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# Activation of a human Ste20-like kinase by oxidant stress defines a novel stress response pathway

**Celia M.Pombo[1,2], Joseph V.Bonventre[2], Arpad Molnar[3], John Kyriakis[3] and Thomas Force[1,4]**

[1]Cardiac, [2]Renal and [3]Diabetes Research Units of the Massachusetts General Hospital and Department of Medicine, Harvard Medical School, Boston, MA, USA

[4]Corresponding author

**Mammalian homologs of the yeast protein kinase, Sterile 20 (Ste20), can be divided into two groups based on their regulation and structure. The first group, which includes PAK1, is regulated by Rac and Cdc42Hs, and activators have been identified. In contrast, very little is known about activators, regulatory mechanisms or physiological roles of the other group, which consists of GC kinase and MST1. We have identified a human Ste20-like kinase from the GC kinase group, SOK-1 (Ste20/oxidant stress response kinase-1), which is activated by oxidant stress. The kinase is activated by autophosphorylation and is markedly inhibited by its non-catalytic C-terminal region. SOK-1 is activated 3- to 7-fold by reactive oxygen intermediates, but is not activated by growth factors, alkylating agents, cytokines or environmental stresses including heat shock and osmolar stress. Although these data place SOK-1 on a stress response pathway, SOK-1, unlike GC kinase and PAK1, does not activate either of the stress-activated MAP kinase cascades (p38 and SAPKs). SOK-1 is the first mammalian Ste20-like kinase which is activated by cellular stress, and the activation is relatively specific for oxidant stress. Since SOK-1 does not activate any of the known MAP kinase cascades, its activation defines a novel stress response pathway which is likely to include a unique stress-activated MAP kinase cascade.**

*Keywords*: MAP kinases/oxidant stress/protein kinases/Ste20

## Introduction

Mitogen-activated protein kinase (MAPK) cascades have been remarkably conserved in evolution. The core of these cascades is a three-tiered module consisting of an MAPK-extracellular signal-regulated kinase kinase (an MEKK), an MEK and an MAPK or extracellular signal-regulated kinase (ERK). In simple eukaryotes, such as the budding yeast, *Saccharomyces cerevisiae*, and the fission yeast, *Schizosaccharomyces pombe*, these cascades are activated predominantly by cellular stresses such as nutritional starvation and osmolar stress (reviewed in Elion, 1995; Herskowitz, 1995; Levin and Errede, 1995). In mammals, these cascades have evolved to allow responses to complex stimuli (e.g. growth factors and inflammatory cytokines). However, in many cases, such as the response to osmolar challenge (Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994), the primitive stress responses remain intact. Epistasis analyses in yeast suggest that upstream of the three-tiered module are Ste20 (Leberer *et al.*, 1993) and related kinases. Recently, mammalian Ste20-like kinases, p21-activated protein kinase (PAK1) (Manser *et al.*, 1994) and germinal center kinase (GC kinase) (Katz *et al.*, 1994), have been shown to be capable of activating mammalian MAPK cascades (Polverino *et al.*, 1995; Pombo *et al.*, 1995; Zhang *et al.*, 1995), further illustrating the remarkable evolutionary conservation. In co-transfection experiments, both kinases activated the stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) cascade, and PAK1 also activated another stress-activated MAPK, p38.

Ste20-like kinases can be divided into two families based on their structure and regulation. Ste20, PAK1 and related PAKs contain a C-terminal catalytic domain and an N-terminal regulatory domain which has a $p21^{cdc42/rac1}$ binding region (Manser *et al.*, 1994; Martin *et al.*, 1995). PAK1 appears to be activated by binding to Cdc42Hs or Rac1. Following binding to the small GTP binding proteins, the kinase undergoes autophosphorylation and is activated. Physiologic activators of PAK1, the chemoattractant peptide, fMetLeuPhe, and interleukin-1, have been identified (Zhang *et al.*, 1995).

In the other family of Ste20-like kinases, the catalytic domain is N-terminal and there is a C-terminal region of unknown function. Besides GC kinase, the only other members of this group reported to date are MST1 (mammalian Ste20-like kinase 1) and Sps1, encoded by the sporulation-specific 1 gene in yeast which is necessary for spore formation in response to nutritional starvation. Very little is known about the regulation of this family of kinases. These kinases lack the Rac/Cdc42Hs binding domain found in the PAKs. MST1 appears to be activated by dephosphorylation. Sps1 and its MAPK, Smk1 (Krisak *et al.*, 1994), are transcriptionally regulated, being expressed only at certain stages of the sporulation process, but it is not known if there are other modes of regulation of Sps1. Since physiological activators of the mammalian kinases have not been identified, their roles in the cell remain unknown.

