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Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6); comparison of the specificities of SAPK3 and SAPK2 (RK/p38)

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Stress-activated protein kinase-3 (SAPK3), a recently described MAP kinase family member with a widespread tissue distribution, was transfected into several mammalian cell lines and shown to be activated in response to cellular stresses, interleukin-1 (IL-1) and tumour necrosis factor (TNF) in a similar manner to SAPK1 (also termed JNK) and SAPK2 (also termed p38, RK, CSBP and Mxi2). SAPK3 and SAPK2 were activated at similar rates *in vitro* by SAPKK3 (also termed MKK6), and SAPKK3 was the only activator of SAPK3 that was induced when KB or 293 cells were exposed to cellular stresses or stimulated with IL-1 or TNF. Co-transfection with SAPKK3 induced SAPK3 activity and greatly enhanced activation in response to osmotic shock. These experiments indicate that SAPKK3 mediates the activation of SAPK3 in several mammalian cells. SAPK3 and SAPK2 phosphorylated a number of proteins at similar rates, including the transcription factors ATF2, Elk-1 and SAP1, but SAPK3 was far less effective than SAPK2 in activating MAPKAP kinase-2 and MAPKAP kinase-3. Unlike SAPK2, SAPK3 was not inhibited by the drug SB 203580. SAPK3 phosphorylated ATF2 at Thr69, Thr71 and Ser90, the same residues phosphorylated by SAPK1, whereas SAPK2 only phosphorylated Thr69 and Thr71. Our results suggest that cellular functions previously attributed to SAPK1 and/or SAPK2 may be mediated by SAPK3.

Keywords: IL-1/MAP kinase kinase/MEK/stress/TNF

Introduction.

Two mitogen-activated protein (MAP) kinase family members have been identified in mammalian cells which are activated by cellular stresses (chemical, heat and osmotic shock, UV radiation, inhibitors of protein synthesis), by bacterial lipopolysaccharide (LPS) and by the cytokines interleukin-1 (IL-1) and tumour necrosis factor (TNF), and have, therefore, been termed stress-activated protein kinases or SAPKs (reviewed in Cohen, 1997). Isoforms of SAPK1 [also termed c-Jun N-terminal kinases (JNKs)] phosphorylate the transcription factors c-Jun (at Ser63 and Ser73) (Pulverer *et al.*, 1991), ATF2 (at Thr69, Thr71 and Ser90) (Gupta *et al.*, 1995; Livingstone *et al.*, 1995) and Elk-1 (Cavigelli *et al.*, 1995) *in vitro*, increasing the transcriptional activity of these proteins. The same sites in these transcription factors also become phosphorylated when cells are exposed to the stresses and cytokines that activate SAPK1, or after co-transfection with protein kinases known to activate SAPK1 (Pulverer *et al.*, 1991; Hibi *et al.*, 1993; Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994; Cavigelli *et al.*, 1995; Gupta *et al.*, 1995; Whitmarsh *et al.*, 1995; Zinck *et al.*, 1995), suggesting that they may be physiological substrates for SAPK1.

SAPK2 [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)] activates two protein kinases, termed MAP kinase-activated protein kinase-2 (MAPKAP-K2, Rouse *et al.*, 1994), and MAPKAP-K3 (McLaughlin *et al.*, 1996), which share 75% amino acid sequence identity, and have similar substrate specificities *in vitro* (Clifton *et al.*, 1996). MAPKAP-K2 and MAPKAP-K3 are activated by the same agonists as SAPK2 (Clifton *et al.*, 1996; McLaughlin *et al.*, 1996), and their activation is prevented by SB 203580, a specific inhibitor of SAPK2 which does not inhibit SAPK1 or p42 and p44 MAP kinases (Cuenda *et al.*, 1995). These observations suggest that MAPKAP-K2 and MAPKAP-K3 are physiological substrates of SAPK2. Further experiments employing SB 203580 (and other evidence) indicate that SAPK2 mediates the stress-induced phosphorylation of the transcription factor CHOP (at Ser78 and Ser81) which enhances its transcriptional activity (Wang and Ron, 1996), and that heat shock protein 27 (HSP27) (Stokoe *et al.*, 1992a; Cuenda *et al.*, 1995; Huot *et al.*, 1995) and the transcription factor CREB (Tan *et al.*, 1996) are physiological substrates for MAPKAP-K2/MAPKAP-K3. The phosphorylation of HSP27 appears to enhance the polymerization of actin and is thought to help repair the actin microfilament network which becomes disrupted during cellular stress, thereby aiding cell survival (Lavoie *et al.*, 1995). The phosphorylation of CREB (at Ser133) is essential to allow this protein to stimulate the transcription of genes that contain cyclic AMP response elements (CREs). The effects of SB 203580 also indicate that activation of the SAPK2–MAPKAP-K2/K3 pathway is rate limiting in the LPS-induced production of IL-1 and TNF in monocytes (Lee *et al.*, 1994), in the TNF-stimulated transcription of the genes encoding IL-6 and granulocyte–macrophage colony-stimulating factor (Beyaert *et al.*, 1996) in fibroblasts, in the IL1-induced stimulation of glucose uptake in epithelial cells (Gould *et al.*, 1995), in collagen-induced platelet aggregation (Saklatvala *et al.*, 1996), in the stress-induced transcription of the genes encoding c-Jun and c-Fos (Hazzalin *et al.*, 1996) in fibroblasts, and in the LPS-induced synthesis of cyclo-oxygenase-2 (COX2, the rate limiting enzyme in prostaglandin synthesis) in RAW 264.7 macrophages (Paul

et al., 1997). Since the c-Fos and COX2 promoters contain CREs, the MAPKAP-K2/K3-mediated phosphorylation of CREB may contribute to the stress- and cytokine-induced transcription of these two genes. The importance of the CRE in the induction of c-*fos* mRNA is well documented (Ginty *et al.*, 1994). The transcription factors ATF2 and Elk-1 are also phosphorylated by SAPK2 *in vitro* and after transfection of mammalian cells with the upstream activators of SAPK2 that do not activate SAPK1 (Raingeaud *et al.*, 1996).

The activation of SAPK1 and SAPK2 is complex, because five chromatographically distinct SAP kinase kinases (SAPKKs) have been identified in mammalian cells (Cuenda *et al.*, 1996; Meier *et al.*, 1996). *In vitro*, SAPKK1 [also termed MKK4 (Dérijard *et al.*, 1995), SEK1 (Sanchez *et al.*, 1994) and XMEK2 (Yashar *et al.*, 1993)] activates both SAPK1 (Sanchez *et al.*, 1994; Dérijard *et al.*, 1995) and SAPK2 (Sanchez *et al.*, 1994; Doza *et al.*, 1995), while SAPKK2 (also termed MKK3, Dérijard *et al.*, 1995) and SAPKK3 (Cuenda *et al.*, 1996) (also called MKK6, Han *et al.*, 1996; Moriguchi *et al.*, 1996; Raingeaud *et al.*, 1996; Stein *et al.*, 1996) activate SAPK2 but not SAPK1, and SAPKK4 and SAPKK5 activate SAPK1 but not SAPK2 (Meier *et al.*, 1996). The sequences of SAPKK4/SAPKK5 are unknown. SAPKK1 and SAPKK2 are the only activators of SAPK2 generated when rat pheochromocytoma (PC12) cells are exposed to chemical stress, osmotic shock, UV irradiation or the protein synthesis inhibitor anisomycin (Meier *et al.*, 1996). However, SAPKK3 is the dominant activator of SAPK2, and SAPKK4/SAPKK5 is/are the dominant activator(s) of SAPK1 when human epithelial (KB) cells are exposed to the same stresses as PC12 cells or stimulated with IL-1, or when human (THP1) monocytes are stimulated with LPS (Cuenda *et al.*, 1996; Meier *et al.*, 1996). The identity of the upstream activators of SAPK1 and SAPK2 may, therefore, vary from cell to cell.

