A comparison of the substrate specificity of MAPKAP kinase-2 and MAPKAP kinase-3 and their activation by cytokines and cellular stress

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Abstract MAPKAP kinase-2 and MAPKAP kinase-3 were both activated in response to cellular stress, interleukin-1 and tumour necrosis factor in KB and HeLa cells, and with identical kinetics. Activation of MAPKAP kinase-3, like MAPKAP kinase-2, was prevented by SB 203580, a specific inhibitor of SAPK-2, the upstream activator of MAPKAP kinase-2. MAPKAP kinase-3 and MAPKAP kinase-2 phosphorylated peptide substrates with similar kinetic constants and phosphorylated the same serine residues in HSP27 at the same relative rates. These results establish that MAPKAP kinase-3 lies 'downstream' of SAPK-2 and that it is likely to have overlapping or identical substrates to MAPKAP kinase-2 in vivo.

Key words: MAPKAP kinase; MAP kinase; Stress; Cytokine; HSP27

1. Introduction

MAP kinase-activated protein kinase-2 (MAPKAP-K2) is a serine/threonine-specific protein kinase that is activated by p42 and p44 MAP kinases in vitro, but distinct from MAPKAP kinase-1 (also known as $p90^{rsk}$) [1,2]. In vivo, however, MAPKAP-K2 is activated by a distinct MAP kinase homologue termed stress-activated protein kinase-2 (SAPK-2), also known as p38 [3], p40 [4], RK [5], CSBP [6] and Mxi2 [7]. SAPK-2 and MAPKAP-K2 become activated within a few minutes when cells are stimulated with the cytokines interleukin-1 (IL-1) [4] or tumour necrosis factor (TNF) [8], with bacterial lipopolysaccharide (LPS) [6,9] or when stressed in a variety of ways, for example by exposure to heat or osmotic shock, UV irradiation, sodium arsenite or anisomycin [3,5,10]. The activation of MAPKAP-K2 by these stimuli is prevented if cells are first incubated with SB 203580, a specific inhibitor of SAPK-2 which does not affect the activity or activation of other MAP kinase family members, such as p42/p44 MAP kinases or SAPK-1 (also termed JNK) [9].

MAPKAP-K2 consists of a proline-rich N-terminal domain, a catalytic domain which is most similar (35–40% identity) to the subfamily that include Ca^{2+}/calmodulin-dependent protein kinases and a C-terminal tail which contains a putative nuclear localisation signal [2]. The activation of MAPKAP-K2 by SAPK-2 in vitro results from the phosphorylation of Thr-222 and Ser-272 in the catalytic domain and Thr-334 in the C-terminal tail. Phosphorylation of any two of these three residues is sufficient for maximal activation [11]. These residues also become phosphorylated in vivo in an SB 203508 sensitive manner when KB cells are stimulated with sodium arsenite [11].

Studies on the substrate specificity of MAPKAP-K2 have established that it preferentially phosphorylates serine residues in sequences where a bulky hydrophobic residue is present at position n-5 and an arginine at position n-3 (where 'n' is the site of phosphorylation) [2]. These findings led to the discovery that heat shock protein (HSP) 27 [9,12] and the transcription factor CREB [13] are physiological substrates for MAPKAP-K2 or a closely related homologue. MAPKAP-K2 phosphorylates the same serine residues in HSP27 and CREB in vitro that become phosphorylated in response to cytokines and cellular stress in vivo, and the phosphorylation of HSP27 and CREB induced by these stimuli is prevented by SB 203580 at concentrations similar to those which prevent the activation of MAPKAP-K2. HSP27 is an actin-binding protein whose phosphorylation appears to stimulate the polymerisation of actin. This is thought to help repair the actin microfilament network that becomes disrupted during cellular stress, thereby aiding cell survival [14]. The phosphorylation of CREB at Serine-133 is essential to allow this transcription factor to transactivate genes whose promoters contain cyclic AMP responsive elements (CREs). The phosphorylation of CREB at Ser-133 may represent one of the mechanisms by which cellular stresses induce the transcription factor c-fos, since the production of c-fos mRNA in response to these stimuli is prevented by SB 203580 [15] and the importance of the CRE in the c-fos promoter for the transcription of this gene has been well documented [16,17].

