phosphorylated for 2 h as in (A), but using [^{32}P]ATP (Cuenda *et al.*, 1997). Aliquots of the reactions were precipitated by the addition of 1 ml of 20% (by mass) TCA, the suspension centrifuged for 5 min at 13 000 *g* and the pellets washed three times with 20% (by mass) TCA, five times with water and dried. The samples were then incubated for 90 min at 110°C in 6 M HCl, electrophoresed on thin layer cellulose at pH 3.5 to resolve phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY), and autoradiographed.

became phosphorylated at a serine residue(s). This did not occur when wild-type SAPK4 was replaced by the catalytically inactive SAPK4-D168A mutant (Figure 6B), indicating that serine phosphorylation is catalysed by SAPK4 itself after it has been activated. Interestingly, the inactive SAPK4 mutant could only be phosphorylated on tyrosine and not on threonine, indicating that this mutation induces a conformational change that affects the recognition of SAPK4 by SKK3.

Activation of SAPK4 by SKK1 (MKK4) in vitro

SAPK2 is not only phosphorylated by SKK2 and SKK3 *in vitro*, but also by SKK1 (MKK4) (see Introduction). SKK1 is activated by cytokines and cellular stresses and it was therefore of interest to investigate whether this enzyme also had the potential to activate SAPK4 *in vitro*. Figure 7 shows that SAPK4 is phosphorylated and activated by SKK1 *in vitro*, but at a much slower rate than SAPK2a under identical conditions. Consistent with this slow rate of activation, SAPK4 was not activated after co-transfection into COS1 cells with MEK kinase (data not shown), an upstream activator of SKK1. This is consistent with the negligible contribution of SKK1 to the SAPK4-activating activity in KB cell extracts (Figure 5).

Comparison of the substrate specificity of SAPK4 with that of SAPK2a, SAPK2b and SAPK3

SAPK4 was more active than SAPK2a or SAPK2b in phosphorylating fusion proteins with glutathione *S*-transferase (GST) linked to the activation domains of the transcription factors Elk-1, ATF2, SAP-1, SAP-2 and p53, while c-Jun was only phosphorylated poorly by the three enzymes (Table I). SAPK4 was far less effective than SAPK2a in activating GST–MAPKAP-K2(5–400) and full-length GST-MAPKAP-K3, the initial rate of activation of MAPKAP-K2 and MAPKAP-K3 and the half-time for maximal activation being 20 times slower (Figure 8). The specificity of SAPK4 *in vitro* was similar to that of SAPK3 (Table I; Cuenda *et al.*, 1997). Consistent with their similar amino acid sequences (Figure 2), the substrate specificities of SAPK2a and SAPK2b were virtually indistinguishable (Table I and Figure 8).

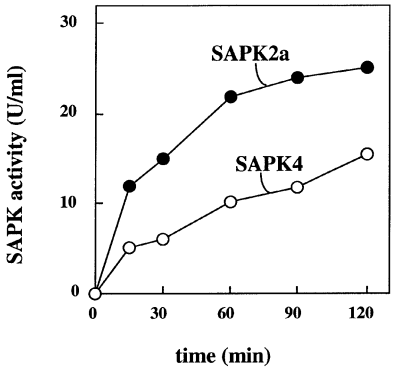


Fig. 7. Activation of SAPK4 and SAPK2a by SKK1 (MKK4). GST–SAPK4 (0.5 μM, open circles) or MalE–Mpk2, the *Xenopus* homologue of SAPK2a (0.5 μM, closed circles) were incubated with 100 U/ml SKK1 and unlabelled ATP and the generation of SAPK activity measured at the times indicated using MBP as substrate.

SAPK4 is not inhibited by SB 203580 or SB 202190

Like SAPK3, bacterially expressed SAPK4 that had been activated by SKK3 *in vitro* was not inhibited by SB 203580 or SB 202190 (Figure 9). In contrast, SAPK2a and SAPK2b were inhibited by both drugs with similar IC_{50} values of 0.3–0.6 μM (Figure 9). SB 203580 and SB 202190 also failed to inhibit the dephosphorylated forms of SAPK3 and SAPK4 which have <0.1% of the activity of the phosphorylated forms (data not shown). In addition, SB 203580 did not prevent the activation of transfected SAPK3 by osmotic shock in KB cells.