The identity of the protein kinases which activate SAPKK1, SAPKK2 and SAPKK3 *in vivo* is unclear. At least five enzymes capable of activating these SAPKKs *in vitro* and/or in co-transfection experiments have been identified, namely MEK kinase (MEKK) (Yan *et al.*, 1994; Lin *et al.*, 1995; Matsuda *et al.*, 1995; Blank *et al.*, 1996), MAP kinase upstream kinase (MUK) (Hirai *et al.*, 1996), mixed lineage kinase-3 (MLK3) (Rana *et al.*, 1996), TGFβ-activated protein kinase-1 (TAK1) (Moriguchi *et el.*, 1996) and the proto-oncogene Tpl2 (Salmeron *et al.*, 1996). MEKK activates co-transfected SAPK1 more effectively than co-transfected SAPK2 or MAP kinase kinase-1 (MKK1, a physiological activator of p42 and of 44 MAP kinases) (Xu *et al.*, 1996). However, SAPKK1 can also be activated by MUK, MLK3 and Tpl2, and SAPKK2 by TAK1, while Tpl2 can also activate MAPKK1. Further work is needed to understand which (if any) of these kinases activate each SAPKK *in vivo* under different conditions.

Recently, a novel MAP kinase family member was cloned in one of our laboratories (Mertens *et al.*, 1996). This enzyme is most similar to SAPK2 (60% identity) and SAPK1 (47% identity) and, therefore, was called SAPK3. Like SAPK2, SAPK3 contains a TGY motif in the activation domain (which is TPY in SAPK1 and TEY in p42 and p44 MAP kinases) and subdomain VII is separated by six amino acids from the activation loop in subdomain VIII (as compared with eight residues in SAPK1 and >12 residues in any other MAP kinase family member). The mRNA encoding SAPK3 is present in all rat tissues examined (Mertens *et al.*, 1996), the highest levels being found in skeletal muscle. Very recently, a human homologue of SAPK3 was cloned and named ERK6 (Lechner *et al.*, 1996). Based on their finding that ERK6 mRNA was only detected in skeletal muscle and that its expression in the skeletal muscle cell line C2C12 enhanced differentiation to myotubes, these investigators proposed that it may play a specialized role in this tissue (Lechner *et al.*, 1996). However, we have also recently cloned human SAPK3 and found its mRNA to have a wide tissue distribution (our unpublished observations), similar to our previous results in rat (Mertens *et al.*, 1996). Here, therefore, we have investigated the mechanism of activation of SAPK3 and its substrate specificity *in vitro*. These studies have established that SAPK3 is activated by the same stimuli that activate SAPK2 and SAPK1, and that SAPKK3 mediates the activation of SAPK3 *in vivo* as well as *in vitro*. The substrate specificity of SAPK3 overlaps with (but is distinct from) SAPK1 and SAPK2, and SAPK3 is not inhibited by SB 203580. Our results raise the possibility that SAPK3, rather than SAPK1 or SAPK2, mediates the phosphorylation of transcription factors like ATF2.

Results

SAPK3 is activated by cellular stresses and cytokines

SAPK3 is most closely related to SAPK2, an enzyme activated by cellular stresses and the cytokines IL-1 and TNF (see Introduction). We therefore investigated whether the same stimuli would activate SAPK3. Human embryonic kidney epithelial 293 cells and human epithelial KB cells were transiently transfected with a myc epitope-tagged SAPK3 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 SAPK2 (or SAPK1) also activate SAPK3. Osmotic shock, the protein synthesis inhibitor anisomycin, a chemical stress (sodium arsenite), UV-C irradiation, IL-1 and TNF all activated SAPK3 5- to 14-fold (Figure 1A and B). Similar results were obtained when SAPK3 was transiently transfected into COS-7 cells and then exposed to osmotic shock, anisomycin or sodium arsenite (data not shown). Epidermal growth factor (EGF) activated transfected SAPK3 more weakly, while insulin-like growth factor-1 (IGF-1) and phorbol esters caused little or no activation (Figure 1A). In contrast, IGF-1 triggered a strong activation of transfected protein kinase B in parallel experiments (Alessi *et al.*, 1996).

Identification of SAPKK3 as the major activator of SAPK3 in epithelial cells

Lysates from 293 cells that had been osmotically shocked for 15 min were chromatographed on Mono S and assayed for activators of SAPK2 and SAPK3. As reported previously for other mammalian cells (Cuenda *et al.*, 1996; Meier *et al.*, 1996), two peaks of SAPK2 activator were identified. The first eluted at the same position as SAPKK1

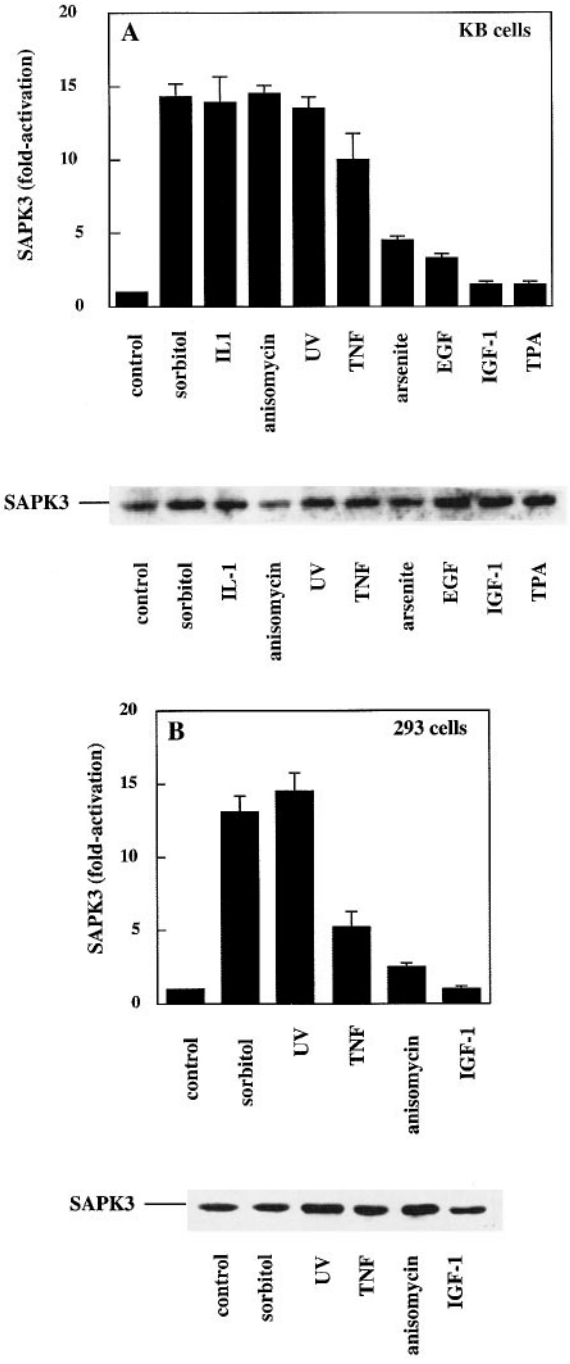

Fig. 1. SAPK3 is activated by cytokines and cellular stresses in KB and 293 cells. KB cells (**A**) or 293 cells (**B**) were transiently transfected with a DNA construct expressing a myc epitope-tagged SAPK3. Cells in DMEM were stimulated for 15 min with 0.5 M sorbitol (an osmotic shock), 20 ng/ml IL-1α, 100 ng/ml TNFα, 0.5 mM sodium arsenite (a chemical stress), 100 ng/ml of EGF or 300 ng/ml TPA, for 30 min with 10 μg/ml anisomycin or 10 min with 100 ng/ml IGF-1. UV-C irradiation of cells was carried out at 60 J/m^2 (KB cells) or 200 J/m^2 (293 cells) and the cells were then left for a further 30 min at 37°C before lysis. The myc epitope-tagged SAPK3 was immunoprecipitated from cell lysates using the 9E10 monoclonal antibody and assayed with MBP as substrate. The figure shows the fold activation of SAPK3 in response to each stimulus and the results are presented as ± SEM for three experiments. After UV-C irradiation, SAPK3 activity in the extracts was 0.18 U/mg (KB cells) and 0.1 U/mg (293 cells). Immunoblotting experiments showed that SAPK3 was expressed at a similar level in each experiment.