A protein kinase, termed MAPKAP-K3, has recently been identified using the yeast 'two hybrid' system to identify proteins that interact with SAPK-2 and shown to be activated by SAPK-2 in vitro [18]. DNA encoding the same protein kinase was also identified while sequencing a region of human chromosome 3 that is frequently deleted in small cell lung carcinomas [19]. The amino acid sequence of MAPKAP-K3 is 75% identical to MAPKAP-K2 and possesses the key features of the latter enzyme, including the proline-rich N-terminus, the nuclear localisation signal at the C-terminus, and conservation of Thr-222, Ser-272 and Thr-334 and the amino acid sequences surrounding these residues. Here we have generated immunoprecipitating antibodies that recognise MAPKAP-K2 and MAPKAP-K3 specifically, and exploited them to make a detailed comparison of the activation of the endogenous enzymes in several cell lines by cellular stresses and cytokines. We have also compared their substrate specificities and find that, in contrast to an earlier report [19], the substrate preferences of MAPKAP-K2 and MAPKAP-K3 are similar.

2. Materials and methods

2.1. Materials

MAPKAP-K2 purified from rabbit skeletal muscle was provided by

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Dr. N. Morrice in the MRC Protein Phosphorylation Unit and CREB was a generous gift from Dr. M. Comb (New England Biolabs, Beverly, MA). TNFα was from Sigma and the sources of other materials are given in [5,9].

2.2. Expression of MAPKAP-K2 and MAPKAP-K3 in E. coli*: purification and activation by SAPK-2*

Escherichia coli strain BL21, transformed with pGEX-5X-CL1 DNA (encoding human MAPKAP-K3 [18]) was induced with 30 μM isopropyl-β-D-thiogalactoside for 20 h at 22°C. The glutathione-S-transferase (GST)-MAPKAP-K3 fusion protein was purified by affinity chromatography on glutathione-Sepharose (Sigma) and showed two bands migrating on SDS/polyacrylamide gels with apparent molecular masses of 67 and 65 kDa. A fusion protein consisting of GST followed by residues 46–400 of MAPKAP-K2 was expressed in *E. coli* and purified as described [11]. This preparation also migrates as two bands with apparent molecular masses of 65 and 62 kDa. Each enzyme was activated by incubation with the *Xenopus* homologue of SAPK-2 [11] which had been expressed in *E. coli* [5] and then activated by the MAP kinase kinase homologue MKK6 from skeletal muscle [20]. Only the 65 kDa band of MAPKAP-K2 [11] and the 67 kDa band of MAPKAP-K3 are phosphorylated by SAPK-2. Activated MAPKAP-K2 and MAPKAP-K3 were mixed with equal volumes of glycerol and stored unfrozen at −20°C.

2.3. Protein kinase assays

MAPKAP-K2 and MAPKAP-K3 were diluted in 20 mM MOPS, pH 7.0, containing 1 mM EDTA, 0.01% Brij-35, 5% (v/v) glycerol, 0.1% 2-mercaptoethanol and 0.2 mg/ml bovine serum albumin and then assayed [2] using the peptide KKLNRTLSVA (30 μM) unless stated otherwise. One unit of activity (U) was that amount which catalysed the phosphorylation of 1 nmol of KKLNRTLSVA in 1 min.

2.4. Cell culture, stimulation and lysis

KB [4] and HeLa [5] cells were cultured as described previously, incubated for 1 h with or without 10 μM SB 203580, then exposed to either chemical (0.5 mM sodium arsenite) or osmotic (0.5 M sorbitol) stress, or stimulated with IL-1 (20 ng/ml), TNFα (100 ng/ml) or anisomycin (10 μg/ml) for the times indicated in the figures. Each 6 cm dish of cells was lysed in 0.2 ml of buffer as described [5], except that 2 μM microcystin was also present in the lysis buffer.

2.5. Chromatography of cell lysates on Mono S

Cell lysates (0.8 mg protein) were chromatographed on Mono S as described [9] using a Pharmacia Smart System.

2.6. Preparation of antibodies and immunoprecipitation

The peptides KQAGSSSASQGCNNQG (corresponding to the C-terminal 15 residues of human MAPKAP-K3) and KEDKERWEDVKEEMTS (corresponding to residues 343–358 of MAPKAP-K2) were synthesized and conjugated to bovine serum albumin and/or keyhole limpet haemocyanin using glutaraldehyde and injected into sheep at the Scottish Antibody Production Unit (Carluke, Ayrshire). The antisera were affinity purified on peptide-agarose columns and the antibodies used to immunoprecipitate MAPKAP-K3 or MAPKAP-K2 essentially as described in [13]. Further details are given in the figure legends.