Discussion

In this paper we report the cloning of the cDNA encoding human SAPK4, a novel MAP kinase family member whose mRNA is widely expressed and whose amino acid sequence is ~60% identical to SAPK2a, SAPK2b and SAPK3. Moreover, SAPK4, like SAPK2a, SAPK2b and SAPK3, contains the dual TGY phosphorylation motif and a six amino acid insertion between subdomain VII and the activation loop in subdomain VIII. SAPK4 is less similar to SAPK1 (45% identity) and p42/p44 MAP

Table I. Comparison of the substrate specificities of SAPK4 with those of SAPK2a, SAPK2b and SAPK3

Substrate (1 μM)	Rates of phosphorylation relative to MBP (%)			
	SAPK2a	SAPK2b	SAPK3	SAPK4
MBP	100	100	100	100
Elk-1	90 ± 10	136 ± 12	181 ± 30	175 ± 25
ATF2	40 ± 2	62 ± 2	107 ± 20	130 ± 2
SAP-1	25 ± 1	27 ± 5	108 ± 15	62 ± 5
MAPKAP-K2	90 ± 5	83 ± 3	25 ± 3	28 ± 5
MAPKAP-K3	87 ± 10	66 ± 6	20 ± 6	18 ± 2
p53	15 ± 3	29 ± 2	30 ± 1	13 ± 1
SAP-2	13 ± 1	18 ± 4	20 ± 3	8 ± 2
c-Jun	4 ± 1	7 ± 2	2 ± 1	6 ± 2

Experiments were carried out with the *Xenopus* homologue of SAPK2a, human SAPK2b and human SAPK4. The SAPKs were activated *in vitro* (Figure 7) and phosphorylation of each protein was studied at a SAPK concentration of 0.15 U/ml. The results are presented as means ± SEM of six determinations (two separate experiments). The data for rat SAPK3 are taken from Cuenda *et al.* (1997).

kinases (41% identity). During the course of this study, we have also sequenced several cDNA clones for SAPK2b. They were found to encode a protein of 364 amino acids that differs from the published p38β sequence (Jiang *et al.*, 1996) in two respects. It lacks an eight amino acid sequence between kinase subdomains V and VI and it shows two amino acid differences in this region. As a result, our SAPK2b sequence aligns with the other SAP kinase sequences without requiring any gaps.

Consistent with its amino acid sequence similarity to SAPK2a, SAPK2b and SAPK3, SAPK4 is activated in response to the same cellular stresses and cytokines. The only activator of SAPK4 that could be detected in extracts prepared from epithelial KB cells exposed to a cellular stress or IL1 was SKK3, the product of the MKK6 gene. SAPK4 also became activated when co-transfected with SKK3 DNA into COS cells. In contrast, SAPK4 was activated poorly by SKK1 (MKK4) *in vitro* and was not activated in co-transfection experiments with MEK kinase, an upstream activator of SKK1 (Yan *et al.*, 1994). SAPK4 was also not activated by MKK1, SKK2 (MKK3), SKK4 or SKK5. Thus, SKK3 which is the dominant activator of SAPK2a (Cuenda *et al.*, 1996), SAPK2b (Jiang *et al.*, 1996) and SAPK3 (Cuenda *et al.*, 1997) in several mammalian cell lines, also appears to be the major activator of SAPK4.

In contrast to SAPK2a and SAPK2b, which are inhibited by SB 203580 or SB 202190 at submicromolar concentrations, SAPK3 and SAPK4 were not affected by these drugs; moreover, SB 203580 did not inhibit the activation of SAPK3. The failure of SB 202190 to inhibit SAPK3 is in disagreement with the work of Li *et al.* (1996) who reported that the basal activity of bacterially expressed SAPK3 was inhibited by this drug. The reason for this discrepancy is unclear, because we failed to find any effect of SB 203580 or SB 202190 on either the basal activity of expressed SAPK3 or on SAPK3 that had been maximally activated by phosphorylation with SKK3. Identical results were obtained using bacterially expressed rat SAPK3 (Cuenda *et al.*, 1997) and human SAPK3.