and SAPKK3 and the second at the same position as SAPKK2. Neither peak was observed in control experiments using lysates from unstimulated cells (Figure 2A). The first peak of activity was SAPKK3 (or a very closely related homologue), because it was immunoprecipitated quantitatively by anti-MKK6 antibodies, but not by anti-MKK4 or anti-MKK3 antibodies (Figure 2B). The second peak was immunoprecipitated by anti-MKK3 antibodies, but not by anti-MKK4 or anti-MKK6 antibodies (Figure 2B). When the same fractions were assayed for activators of SAPK3, only a single peak was detected which co-eluted with SAPKK3 (Figure 2C) and was immunoprecipitated by anti-MKK6, but not by anti-MKK4 or anti-MKK3 antibodies (Figure 2D). Identical results were obtained when the cells were stimulated with anisomycin (data not shown). No SAPK3 activator was observed in control experiments using lysates from unstimulated cells (Figure 2C).

We have shown previously that SAPKK3 accounts for 95–100% of the SAPK2 activator detected after Mono S chromatography of lysates from KB cells that have been stressed in several ways or stimulated with IL-1 (Cuenda *et al.*, 1996; Meier *et al.*, 1996). As shown in Figure 3, the single peak of SAPK3 activator detected after subjecting KB cells to osmotic shock or anisomycin co-migrated with SAPKK3 on Mono S and was immunoprecipitated quantitatively and specifically by anti-MKK6 antibodies. This experiment also demonstrated that SAPKK4 and SAPKK5, which are also activated in KB cells by these stimuli, and which elute from Mono S at a higher NaCl concentration than SAPKK3 (Meier *et al.*, 1996), do not activate SAPK3.

Further evidence that SAPKK3 can activate SAPK3 *in vivo* was obtained by co-transfection into COS cells. These experiments showed that SAPK3 activity was elevated 10-fold by co-expression with SAPKK3, and that exposure to osmotic shock triggered a 30-fold greater activation of transfected SAPK3 in cells where SAPKK3 had been co-transfected (Figure 4).

Activation of SAPK3 by SAPKK3 in vitro

SAPK3 was activated *in vitro* by a highly purified preparation of SAPKK3 from skeletal muscle (Figure 5), but could not be activated by MKK1 under conditions where p42 MAP kinase was activated maximally (data not shown). The rate of activation of SAPK3 by SAPKK3 was similar to that of SAPK2, and both enzymes attained the same specific activity towards myelin basic protein (MBP) after 1 h (Figure 5A and B). The activity of SAPK2 and SAPK3 towards MBP is 25- to 50-fold lower than the activity of p42 MAP kinase towards this substrate (Stokoe *et al.*, 1992b). No activation or phosphorylation of SAPK2 or SAPK3 occurred when SAPKK3 was inactivated by protein phosphatase 2A prior to incubation with SAPK2 and SAPK3 (Figure 5A and B). The activation of SAPK2 and SAPK3 was accompanied by the appearance of phosphotyrosine and phosphothreonine as expected, both residues being phosphorylated to a similar extent at low or high levels of activation of SAPK3 and SAPK2 (Figure 5C). Interestingly, SAPK3 (but not SAPK2) also became phosphorylated at a serine residue(s). This did not occur when wild-type SAPK3 was replaced by an inactive mutant (Figure 5D), suggesting that serine

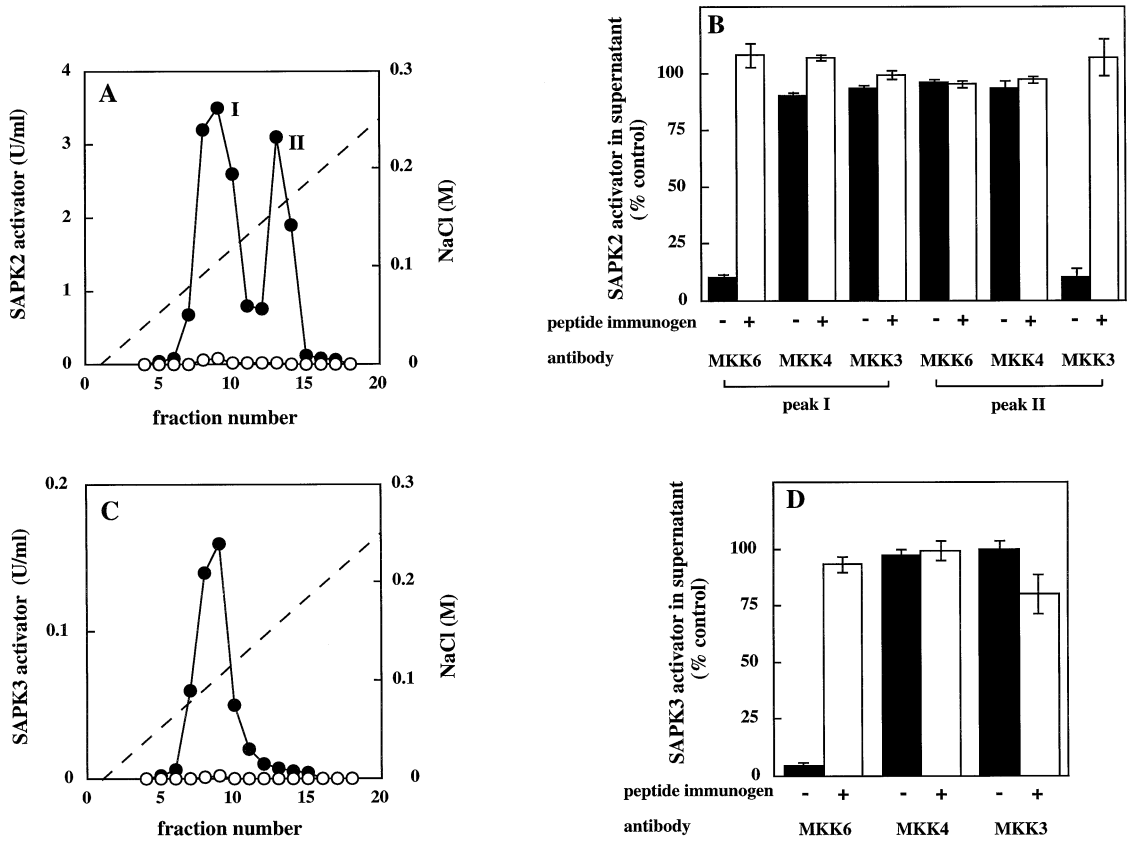

Fig. 2. SAPKK3 is the major SAPK3 activator in 293 cells. Cell lysates (0.8 mg of protein) from unstimulated 293 cells (○) or cells shocked osmotically for 15 min with 0.5 M sorbitol (●) were chromatographed on Mono S (5×0.16 cm) using a Pharmacia Smart System (Cuenda *et al.*, 1995). The fractions were assayed for activators of SAPK2 (**A**) or SAPK3 (**C**) as described in Materials and methods. The broken line shows the NaCl gradient. Similar results were obtained in three different experiments. The two major SAPK2 activators from (A) (I and II) were pooled separately and immunoprecipitated using anti-MKK6 antibodies, anti-MKK4 antibodies or anti-MKK3 antibodies. In (**B**), the amount of SAPK2 activator remaining in the supernatant is shown and in (**D**) the amount of SAPK3 activator remaining in the supernatant after immunoprecipitation in the presence (+) or absence (–) of the appropriate competing peptide immunogen (0.6 mM) is shown relative to control incubations (100%) where antibody was omitted. The results presented are the average ± SEM for three separate experiments.

phosphorylation is an autophosphorylation event catalysed by SAPK3 itself after it has been activated. The site of autophosphorylation is likely to be at Ser3 and/or Ser281, which are the only two serine residues in SAPK3 that are followed by proline (Mertens *et al.*, 1996).