3. Results

3.1. Generation of antibodies that immunoprecipitate MAPKAP-K2 and MAPKAP-K3 specifically

Antibodies raised against residues 343–358 of human MAPKAP-K2 immunoprecipitated MAPKAP-K2 activity specifically but did not immunoprecipitate MAPKAP-K3 activity. Conversely, antibodies raised against the last 15 residues of

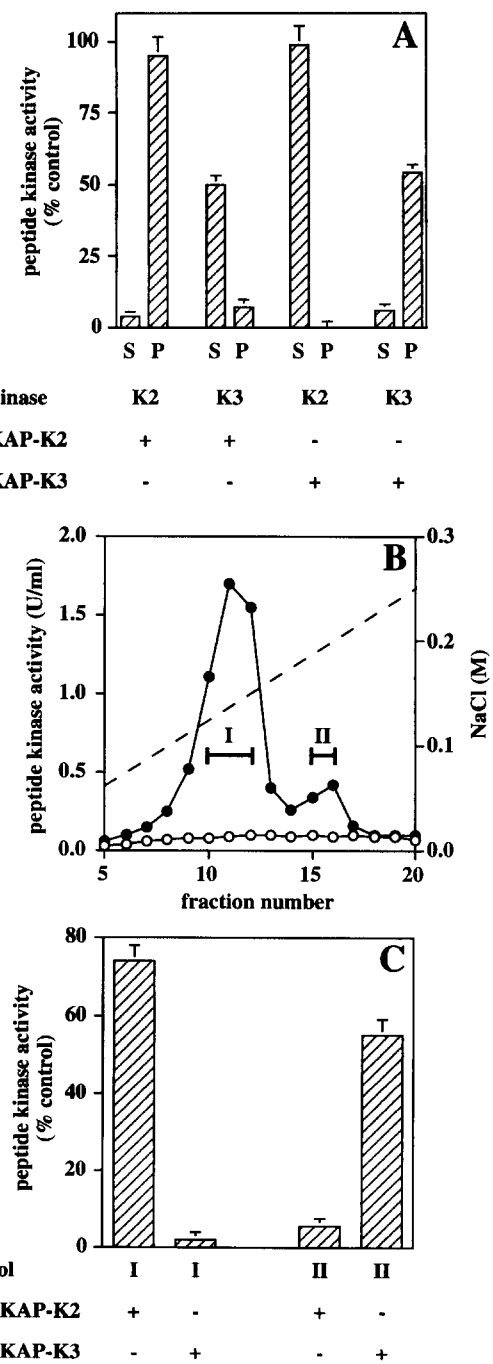

Fig. 1. Generation of antibodies that immunoprecipitate MAPKAP-K2 and MAPKAP-K3 specifically. (A) MAPKAP-K2 (K2) and MAPKAP-K3 (K3), expressed in *E. coli* and activated by SAPK-2 (Section 2.2), were immunoprecipitated with anti-MAPKAP-K2 (3 μg) or anti-MAPKAP-K3 (10 μg) antibodies. The peptide kinase activity towards KKLNRTLSVA remaining in the supernatant (S) or recovered in the immunoprecipitate (P) is presented as a percentage of the activity measured in control incubations where antibodies were replaced by buffer. The results are presented as standard errors for three experiments. (B) A lysate from anisomycin-stimulated KB cells (0.8 mg protein) was chromatographed on Mono S (Section 2.5) at a flow rate of 0.2 ml/min. Fractions (0.1 ml) were assayed for peptide kinase activity using KKLNRTLSVA (●). The open circles (○) show the result of a separate experiment in which anisomycin was omitted. The broken line (– – –) shows the NaCl gradient and the horizontal bars the fractions pooled. (C) Pools I and II from B were immunoprecipitated with anti-MAPKAP-K2 (1 μg) and anti-MAPKAP-K3 (10 μg) antibodies and the figure shows activity (measured as in A) recovered in the immunoprecipitates relative to control incubations in which the antibodies were replaced by buffer. The results are presented as standard errors for three experiments.