The substrate specificity of SAPK4 *in vitro* resembled that of SAPK3 in that, while both enzymes phosphorylated a number of proteins (including the activation domains of several transcription factors) at similar rates to SAPK2a and SAPK2b, they were far less effective in activating

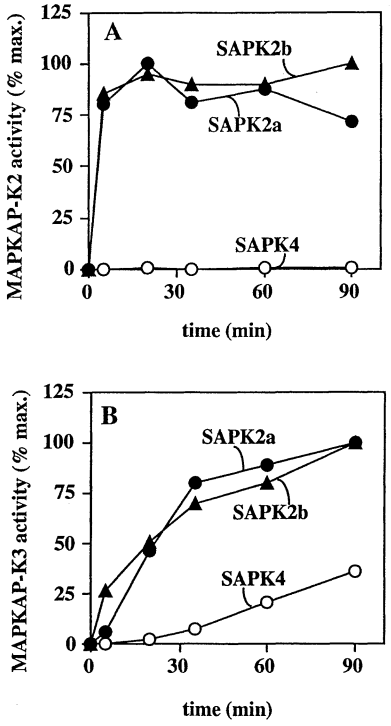


Fig. 8. Activation of MAPKAP-K2 and MAPKAP-K3 by SAPK2a, SAPK2b and SAPK4. (**A**) GST–MAPKAP-K2(5–400) or (**B**) GST–MAPKAP-K3 (0.2 mg/ml) were incubated with unlabelled ATP and 5 U/ml of the *Xenopus* homologue of SAPK2a (closed circles), 5 U/ml of SAPK2b (closed triangles) or 5 U/ml of SAPK4 (open circles) and MAPKAP-K2/K3 activity measured at the times indicated, as described in Materials and methods. There was no phosphorylation or activation of GST–MAPKAP-K2(5–400) or GST–MAPKAP-K3 when SAPK2a, SAPK2b or SAPK4 were omitted.

MAPKAP-K2 and MAPKAP-K3 than either SAPK2a or SAPK2b. The last mentioned result is consistent with the finding that SB 203580 suppresses the stress- and cytokine-induced activation of MAPKAP-K2 and MAPKAP-K3 by 80–95% in every mammalian cell so far examined (Cuenda *et al.*, 1995; Beyaert *et al.*, 1996; Clifton *et al.*, 1996; McLaughlin *et al.*, 1996). Moreover, although SAPK3 and SAPK4 phosphorylate the activation domain of the transcription factor Elk-1 efficiently *in vitro*, neither enzyme appears to be rate-limiting for Elk-1 phosphorylation *in vivo*, because Elk-1 phosphorylation induced by