Activation of SAPK3 by SAPKK1(MKK4) in vitro

SAPK2 is not only phosphorylated by SAPKK2 and SAPKK3 *in vitro*, but also by SAPKK1 (MKK4) (see Introduction). SAPKK1 is itself activated by cytokines and cellular stresses and it was of interest, therefore, to investigate whether this enzyme had the potential to activate SAPK3 *in vitro*. Figure 6 shows that SAPK3 can be activated by SAPKK1 *in vitro*, but the initial rate of activation of SAPK3 is much slower than that of SAPK2 under identical conditions (Figure 6). Consistent with this slow rate of activation, SAPK3, like SAPK2, was not activated after co-transfection in COS-1 cells with MEK kinase (Figure 4), an upstream activator of SAPKK1. This is consistent with the negligible contribution of SAPKK1 to the total SAPK3-activating activity observed in KB and COS cells.

Comparison of the substrate specificities of SAPK2 and SAPK3

SAPK3 was just as active as SAPK2 in phosphorylating the transcription factors Elk-1, ATF2, SAP1, SAP2 and p53, while c-Jun was only poorly phosphorylated by both enzymes (Table I). SAPK1 has been shown to phosphorylate ATF2 at Thr69, Thr71 and Ser90 (Livingstone *et al.*, 1995) and the phosphorylation of ATF2 by SAPK2 and SAPK3 was therefore studied in greater detail using wild-type ATF2 and mutants in which these residues had been changed to Ala. Phosphoamino acid analysis showed that, after phosphorylation by SAPK3, wild-type ATF2 was phosphorylated on threonine and serine residues, the phosphothreonine content being twice as high as the phosphoserine content (data not shown). In contrast, after phosphorylation by SAPK2, ATF2 was only phosphorylated on threonine (data not shown). Consistent with these experiments, a mutant ATF2 in which Thr69 and Thr71 were changed to alanine was phosphorylated by SAPK3, but not by SAPK2 (Figure 7) and phosphorylation of this mutant by SAPK3 occurred only on serine (data not shown). A mutant ATF2, in which Thr69, Thr71 and Ser90 were all changed to Ala, was not phosphorylated by either SAPK2 or SAPK3, and a mutant in which Ser90 was changed to Ala was phosphorylated at threonine residues by both SAPK2 and SAPK3 (Figure 7). These experiments indicate that SAPK2 and SAPK3 phosphorylate ATF2 at Thr69 and Thr71, and that only SAPK3 phosphorylates Ser90.

SAPK3 was far less effective than SAPK2 in activat-

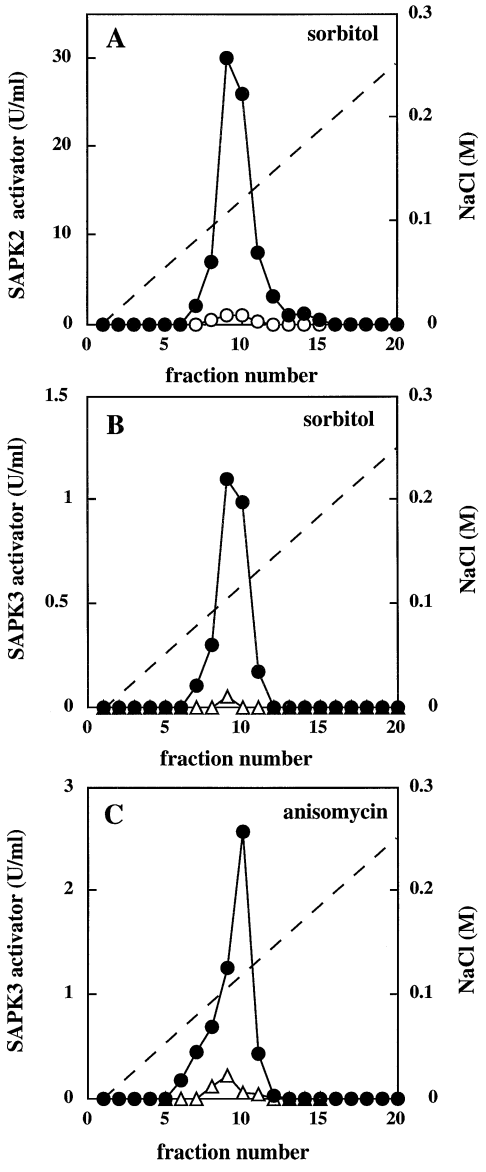

Fig. 3. SAPKK3 is the major activator of SAPK3 in KB cells. (**A**) and (**B**) Cell lysates (0.4 mg of protein) from unstimulated KB cells (○) or cells stimulated with 0.5 M sorbitol (●) were chromatographed on Mono S, and the fractions assayed for activators of SAPK2 (A) or for activators of SAPK3 (B). Aliquots of each fraction were immunoprecipitated using anti-MKK6 antibodies and the supernatants reassayed for activators of SAPK3 (△). No SAPK3 activator was detected in unstimulated cells (see Figure 1A). The broken lines show the NaCl gradient. Similar results were obtained in three different experiments. (**C**) Cells were stimulated for 30 min with 10 μg/ml anisomycin instead of sorbitol and assayed for SAPK3 activators before (●) and after (△) immunoprecipitation of SAPKK3.

ing GST–MAPKAP-K2(46–400) 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 8A and B). We have reported previously that p42 MAP kinase can activate GST–MAPKAP-K2(46–400) *in vitro*, but cannot activate a nearly full-length MAPKAP-K2 [GST–MAPKAP-K2(5–400)] (Ben-Levy *et al.*, 1995). SAPK3 was also unable to activate GST–MAPKAP-K2(5–400) significantly (Figure 8C).

The rate of phosphorylation of MAPKAP-K2 and

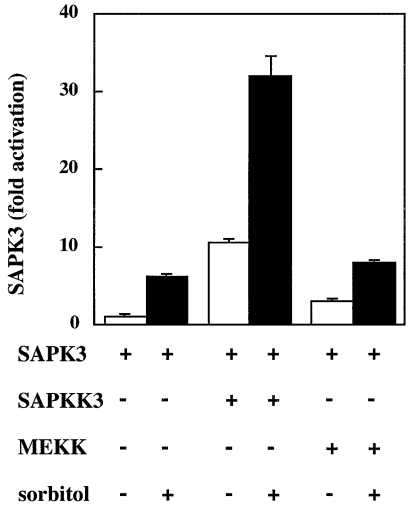

Fig. 4. SAPKK3 activates SAPK3 in transfected cells. COS-1 cells were transiently transfected with DNA expressing SAPK3 or co-transfected with either SAPKK3 or MEK kinase (MEKK). Cells were stimulated in DMEM for 15 min with 0.5 M sorbitol. After stimulation and lysis, SAPK3 was immunoprecipitated from the cell lysates using an anti-SAPK3 antibody raised against the C-terminal 16 residues of SAPK3 (see Materials and methods) and activity measured using MBP as the substrate. The figure shows the fold activation of SAPK3 in response to sorbitol and/or co-transfection with SAPKK3 relative to unstimulated cells transfected with SAPK3 alone.