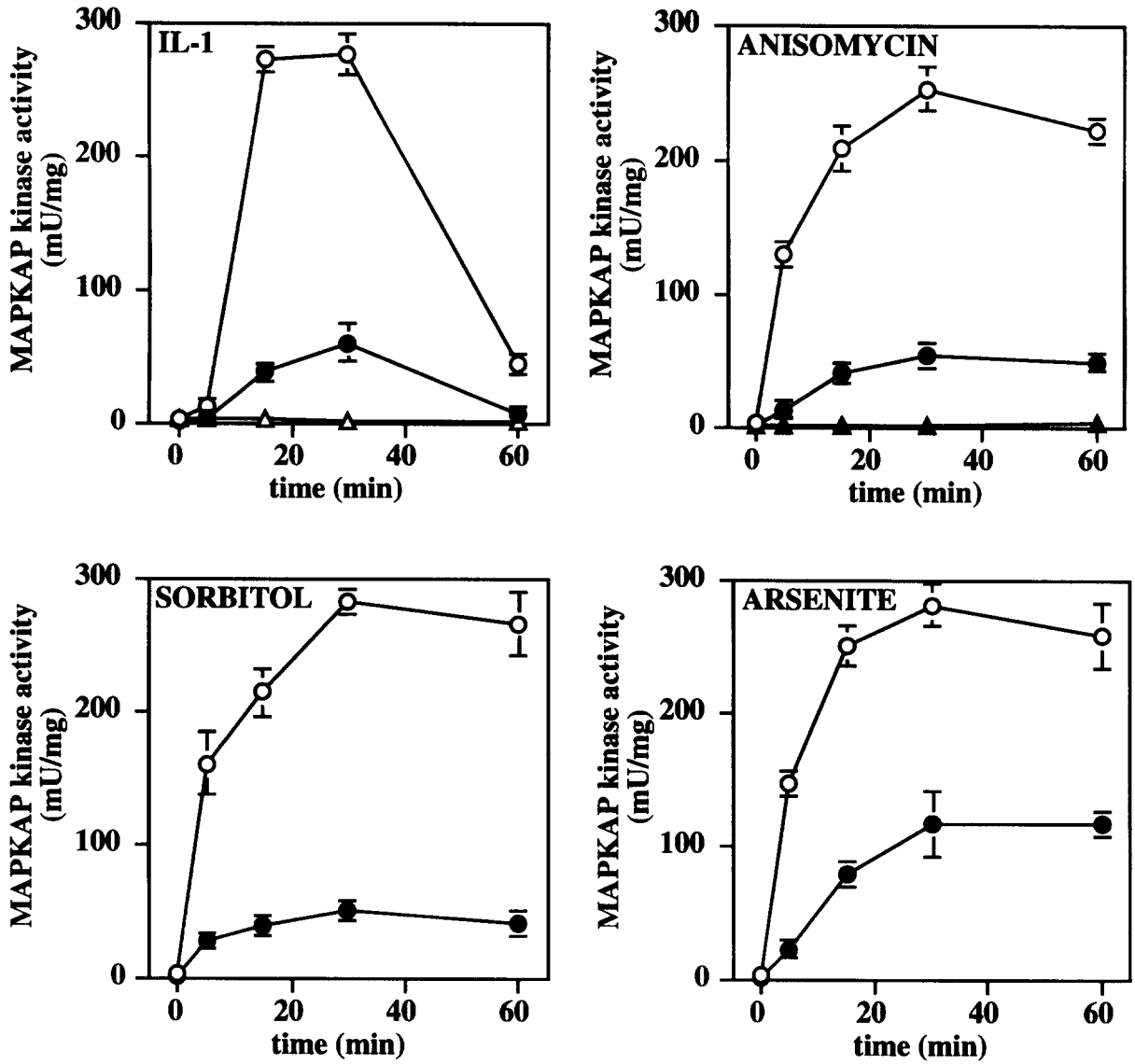

Fig. 2. Activation of MAPKAP-K2 and MAPKAP-K3 by IL-1 and cellular stresses in KB cells. Cells were stimulated for the indicated times with 20 ng/ml IL-1, 10 μg/ml anisomycin, 0.5 M sorbitol or 0.5 mM sodium arsenite. MAPKAP-K2 (○) and MAPKAP-K3 (●) were assayed after immunoprecipitation of these enzymes from cell lysates (50 μg protein) with 3 μg of anti-MAPKAP-K2 and 10 μg of anti-MAPKAP-K3. The open triangles (△) show MAPKAP-K2 activity and the closed triangles (▲) MAPKAP-K3 activity in cell lysates from unstimulated cells. The results are shown as standard errors for four separate immunoprecipitations using two dishes of cells.