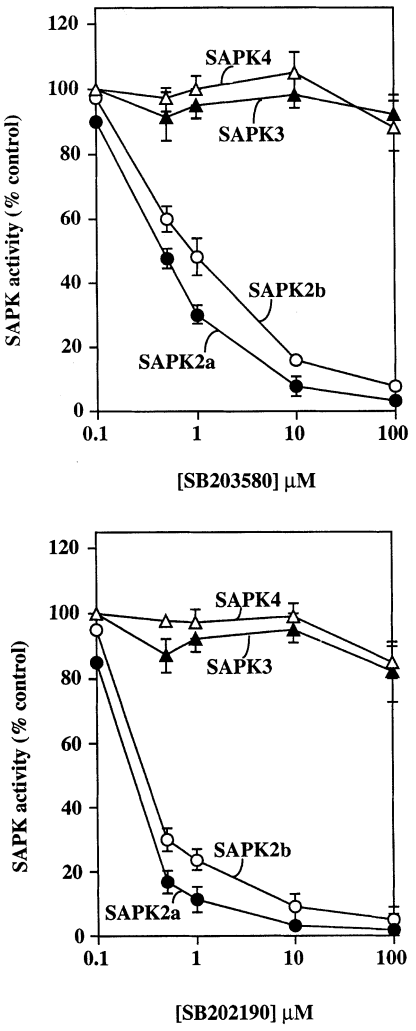


Fig. 9. Effects of SB 203580 or SB 202190 on SAPK2a, SAPK2b, SAPK3 and SAPK4. The *Xenopus* homologue of SAPK2a (closed circles), human SAPK2b (open circles), human SAPK3 (closed triangles) and human SAPK4 (open triangles) were activated *in vitro* with purified SKK3, as in Figure 6, and then assayed after a 10 min incubation with the indicated concentrations of SB 203580 or SB 202190. The results are presented relative to control incubations in which the inhibitor was omitted.

cellular stresses can be prevented by SB 203580 in fibroblast cell lines (Hazzalin *et al.*, 1996) or by a combination of SB 203580 and PD 98059 in HeLa cells (Price *et al.*, 1996). PD 098059, a specific inhibitor of the activation of MKK1 (Alessi *et al.*, 1995), prevents the activation of p42/p44 MAP kinases. Candidates as physiological substrates for SAPK3 and SAPK4 are proteins whose phosphorylation/activation triggered by cellular stresses and/or pro-inflammatory cytokines is not prevented by SB 203580. Such proteins include the transcription factors c-Jun, ATF2 and NFκB (Beyaert *et al.*, 1996; Hazzalin *et al.*, 1996). However, c-Jun is phosphorylated very poorly by SAPK3 and SAPK4 and isoforms of SAPK1 are likely to phosphorylate this protein *in vivo* (see Introduction).

In summary, the number of MAP kinase family members which are activated by cellular stresses and/or pro-inflammatory cytokines is much greater than was realized previously (shown schematically in Figure 10). In addition