MAPKAP-K3 by SAPK2 was similar to that of MBP under the same conditions used in Figure 1. Although SAPK3 was a much poorer activator of MAPKAP-K2 and MAPKAP-K3 than SAPK2, the initial rate of phosphorylation of MAPKAP-K2 and MAPKAP-K3 by SAPK3 was only 2- to 3-fold slower than by SAPK2. This implies that, like p42 MAP kinase, SAPK3 can phosphorylate sites on MAPKAP-K2 and MAPKAP-K3 that do not cause activation

SAPK3 is not inhibited by SB 203580

SAPK3 that had been expressed in *Escherichia coli* and activated by SAPKK3 (Figure 9), or myc epitope-tagged SAPK3 that had been immunoprecipitated from the lysates of osmotically shocked KB cells (data not shown) was not inhibited significantly by SB 203580, even at a concentration (0.1 mM) that was >100-fold higher than the IC_{50} for SAPK2 (0.6 μM).

Discussion

In this study, we demonstrate that SAPK3 is activated in response to the same cellular stresses and cytokines as SAPK1 and SAPK2. In two epithelial cell lines, the only activator of SAPK3 that could be detected was SAPKK3, the product of the *MKK6* gene that we have shown previously to be the dominant activator of SAPK2 in extracts prepared from monocytes and muscle, as well as epithelial cells (Cuenda *et al.*, 1996). SAPKK3 activated SAPK3 and SAPK2 at similar rates *in vitro*. Moreover, the stress-induced activation of SAPK3 was greatly enhanced by co-expression with SAPKK3. These observations indicate that SAPKK3 mediates the activation of SAPK3 by cellular stresses and cytokines in epithelial cells. SAPK3 was not activated at a significant rate by

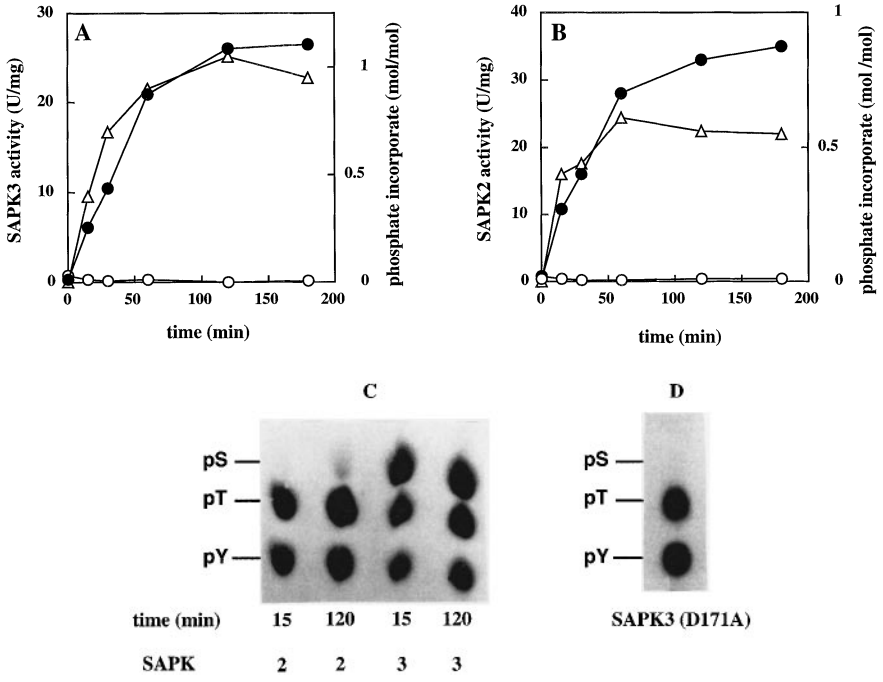

Fig. 5. Phosphorylation and activation of SAPK3 and SAPK2 by purified SAPKK3. (**A**) GST–SAPK3 (0.5 μM) and (**B**) the *Xenopus* homologue of SAPK2 (MalE–Mpk2, 0.5 μM) were incubated with unlabelled MgATP and 100 U/ml SAPKK3 and the generation of activity (●) measured at the times indicated using MBP as substrate. No activation of either GST–SAPK3 or MalE–Mpk2 occurred when SAPKK3 was first inactivated with PP2A (○). Phosphorylation of GST–SAPK3 or MalE–Mpk2 (△) was determined in parallel incubations using [γ-^{32}P]ATP (10^6 c.p.m. per nmol) and in (**C**) the 15 and 120 min time points from (A) and (B) were partially hydrolysed for 90 min in 6 M HCl at 110°C, electrophoresed on thin layer cellulose at pH 3.5 and autoradiographed to identify phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY). (**D**) Same as the 120 min time point in (C), except that wild-type SAPK3 was replaced by a 'kinase-inactive' mutant in which Asp171 was changed to Ala.

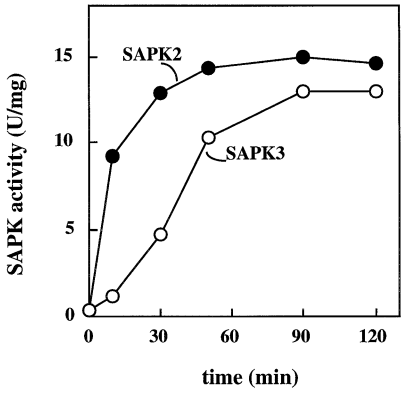

Fig. 6. Activation of SAPK3 and SAPK2 by SAPKK1 (MKK4). GST–SAPK3 (0.5 μM, ○) or a MalE fusion protein of the *Xenopus* homologue of SAPK2 (0.5 μM, ●) was incubated with unlabelled MgATP and 100 U/ml SAPKK1 and the generation of activity measured at the times indicated using MBP as substrate.

Table I. Comparison of substrate specificities of SAPK2 and SAPK3

Substrate (1 μM)	Rate of phosphorylation relative to MBP (%)	
	SAPK2	SAPK3
MBP	100	100
Elk-1	122 ± 22	181 ± 30
SAP1	55 ± 13	108 ± 15
ATF2	86 ± 20	107 ± 20
p53	13 ± 2	30 ± 1
SAP2	13 ± 3	20 ± 3
c-Jun	2 ± 1	2 ± 1

SAPK2 and SAPK3 were activated *in vitro* (Figure 5) and phosphorylation of each protein studied at a SAPK concentration of 0.1 U/ml.

MKK1, SAPKK1 (MKK4), SAPKK2 (MKK3), SAPKK4 or SAPKK5.

Although the amino acid sequence of SAPK3 is 60% identical to SAPK2, and both enzymes share the TGY phosphorylation motif and a six amino acid insertion between subdomain VII and the activation loop in subdomain VIII (see Introduction), the properties of SAPK3 differed from SAPK2 in several respects. Firstly, SAPK3 was not inhibited by SB 203580 (Figure 9). Secondly, SAPK3 but not SAPK2 phosphorylated ATF2 at Ser90 (Figure 7). Thirdly, SAPK3 was far less effective than SAPK2 in activating MAPKAP-K2 and MAPKAP-K3, 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 examined so far (Cuenda *et al.*, 1995; Beyaert *et al.*, 1996; Clifton *et al.*, 1996; McLaughlin *et al.*, 1996).