Herein we report the cloning and characterization of human Ste20/oxidant stress response kinase, SOK-1, which belongs to the Sps1/GC kinase group of Ste20-like kinases with N-terminal catalytic domains. The kinase is positively regulated by phosphorylation and negatively regulated by its C-terminal non-catalytic region. SOK-1 is not part of a generalized stress response pathway, but is activated relatively specifically by oxidant stress and does not activate any of the known MAPK pathways

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**Fig. 1.** Nucleotide and predicted amino acid sequence of SOK-1. For the nucleotide sequence, numbers refer to the position of the codon relative to the initiator ATG. The predicted translation product is indicated below the nucleotide sequence, and the numbers refer to the position of the amino acid relative to the initial methionine. The stop codon 15 bp 5′ of the putative initiation codon is in bold and the kinase domain is underlined.

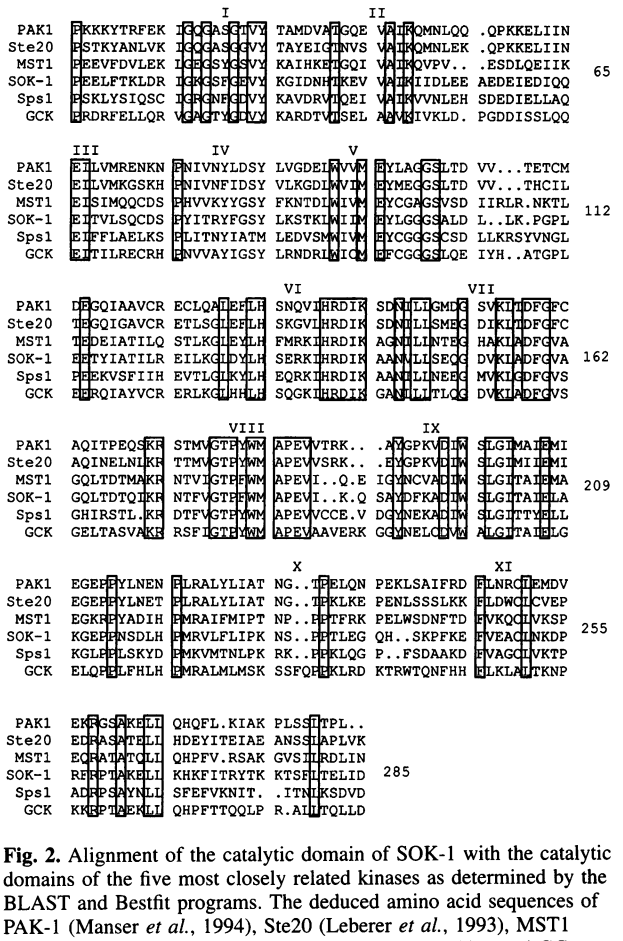


**Fig. 2.** Alignment of the catalytic domain of SOK-1 with the catalytic domains of the five most closely related kinases as determined by the BLAST and Bestfit programs. The deduced amino acid sequences of PAK-1 (Manser *et al.*, 1994), Ste20 (Leberer *et al.*, 1993), MST1 (Creasy and Chernoff, 1995), Sps1 (Friesen *et al.*, 1994) and GC kinase (Katz *et al.*, 1994) were aligned by eye after being aligned with the Pileup program. Gaps, which were introduced to maintain alignment, are denoted by dots. Roman numerals indicate the 11 protein serine/threonine kinase subdomains (Hanks and Quinn, 1991). Residues that are conserved in all family members are enclosed in boxes.

including SAPK, p38 or ERK-1/-2. These data place SOK-1 on a novel stress response pathway and suggest it resembles in function yeast Ste20s which transduce signals in response to environmental stress.

## Results

### *Cloning and sequencing of SOK-1*

Degenerate oligonucleotide primers based on the DNA sequence from within highly conserved regions of subdomains II and VI of protein kinases (Lange-Carter *et al.*, 1993) were used to amplify cDNA from human placenta. The sequence of one product was consistent with a novel protein kinase. This fragment was used to probe a human B cell cDNA library. Screening identified two clones of 1.8 and 2.0 kb. The longer one contained a Kozak sequence (gcggccatgg) (Kozak, 1991) at a candidate initiation codon (Figure 1). There is an in-frame stop codon 15 bp 5′ of this initiation codon. There are no other candidate initiation codons between this stop codon and the ATG, which suggests that this codon is the true translation start site. A poly(A) tail was present at the 3′ end of the 1.8 kb fragment.