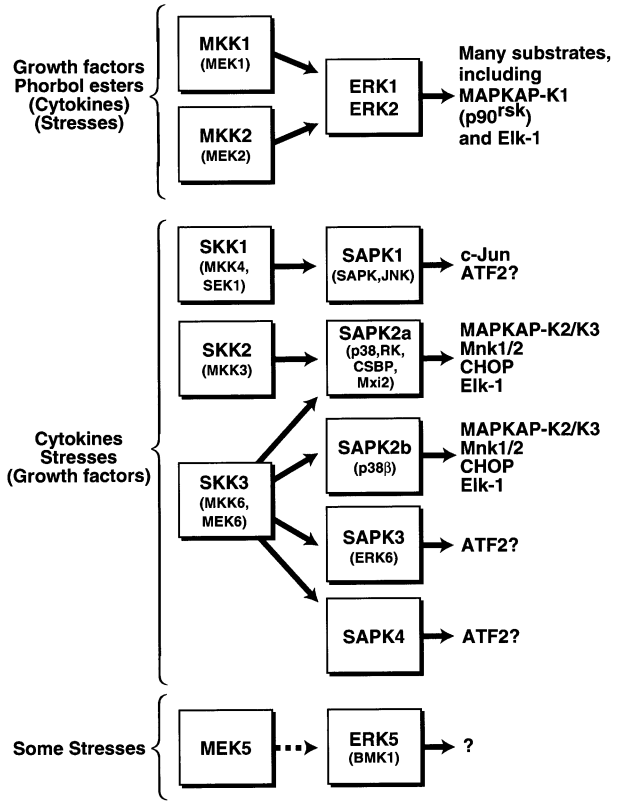


Fig. 10. Schematic representation of mammalian MAP kinase and SAP kinase signal transduction pathways. Following growth factor or phorbol ester stimulation, the MAP kinase kinases MKK1 and MKK2 (also called MEK1 and MEK2) activate the ERK1 and ERK2 group of MAP kinases. ERK1 and ERK2 (also termed p44 and p42 MAP kinases, respectively) phosphorylate a number of substrates, including the protein kinase MAPKAP-K1 (also called $p90^{rsk}$) and the transcription factor Elk-1. In some cellular backgrounds, pro-inflammatory cytokines and cellular stresses also activate the MAP kinase pathway, albeit more weakly than growth factors or phorbol esters (indicated by brackets). SAP kinase (SAPK) pathways are strongly activated by pro-inflammatory cytokines and a large number of stressful stimuli. In some cells they are also activated more weakly by certain growth factors (indicated by brackets). The SAP kinase kinase SKK1 (also termed MKK4 and SEK1) activates SAPK1 (also called SAPK or JNK) which phosphorylates the transcription factor c-Jun. The highly homologous SAPK1 isoforms are the products of three different genes. The SAP kinase kinase SKK2 (also called MKK3) activates SAPK2a (also termed p38, RK, CSBP and Mxi2). The SAP kinase kinase SKK3 (also called MKK6 or MEK6) activates SAPK2a, SAPK2b (also termed p38β), SAPK3 (also called ERK6 and p38γ) and SAPK4. Additional forms of SAPK1, SAPK2a and SAPK3 are produced by alternative mRNA splicing. Studies with the specific inhibitor SB 203580 indicate that SAPK2a (and probably SAPK2b) phosphorylate the protein kinases MAPKAP-K2/3 and Mnk1/2, as well as the transcription factors CHOP and Elk-1. The reason ATF2 is an unlikely physiological substrate for SAPK2a and SAPK2b is given in the text, and this transcription factor may therefore be phosphorylated by SAPK1, SAPK3 and/or SAPK4 *in vivo*. SKK1 activates SAPK2a and SAPK2b *in vitro* and in transfection experiments, but is not thought to activate these enzymes *in vivo*. SKK1 also activates SAPK3 and SAPK4 weakly *in vitro*, but not in transfection experiments. Oxidative and osmotic stresses activate ERK5 (also called BMK1) which is believed to be activated by MEK5, although this has not been shown directly (indicated by broken arrow). Question marks indicate that a given SAPK phosphorylates a target protein *in vitro*, with no current evidence for a corresponding role *in vivo*. Although SAPK1 phosphorylates Elk-1 *in vitro*, it is not rate-limiting for the phosphorylation of this protein *in vivo*, since the phosphorylation of Elk-1 and the transcription of c-fos are completely suppressed by SB 203580 and PD 098059 (Hazzalin *et al.*, 1996; Price *et al.*, 1996). PD 098059, a specific inhibitor of the activation of MKK1 (Alessi *et al.*, 1995), prevents activation of ERK1 and ERK2.

to SAPK1, SAPK2a, SAPK2b, SAPK3 and SAPK4, the p42/p44 MAP kinases, which are strongly activated by growth factors, are also activated by stressful stimuli and pro-inflammatory cytokines in some cellular backgrounds, albeit more weakly. Moreover, the MAP kinase family member ERK5 (also called BMK1) is activated by osmotic and oxidative stresses (Abe *et al.*, 1996). The development of specific inhibitors for each of these MAP kinase family members would greatly facilitate the elucidation of their physiological roles.

Materials and methods

Materials

SB 203580 and SB 202190 were generous gifts from Dr John Lee (SmithKline Beecham, King of Prussia, PA, USA) and dissolved in DMSO. Anti-MKK6 antibodies (raised against the peptide CNPGLKEAFEQPQTS corresponding to a sequence near the N-terminus of human MKK6) were generated and purified as described previously (Cuenda *et al.*, 1996; Meier *et al.*, 1996). MalE–Mpk2, the *Xenopus* homologue of SAPK2a (Rouse *et al.*, 1994) and rat GST–SAPK3 (Cuenda *et al.*, 1997) were expressed in *Escherichia coli* and purified as described previously. Sources of all other materials, enzymes, fusion proteins and methods are given in Cuenda *et al.* (1997).