Despite the differences between SAPK3 and SAPK2 outlined above, these enzymes phosphorylated a number of proteins *in vitro* at similar rates (Table I), including the transcription factor ATF2. ATF2 has been suggested to be a physiogical substrate of SAPK1 and SAPK2, since it is phosphorylated in cells co-transfected with SAPKK1, MEKK (an upstream activator of SAPKK1) or SAPKK3 (see Introduction). However, transfection with upstream activators may not mimic the *in vivo* situation, for example by generating abnormally high levels of SAPK1 and SAPK2 activity. Moreover, the present work has raised another problem in the interpretation of these experiments

by demonstrating that SAPKK3 activates SAPK3 as well as SAPK2. Further experiments employing SB 203580 are essential, therefore, to evaluate whether SAPK2 or SAPK3 mediates the phosphorylation of ATF2 triggered by transfection with SAPKK3. However, even if SB 203580 prevents the phosphorylation of ATF2 induced by transfection with SAPKK3, it is unlikely that SAPK2 activity is rate limiting for ATF2 phosphorylation *in vivo*, because neither the TNF-induced (Beyaert *et al.*, 1996) nor the stress-induced (Hazzalin *et al.*, 1996) phosphorylation of the endogenous ATF2 in fibroblastic cell lines is affected by SB 203580 under conditions where this drug prevents the activation of MAPKAP-K2 and the induction of IL-6 and c-*fos* mRNA. Further work is needed to clarify whether the phosphorylation of ATF2 occurring under these conditions is catalysed by SAPK1, SAPK3 or another (as yet unidentified) SAPK.

SAPK3 and SAPK2 phosphorylated the transcription factor Elk-1 at similar rates (Table I) and, like ATF2, Elk-1 has been suggested to be a physiogical substrate of SAPK1 and SAPK2, because it becomes phosphorylated in cells that have been co-transfected with SAPKK1, MEKK or SAPKK3 (see Introduction). These experiments are subject to the same reservations as outlined above for ATF2, but a further caveat with Elk-1 is that it is also an effective substrate for p42 and p44 MAP kinases which are activated by several of the stresses and cytokines that activate SAPKs. Elk-1 (like SAP1 and SAP2) is an Ets domain protein and a member of the TCF family of transcription factors that form ternary complexes with the serum response factor (SRF) and bind to the serum response element (SRE) found in the promoters of a number of genes including c-Fos. The induction of c-*fos* mRNA in response to anisomycin or UV irradiation is largely suppressed by SB 203580 in C3HT101/2 fibroblasts (Hazzalin *et al.*, 1996), while in HeLa cells and NIH 3T3 cells the induction of c-Fos induced by UV irradiation was partially inhibited by SB 203580 and by PD 98059 [a specific inhibitor of the activation of MKK1 (Alessi *et al.*, 1995a)] and almost completely suppressed in the presence of both inhibitors (Price and Treisman, 1996). These observations demonstrate that SAPK2 activity is rate limiting in the transcriptional activation of the c-*fos* gene and that the activation of both SAPK2 and ERK1/ERK2 contributes to the induction of c-*fos* mRNA in UV-irradiated cells. However, whether the suppression of c-*fos* mRNA production by SB 203580 results from inhibition of the phosphorylation of Elk-1 or another TCF like SAP1 or SAP2, or by preventing the MAPKAP-K2-mediated phosphorylation of CREB (see Introduction), has not yet been established. The phosphorylation of Elk-1 induced by co-transfection with MEKK was prevented by PD 98059, indicating that ERK1/ERK2 mediate the phosphorylation of Elk-1 under these conditions. Earlier reports had claimed that SAPK1 was activated specifically when cells were transfected with low levels of MEKK DNA (Minden *et al.*, 1994), but the effects of PD 98059 (which has no effect on the activation of SAPK1; Alessi *et al.*, 1995a; Price and Treisman, 1996) indicate that this is not the case. Taken together, the results of Price and Treisman (1996) indicate that neither SAPK1 nor SAPK3 are rate

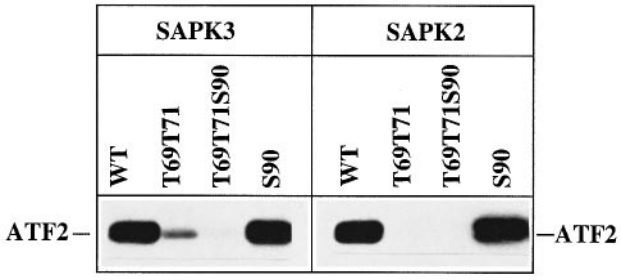

Fig. 7. Identification of the sites on ATF2 phosphorylated by SAPK3 and SAPK2. GST–SAPK3 and the *Xenopus* homologue of SAPK2 were matched for activity against MBP and each enzyme (0.4 U/ml) incubated for 30 min at 30°C with 1 μM wild-type (WT) or mutant GST–ATF2(19–96) and Mg[γ-^{32}P]ATP in a total volume of 50 μl. The reactions were stopped by adding 5 μl of 6% (by mass) SDS and the samples electrophoresed on 10% SDS–polyacrylamide gels and autoradiographed. Abbreviations: T69T71, mutant in which Thr69 and Thr71 were mutated to Ala; T69T71S90, mutant in which Thr69, Thr71 and Ser90 were mutated to Ala; S90, mutant in which Ser90 was mutated to Ala.

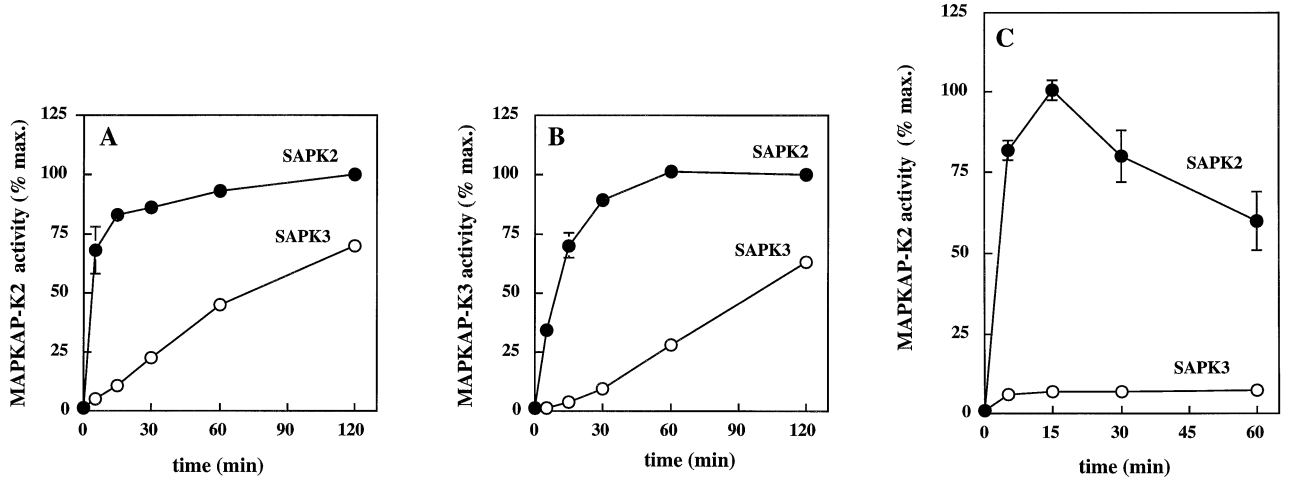

Fig. 8. Activation of MAPKAP-K2 and MAPKAP-K3 by SAPK2 and SAPK3. (**A**) GST–MAPKAP-K2(46–400), (**B**) GST–MAPKAP-K3 or (**C**) GST–MAPKAP-K2(5–400) (0.2 mg/ml), were incubated with unlabelled MgATP and 0.3 U/ml SAPK2 (●) or 0.3 U/ml SAPK3 (○) and activation measured at the times indicated. There was no phosphorylation or activation of MAPKAP-K2 or GST–MAPKAP-K3 when SAPK2 and SAPK3 were omitted.