MAPKAP-K3 immunoprecipitated MAPKAP-K3 activity specifically, but did not immunoprecipitate MAPKAP-K2 activity (Fig. 1A).

In order to further establish the specificity of each antibody and to identify MAPKAP-K3, lysates from anisomycin-stimulated KB cells were chromatographed on Mono S and the fractions assayed for protein kinase activity towards KKLNRTLSVA, the standard substrate for MAPKAP-K2 (Fig. 1B). These experiments demonstrated that the major peak of activity corresponded to MAPKAP-K2 and the minor peak, eluting at a higher salt concentration, to MAPKAP-K3 (Fig. 1C).

3.2. Activation of MAPKAP-K3 and MAPKAP-K2 by cellular stress and cytokines

MAPKAP-K3 and MAPKAP-K2 were immunoprecipitated from the lysates of KB cells and HeLa cells previously stimulated with cytokines or exposed to cellular stress. These experiments demonstrated that MAPKAP-K3 was activated by several stimuli known to activate MAPKAP-K2 with similar kinetics in each case (Figs. 2 and 3). Consistent with the results presented in Fig. 1B, MAPKAP-K3 accounted for 15–20% and MAPKAP-K2 for 80–85% of the activity immunoprecipitated from the lysates with every stimulus, except for arsenite which induced a higher level of activation of MAPKAP-K3 (Figs. 2 and 3). The activation of MAPKAP-K3, like MAPKAP-K2, was largely prevented by SB 203580 in both KB cells (Fig. 4) and HeLa cells (data not shown).

3.3. Comparison of the substrate specificity of MAPKAP-K3 with that of MAPKAP-K2

In the present study, we activated MAPKAP-K3 and MAPKAP-K2 with SAPK-2 and compared their specificities towards a panel of synthetic peptides previously used to study

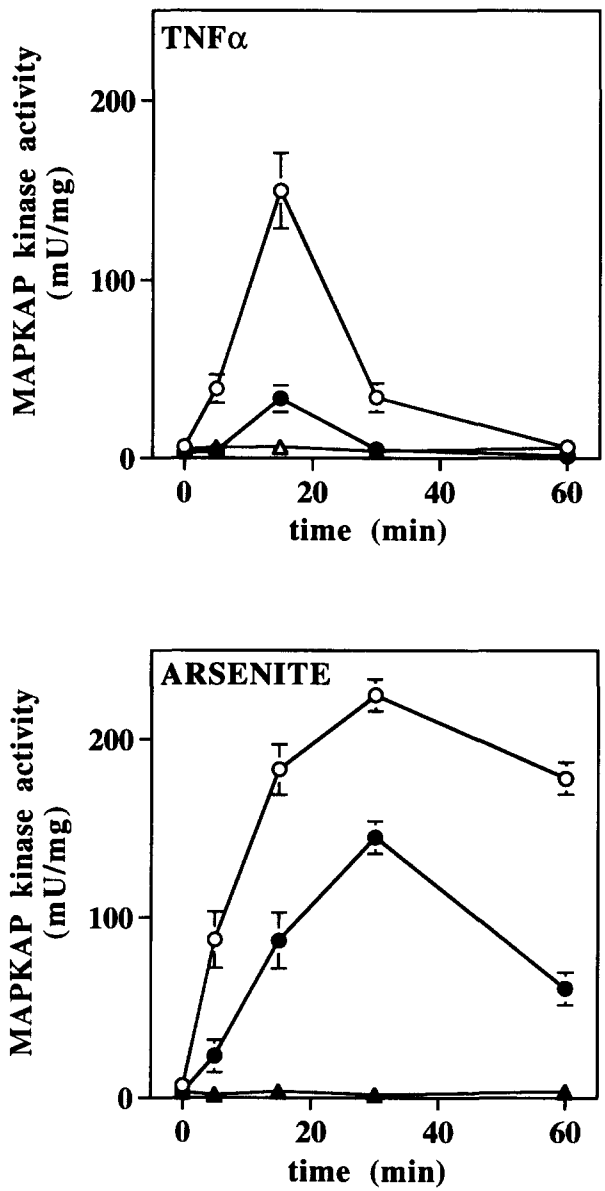

Fig. 3. Activation of MAPKAP-K2 and MAPKAP-K3 by TNF and cellular stress in HeLa cells. Cells were stimulated for the indicated times with 100 ng/ml TNF or 0.5 mM sodium arsenite. MAPKAP-K2 (○) and MAPKAP-K3 (●) were assayed after immunoprecipitation of these enzymes from cell lysates as in Fig. 2. The open triangles (△) show MAPKAP-K2 activity and the closed triangles (▲) MAPKAP-K3 activity in cell lysates from unstimulated cells. The results are shown as standard errors for four separate immunoprecipitations using two dishes of cells.