cDNA cloning and sequencing

The *Not*I–*Eco*RI insert from EST clone 156272 (DDBJ/EMBL/GenBank accession number R72662), which encodes a portion of SAPK2b (p38β, Jiang *et al.*, 1996), was used as the probe to screen a human pituitary gland cDNA library (Clontech, Palo Alto, CA) at high-stringency. Several partial ERK5 clones, a number of SAPK2b clones, some of which were full-length, and one partial SAPK4 clone were obtained after screening 2×10^6 phage. Following sequencing, the SAPK4 insert was used as the probe to screen the pituitary gland cDNA library under high-stringency conditions. A total of 16 hybridization-positive clones were obtained after screening 3×10^6 plaques; they were isolated, the *Eco*RI inserts subcloned into M13mp18 and sequenced. Three of the 16 clones were full-length. 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 hSAPK412. The NCBI sequence databases were searched using the blast algorithm (blast@ncbi.nlm.nih.gov). A multiple alignment of SAPK4, SAPK3, SAPK2a, SAPK2b and HOG1 was built up by eye.

RNA blot analysis

RNA blots were performed using human multiple tissue Northern blots from Clontech with 2 μg poly(A)$^+$ RNA per lane. Probes were labelled with [^{32}P]dCTP by random priming and hybridized under high-stringency conditions. The SAPK4 probe was prepared from the gel-purified insert of cDNA clone hSAPK412. The human β-actin probe was purchased from Clontech.

SAPK2b, SAPK3 and SAPK4 expression plasmids

For bacterial expression, the open reading frames of human cDNA clones hSAPK2B2, hSAPK32 and hSAPK412 were amplified by PCR and subcloned as *Eco*RI fragments into expression vector pGEX4T-1 (Pharmacia), followed by transformation into *E.coli* strain BL21(DE3). The transformed bacteria were grown to an absorbance of 0.6 at 600 nm and induced with 0.4 mM isopropyl-1-thio-β-galactopyranoside (IPTG). Human GST–SAPK2b, GST–SAPK3 and GST–SAPK4 were purified by affinity chromatography on glutathione-agarose. For expression of c-myc epitope-tagged SAPK4, PCR was used to introduce the nucleotide sequence encoding the amino acid sequence MEQKLISEEDLN at the carboxy-terminus of SAPK4, followed by a stop codon. The resulting *Eco*RI–*Not*I fragment was subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen). Substitution of Asp168 by Ala in SAPK4 to produce a kinase-inactive mutant was performed by site-directed mutagenesis. PCR fragments were verified by DNA sequencing. Transfections into COS1 cells were carried out as described (Cuenda *et al.*, 1997).

Protein kinase assays

SAPK4, SAPK2a and SAPK2b were assayed routinely by the phosphorylation of MBP, exactly as described previously for SAPK3 (Cuenda *et al.*, 1997). One unit of activity was the amount of enzyme which incorporated 1 nmol of phosphate into MBP in 1 min. SAPK4 activators were assayed by their ability to activate GST–SAPK4, as described previously for GST–SAPK3 (Cuenda *et al.*, 1997). Control experiments were carried out in which GST–SAPK4 was omitted. One unit of SAPK4 activator was the amount which increased SAPK4 activity by 1 U/min. MAPKAP-K2 and MAPKAP-K3 were assayed using the peptide KKLNRTLSVA as substrate (Rouse *et al.*, 1994) and one unit of activity was the amount which catalysed the phosphorylation of 1 nmol of peptide substrate in 1 min. SAPK2a activators were assayed by their ability to activate the *Xenopus laevis* homologue of SAPK2a (Meier *et al.*, 1996). Activated SAPK2a itself was measured by the activation of GST–MAPKAP-K2(46–400). 0.1% DMSO was present in all experiments where the effects of SB 203580 and SB 202190 were being studied. This concentration of DMSO inhibited each SAPK by ~10%.

Immunoprecipitation of SAPK4

Lysates of cells transfected with myc epitope-tagged SAPK4 were centrifuged at 4°C for 10 min at 13 000 *g*. Aliquots of the supernatant (100 μg protein) were incubated for 2 h on a shaking platform with 5 μl of protein G–Sepharose coupled to 3 μg of monoclonal antibody 9E10 which recognizes the c-myc epitope. The immunoprecipitates were washed and assayed as described (Cuenda *et al.*, 1997).

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