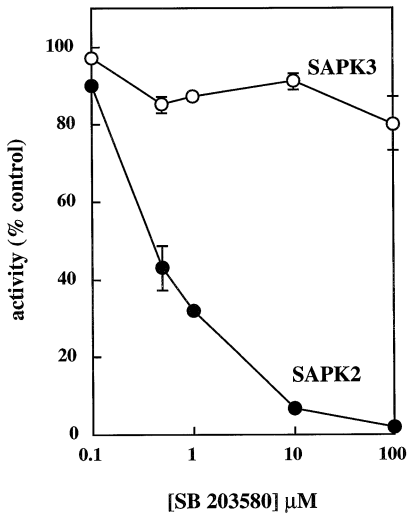

Fig. 9. Effect of SB 203580 on the activity of SAPK3 and SAPK2. Bacterially expressed SAPK3 (○) and SAPK2 (●) were activated *in vitro* with purified SAPKK3 (Figure 5) and then assayed in the presence of the indicated concentrations of SB 203580. The results are presented relative to control incubations in which the inhibitor was omitted.

limiting for the phosphorylation of Elk-1 and induction of c-*fos* mRNA in UV-irradiated HeLa cells. The development of specific inhibitors of the activity or activation of SAPK1 and SAPK3 will be needed to elucidate the physiological roles of these enzymes.

While this manuscript was in preparation, the amino acid sequence of a novel MAP kinase homologue was reported (Jiang *et al.*, 1996). This enzyme, called p38β, is 74% identical to SAPK2. Transfected p38β was activated in response to IL-1, TNF or stresses and appeared to be a relatively specific substrate of SAPKK3 (MKK6). Moreover, when activated, it was able to phosphorylate MAPKAP·K2 and was inhibited by SB 202190 (an analogue of SB 203580) at concentrations similar to those which inhibit SAPK2. In view of these similarities, we suggest that SAPK2 and p38β be called SAPK2A and SAPK2B, respectively (Figure 10), since they are uniquely sensitive to the cytokine synthesis anti-inflammatory drugs (CSAIDs) that include SB 203580 and SB 202190.

Materials and methods

Materials

Tissue culture reagents, microcystin-LR, Lipofectin reagent, MBP and human IGF-1 were purchased from Gibco-BRL (Paisley, UK), protein G–Sepharose from Pharmacia (Milton Keynes, UK), and recombinant human IL-1α and EGF from Boehringer; anisomycin, sorbitol and TNFα from Sigma (Poole, UK). PKI, the specific peptide inhibitor of cAMP-dependent protein kinase (TTYADFIASGRTGRRNAIHD) and all other peptides were synthesized by Mr F.B.Caudwell in the MRC Protein Phosphorylation Unit. SB 203580, a generous gift from Dr J.Lee (SmithKline Beecham, King of Prussia, USA), was dissolved in dimethylsulfoxide to give a final concentration of 20 mM. Other reagents were of analytical grade or better, and purchased from BDH Chemicals or Sigma Chemical Co. (Poole, UK).

Enzymes and transcription factors

SAPKK3 was purified from rabbit skeletal muscle (Cuenda *et al.*, 1996). The *E.coli* plasmids encoding glutathione *S*-transferase (GST) fusion proteins were kindly provided by the investigators shown in parentheses, expressed in *E.coli* and purified by affinity chromatography on glutathione–agarose: GST–MAPKAP-K2(5–400) and GST–MAPKAP-K2 (46–400) (Dr R.Ben-Levy and Dr C.Marshall, Institute for Cancer Research, London, Ben Levy *et al.*, 1995), GST–MAPKAP-K3 (Dr J.Young, SmithKline Beecham, King of Prussia, USA), GST–MKK1 (Dr C.Marshall), GST–MKK4 (Dr J.Woodgett, Ontario Cancer Institute, Canada), GST–SAP1(267–431), GST–SAP2(221–417), GST–Elk-1(307–428), GST–cJun(1–194), GST–ATF2(19–96) and various derivatives of GST–ATF2(19–96) in which Thr69, Thr71 and/or Ser90 were mutated to Ala (Drs N.Jones and R.Treisman, ICRF, London). MalE fusion proteins expressing the C-terminal kinase domain of MEK kinase (MalE–MEKK) and the *Xenopus* homologue of SAPK2 (MalE–Mpk2) were generous gifts from Dr A.R.Nebreda (EMBL, Heidelberg, Germany). DNA expressing the C-terminal kinase domain of MEK kinase for transfection studies (Olson *et al.*, 1995) was provided by Dr A.Ashworth (Institute of Cancer Research, London). p53 (Helps *et al.*, 1995) was provided by Dr N.Helps in the MRC Protein Phosphorylation Unit.

SAPK3 and SAPKK3 expression plasmids

For bacterial expression, the open reading frame (ORF) of cDNA clone rSAPK37 (Mertens *et al.*, 1996) was amplified by PCR and subcloned as an *Eco*RI fragment into expression vector pGEX-4T1 (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). GST–SAPK3 was purified by affinity chromatography on glutathione–agarose. For transfections, the ORF of cDNA clone rSAPK37 was amplified by PCR and subcloned as an *Eco*RI fragment into the mammalian expression vector pSG5 (Stratagene). Alternatively, PCR was used to introduce a nucleotide sequence encoding the c-myc tag MEQKLISEEDLN at the carboxy-terminus of rSAPK3, followed by a stop condon. The resulting fragment was then ligated into pSG5. A bacterial expression construct encoding human SAPKK3 (Cuenda *et al.*, 1996) was a kind gift from Dr A.R.Nebreda. The ORF of human SAPKK3 was amplified by PCR and subcloned as a *Hin*dIII fragment into the mammalian expressing plasmid pcDNA3.1 (Invitrogen). Substitution of Asp171 by Ala in SAPK3 to produce a kinase-inactive mutant was performed by site-directed mutagenesis. PCR fragments were verified by DNA sequencing.

Cell culture and transient transfections

KB, 293 and COS-7 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), at 37°C, in an atmosphere of 5% CO_2. Transfections of 293 cells were carried out using the calcium phosphate method. Cells were split to a density of 2×10^6 per 10 cm dish and, after 12 h at 37°C, 10 μg of plasmid DNA in 0.45 ml of sterile water was added to 50 μl of sterile $CaCl_2$, and then 0.5 ml of sterile buffer composed of 50 mM *N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid/HCl pH 6.96, 0.28 M NaCl and 1.5 mM Na_2HPO_4 was added. The resulting mixture was vortexed for 1 min, allowed to stand at room temperature for 20 min, and then added dropwise to a 10 cm dish of 293 cells. The cells were placed in an atmosphere of 3% CO_2 for 16 h at 37°C, then the medium was aspirated and replaced with new DMEM containing 10% FCS. The cells were incubated for 24 h at 37°C in an atmosphere of 5% CO_2 before stimulation. Plasmid DNA was transfected into KB cells and COS-1 cells by the lipofectin method, as recommended by the suppliers. After splitting cells to a density of 1×10^6 per 6 cm dish and incubation for 24 h at 37°C in an atmosphere of 5% CO_2, the cells were washed once with 2 ml of serum-free growth medium, and 2 μg of DNA (previously incubated for 15 min at room temperature with 15 μg of lipofectin reagent) was added. After 6 h at 37°C in an atmosphere of 5% CO_2, the DNA-containing medium was replaced with new DMEM containing 10% FCS. After a further 48 h, the cells were stimulated with the agonists indicated in the figure legends. Cells were incubated in DMEM for 12 h in the absence of serum before stimulation with IGF-1 or for 1 h before exposure to other stimuli. Cells were lysed as described (Rouse *et al.*, 1994), except that 2 μM microcystin was present in the lysis buffer.