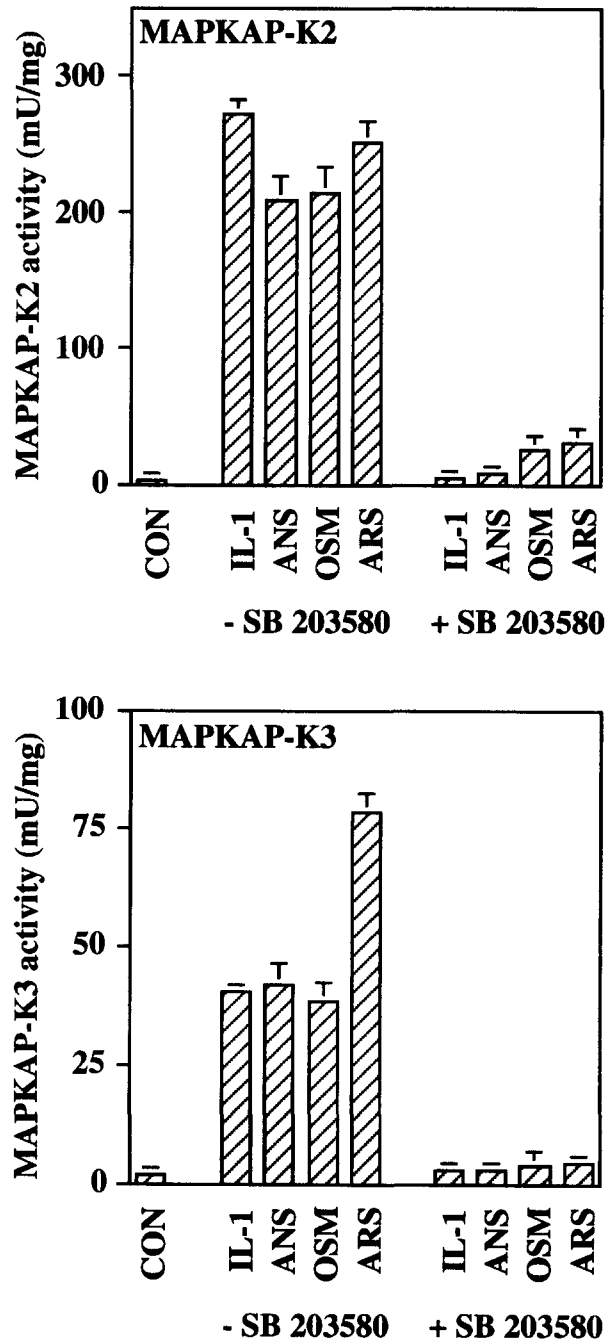

Fig. 4. SB 203580 prevents the activation of MAPKAP-K2 and MAPKAP-K3 by cellular stress and IL-1 in KB cells. Cells were incubated for 1 h in the absence or presence of 10 μM SB 203580 and then for 15 min with 20 ng/ml IL-1, 10 μg/ml anisomycin (ANS), 0.5 M sorbitol (osmotic shock-OSM) or 0.5 mM sodium arsenite (ARS) in the continued presence of SB 203580. MAPKAP-K2 and MAPKAP-K3 were assayed after immunoprecipitation from the cell lysates as in Fig. 2. The results are shown as standard errors for four separate immunoprecipitations using two dishes of cells.

MAPKAP-K2 [2]. These experiments established that, like MAPKAP-K2, MAPKAP-K3 requires a bulky hydrophobic residue at position n-5 to phosphorylate peptides with a low Km value (Leu=Phe > Val ≫ Ala) and that MAPKAP-K3, like MAPKAP-K2, cannot tolerate replacement of Arg by Lys at n-3, or Val by either Pro or Lys at position n+1 (Table 1). However, MAPKAP-K3 was able to tolerate replacement of Ser by Thr at position 'n' to a somewhat greater extent than MAPKAP-K2 (Table 1). MAPKAP-K2 expressed in *E. coli* and then activated with SAPK-2 phosphorylated each peptide with similar kinetic constants to MAPKAP-K2 isolated from rabbit skeletal muscle (data not shown).

Activated MAPKAP-K3 phosphorylated HSP27 at a similar rate to activated MAPKAP-K2 when activities were matched towards the standard peptide KKLNRTLSVA. The three serine residues in HSP27 phosphorylated by MAPKAP-K2 [12] were also phosphorylated at the same relative rates by

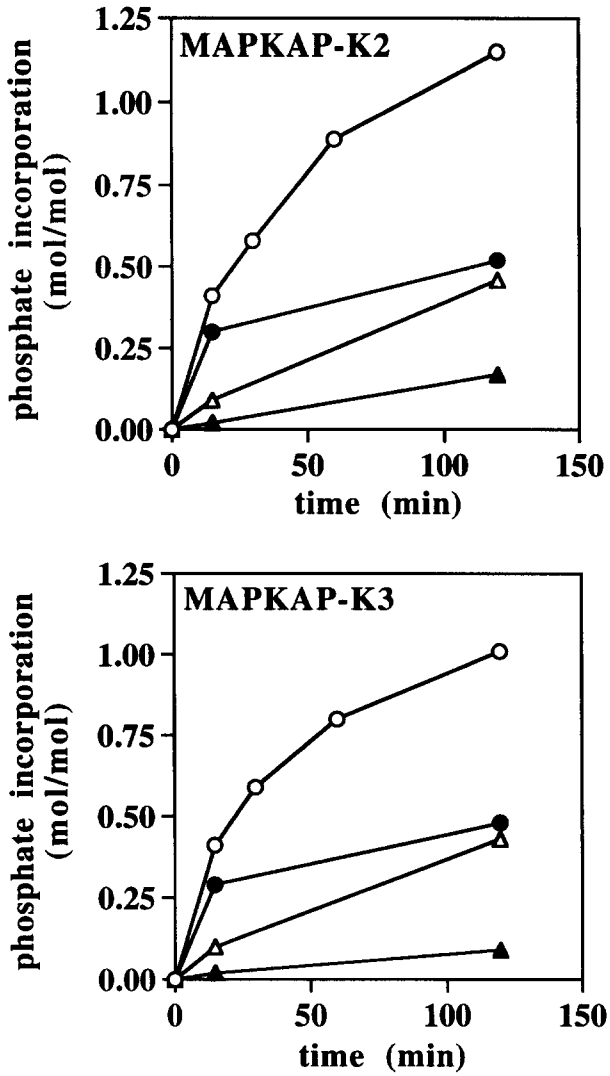

Fig. 5. Phosphorylation of HSP27 by MAPKAP-K2 and MAPKAP-K3. HSP 27 (2 μM) was incubated at 30°C in 0.08 ml of 50 mM sodium β-glycerophosphate pH 7.4, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 10 mM magnesium acetate/0.1 mM [γ^{32}P]ATP (6×10^6 cpm/nmol), with or without 10 U/ml MAPKAP-K2 or MAPKAP-K3. After incubation for the indicated times, an aliquot (15 μl) was removed and the incorporation of phosphate into HSP27 protein (○) measured after precipitation with trichloroacetic acid [12]. The precipitated HSP27 was digested with trypsin, chromatographed on a Vydac C_{18} column to resolve the different phosphopeptides as described [12] and the incorporation of phosphate into the peptides containing Ser-82 (●), Ser-78 (△) and Ser15 (▲) was quantitated.

MAPKAP-K3 (Ser-82 ≫ Ser-78 > Ser-15; Fig. 5). The two protein kinases also phosphorylated CREB at similar rates (data not shown).