Protein kinase assays

SAPK3 was assayed routinely by the phosphorylation of MBP. SAPK3 (0.5 or 1 μM) in 40 μl of 25 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 0.1 mM sodium orthovanadate, 1 μM PKI and 0.33 mg/ml MBP was incubated for 3 min at 30°C before initiating the reaction with 10 μl of 50 mM magnesium acetate–0.5 mM [γ-^{32}P]ATP. After 20 min at 30°C, 40 μl aliquots were withdrawn, spotted on to 1.5×1.5 cm squares of Whatman P81 phosphocellulose paper and immersed in 75 mM phosphoric acid. After washing and drying the papers (Alessi *et al.*, 1995b),

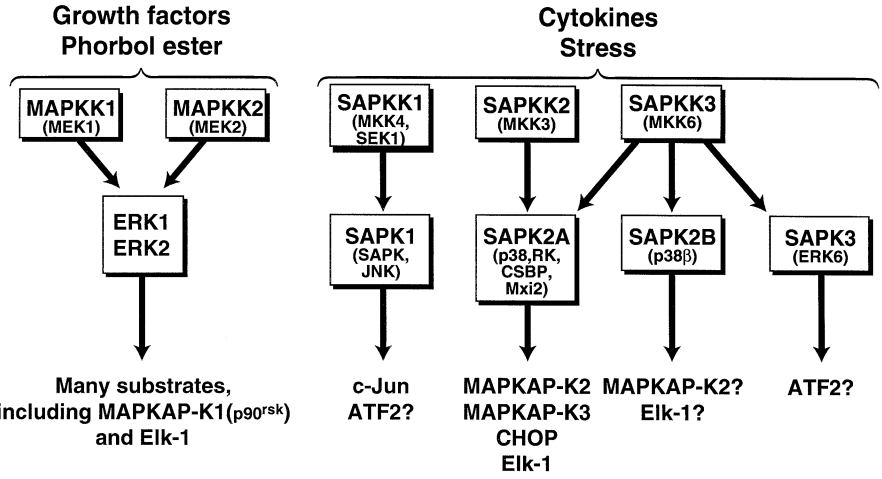

Fig. 10. Schematic representation of mammalian MAP kinase and SAP kinase signal transduction pathways. Following growth factor or phorbol ester stimulation, the MAP kinase kinases MAPKK1 and MAPKK2 (also termed MEK1 and MEK2) activate the ERK1 and ERK2 group of MAP kinases. ERK1 and ERK2 (also termed p44 and p42 MAP kinases, respectively) phosphorylate a number of substrates, including the protein kinase MAPKAP-K1 (also termed $p90^{rsk}$) and the transcription factor Elk-1. SAP kinase (SAPK) pathways are activated by proinflammatory cytokines and a number of stressful stimuli. The SAP kinase kinase SAPKK1 (also termed MKK4 and SEK1) activates SAPK1 (also termed SAPK or JNK) which phosphorylates the transcription factor c-Jun. SAPKK2 (also termed MKK3) activates SAPK2A (also termed p38, RK, CSBP and Mxi2), which phosphorylates the protein kinases MAPKAP-K2 and MAPKAP-K3, as well as the transcription factors CHOP and Elk-1. The SAP kinase kinase SAPKK3 (also termed MKK6) activates SAPK2A, SAPK2B (also termed p38β) and SAPK3 (also termed ERK6). Studies with the specific inhibitor SB 203580 indicate that SAPK2A (and perhaps SAPK2B) phosphorylate the protein kinases MAPKAPK-2 and MAPKAPK-3, as well as the transcription factors CHOP and Elk-1. The reason why 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 and/or SAPK3 *in vivo*. SAPKK1 activates SAPK2A and SAPK2B *in vitro* and in co-transfection experiments, but is not thought to activate these enzymes *in vivo*. Question marks indicate that a given SAPK phosphorylates a target protein *in vitro*, with no current evidence for a corresponding role *in vivo*.

^{32}P radioactivity incorporated into MBP was measured. One unit of activity was that amount of enzyme which incorporated 1 nmol of phosphate into MBP in 1 min.

SAPK3 activators were assayed by their ability to activate GST–SAPK3. A 15 µl Mono S fraction was incubated for 3 min at 30°C with 2.5 µl of 10 µM GST–SAPK3 in 50 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 0.03% (by mass) Brij-35, 0.1% (by vol.) 2-mercaptoethanol and 5% (by vol.) glycerol, and the reaction was initiated with 2.5 µl of 80 mM magnesium acetate–0.8 mM unlabelled ATP. After 30 min, an aliquot (10 µl) was withdrawn and assayed for SAPK3 activity as described above. Control experiments were carried out in which GST–SAPK3 was omitted from the incubation mixture. One unit of SAPK3 activator was that amount which increased the activity of SAPK3 by 1 U/min.

MAPKAP-K2 and MAPKAP-K3 were assayed using the peptide KKLNRTLSVA as substrate (Stokoe *et al.*, 1993) and one unit of activity was that amount which catalysed the phosphorylation of 1 nmol of peptide substrate in 1 min. SAPK2 was measured by the activation of GST–MAPKAP-K2(46–400) and SAPK2 activators by their ability to activate MalE–Mpk2 (the *Xenopus* homologue of SAPK2) (Meier *et al.*, 1996).

Activation and stoichiometric phosphorylation of GST–SAPK3 and MalE–Mpk2 by SAPKK3 and SAPKK1

The incubations contained 25 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 1 µM PKI, 0.1% (by vol.) 2-mercaptoethanol, 0.5 µM GST–SAPK3 or MalE–Mpk2, purified SAPKK3 or SAPKK1 (100 U/ml, see Cuenda *et al.*, 1996 for definition of units), 10 mM magnesium acetate and 0.1 mM ATP. The reaction was initiated with MgATP after pre-incubating the other components for 3 min at 30°C. At various times, aliquots were removed and assayed for SAPK3 or SAPK2 activity as described above. To determine the extent of phosphorylation of SAPK3 and SAPK2, parallel incubations were carried out in which unlabelled ATP was replaced by [γ-^{32}P]ATP (2×10^6 c.p.m./nmol). At various times, aliquots of the reaction were added to a 1 ml of 20% (by mass) trichloroacetic acid (TCA). After centrifugation for 5 min at 13 000 *g*, the supernatant was discarded and the pellet washed three times with 25% TCA and analysed by Cerenkov counting. Phosphorylation stoichiometries were determinated using the calculated molecular masses of each fusion protein and the protein concentration determined according to Bradford (1976).

Immunoprecipitation of SAPK3

Lysates of cells transfected with SAPK3 were centrifuged at 4°C for 10 min at 13 000 *g*. Aliquots of the supernatant (100 µg protein) were incubated for 120 min on a shaking platform with 5 µl of protein G–Sepharose coupled to either 3 µg of 9E10 monoclonal antibody which recognizes the myc epitope (for myc-epitope tagged SAPK3) or 5 µg of affinity-purified polyclonal antibody raised in sheep against the C-terminal sequence of rat SAPK3 (KPPRNLGARVPKETAL) (for untagged SAPK3). The suspension was centrifuged for 1 min at 13 000 *g*, and the pellet washed twice with 1 ml of lysis buffer containing 0.5 M NaCl, and twice with lysis buffer, and the immunoprecipitate assayed for SAPK3 activity as described above, except that the reactions were carried out on a shaking platform to ensure that the agarose beads remained in suspension and therefore had access to the substrate.

Antibodies and immunoprecipitation of SAPKKs

Polyclonal anti-MKK3 antibodies (raised against the peptide RNLDSRTFITIGDRN corresponding to a sequence near the N-terminus of MKK3), anti-MKK4 antibodies (raised against the peptide EQMPVSPSSPMYVD corresponding to the C-terminal 14 residues of XMEK2) and 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). Aliquots (30 µl) of Mono S-purified SAPK2 activators or SAPK3 activator were incubated at 4°C on a shaking platform with 5 µl of protein G–Sepharose coupled to 5 µg of anti-MKK3, 5 µl of anti-MKK4 or 2 µg of anti-MKK6 antibodies. After mixing for 90 min, the suspensions were centrifuged for 2 min at 13 000 *g* and the supernatants assayed for SAPK3 activator. In control experiments, antibodies bound to protein G–Sepharose were incubated for 30 min at 4°C with 50 nmol of the appropriate peptide immunogen prior to immunoprecipitation.

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