4. Discussion

In this paper we present the first comparison of the activation of MAPKAP-K2 and MAPKAP-K3 in vivo and the substrate specificities of these enzymes in vitro. These studies demonstrate that MAPKAP-K2 and MAPKAP-K3 are activated by the same stimuli and with identical kinetics (Figs. 2 and 3), and establish that MAPKAP-3, like MAPKAP-K2, is a stress and cytokine-activated protein kinase which lies 'downstream' of SAPK-2 (Fig. 4). The activation of an epitope-tagged version of MAPKAP-K3 by TNF or osmotic shock and blockade of its activation by SB 203580 has been demonstrated previously after its transfection into HeLa cells [18]. However, by raising antibodies that immunoprecipitate either MAPKAP-K2 or MAPKAP-K3, we have been able to study the activation of the endogenous enzymes in the same cell lysates. This has avoided expression/overexpression of epitope-tagged proteins which has the potential to produce misleading results. For example, the overexpression of MEK kinase triggers the activation of the growth factor-stimulated MAP kinase cascade as well as the stress-activated pathway leading to the activation of SAPK-1, although at lower levels of expression MEK kinase is reported to activate the latter pathway relatively specifically [21,22]. Moreover, MAPKAP-K2 transfected into COS cells can be activated by the growth factor stimulated MAP kinase pathway, in contrast to the endogenous MAPKAP-K2 which is only activated by SAPK-2 (R. BenLevy and C.J. Marshall, pers. Commun., 1996).

In KB and HeLa cells, MAPKAP-K3 only accounted for 15–20% of the SB 203580 sensitive MAPKAP kinase activity observed after stimulation with IL-1, TNF, anisomycin or osmotic shock and for 30–40% of the activity observed after exposure to sodium arsenite (Figs. 2 and 3). It is not yet clear whether this difference reflects the relative levels of these kinases in vivo or whether MAPKAP-K2 is activated preferentially by SAPK-2. The greater activation of MAPKAP-K3 after stimulation with sodium arsenite (Figs. 2 and 3) suggests that the latter explanation is correct. In other cells, MAPKAP-K3 made an even lower contribution accounting for only 11% of the total MAPKAP-K2+MAPKAP-K3 activity in arsenite-stimulated human A431 fibroblasts, 6% in arsenite-stimulated mouse Swiss-3T3 cells and a negligible proportion in arsenite-stimulated (or LPS-stimulated [9]) human THP1 monocytes and rat PC12 cells (A. Clifton, unpublished experiments). The last mentioned observation is consistent with the very low levels of MAPKAP-K3 mRNA found in the brain [19].

Our results demonstrate that the substrate specificity of MAPKAP-K3 is similar to that of MAPKAP-K2. In a side by side comparison, we found that both kinases phosphorylated a panel of synthetic peptides with similar kinetics (Table 1), and phosphorylated HSP27 and CREB at similar rates. Moreover, the three serine residues in HSP27 were also phosphorylated at the same relative rates (Fig. 5). This demonstrates that MAPKAP-K3, like MAPKAP-K2, preferentially phosphorylates the sequence Hyd-Xaa-Arg-Xaa-Xaa-Ser-Xaa, where Hyd is a bulky hydrophobic residue. However, MAPKAP-K3 can tolerate replacement of serine by threonine at the site of phosphorylation better than can MAPKAP-K2. In contrast, Sithanandam et al. [19], using the same panel of synthetic peptides, reported that MAPKAP-K3 had a novel specificity different from MAPKAP-K2. The reason for this discrepancy with our work is unclear, although it should be noted that no side by side comparison with MAPKAP-K2 was made in their study. In summary, the finding that MAPKAP-K2 and MAPKAP-K3 are activated by the same signal transduction pathway in vivo and that they have similar specificities in vitro, suggests that they are also likely to have overlapping or identical substrate specificities in vivo.

Table 1
Comparison of the specificities of MAPKAP-K2 and MAPKAP-K3 towards synthetic peptides

Peptide	MAPKAP-K2		MAPKAP-K3	
	K_m (μM)	V_{max}	K_m (μM)	V_{max}
KKLNRTLSVA	3.8± 0.9	100	2.5± 0.2	100
KKFNRTLSVA	5.9± 1.7	113±19	2.8± 0.1	104±3
KKVNRTLSVA	18 ± 3	102±24	7.2± 0.6	90±2
KKANRTLSVA	274 ±54	91±12	86 ±14	81±5
KKLNRTLTVA	170 ±17	31± 1	53 ± 1	65±1

Peptide	Activity at 30 μM relative to KKLNRTLSVA	
	MAPKAP-K2	MAPKAP-K3
KKLNRTLSVA	100	100
KKKNRTLSVA	6	11
KKENRTLSVA	5	8
KKLNKTLSVA	3	6
KKLNRTLTVA	6	25
KKLNRTLSPA	4	6
KKLNRTLSKA	<1	<1

The data are presented as standard deviations for three separate experiments with each peptide except KKLNRTLTVA which shows the average of two independent experiments. V_{max} is the maximal activity at saturating substrate concentration relative to KKLNRTLSVA.

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