The open reading frame encodes a protein which is 426 amino acids in length and has a predicted $M_r$ of 48 041 Da. The kinase domain is located in the N-terminal half of the protein and contains all 11 subdomains of serine/threonine kinases (Figure 2) (Hanks and Quinn, 1991). Comparison of the amino acid sequence of the catalytic domain with other protein kinases using the BLAST program identified three Ste20-like kinases, the yeast kinase, Sps1 (Friesen *et al.*, 1994), and the mammalian kinases, MST1 (Creasy and Chernoff, 1995) and GC kinase (Katz *et al.*, 1994), as most closely related. Within the catalytic domain, SOK-1 was 50% identical and 68% similar to Sps1, 56% identical and 73% similar to MST1, and 51% identical and 68% similar to GC kinase. SOK-1 was 42% identical to Ste20 (Leberer *et al.*, 1993) and 46% identical to PAK-1 (Manser *et al.*, 1994). Alignment of the amino acid sequence of the catalytic domains of Sps1 and Ste20 with SOK-1 indicates a high degree of evolutionary conservation (Figure 2). Comparison of the amino acid sequence of the C-terminal non-catalytic region of SOK-1 with the database using the BLAST, BEAUTY and BLASTPAT programs failed to identify regions of significant homology.

Northern blot analysis of rat organ mRNA, using a probe from the C-terminal non-catalytic region of SOK-1,

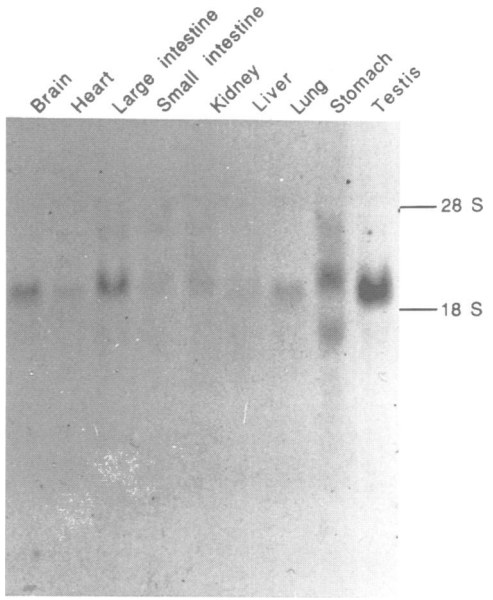


**Fig. 3.** Tissue expression of SOK-1. The expression of SOK-1 was examined by Northern blot analysis of total RNA isolated from various rat tissues. The probe was derived from the non-catalytic C-terminal region of SOK-1. Equal loading was verified by ethidium bromide staining.

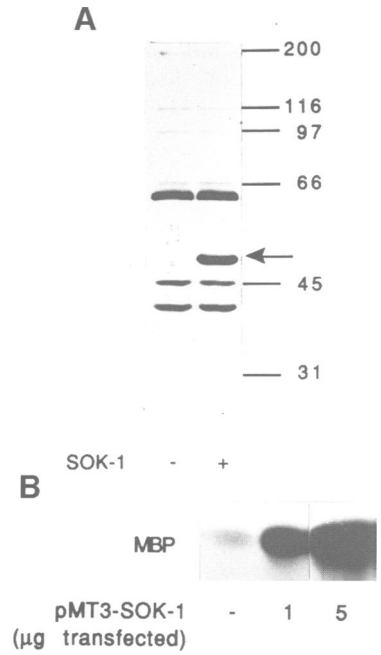


**Fig. 4.** Characterization of SOK-1. (**A**) Western blot of HA-SOK-1 after transfection of COS7 cells with pMT3-SOK-1 encoding SOK-1 with a nine amino acid HA epitope tag (SOK-1 +) or vector alone (SOK-1 –). The blot was probed with monoclonal anti-HA antibody. The arrow identifies SOK-1. (**B**) Kinase activity of SOK-1. COS7 cells were transfected with pMT3 vector alone (–) or with 1 or 5 μg of pMT3-SOK-1. Forty-eight hours later, cells were harvested and lysates were subjected to immunoprecipitation with anti-HA antibody followed by immune complex kinase assay using MBP as substrate.

demonstrated ubiquitous expression of a 2300 bp mRNA, except in the stomach where the probe hybridized to two transcripts, one of ~2600 bp and one of 1500 bp (Figure 3). The highest levels of expression were in testis, large intestine, brain and stomach. Intermediate levels of expression were seen in the heart and lung. The kinase was expressed in the two human B cell lines examined, Ramos, a Burkitt lymphoma cell line that has features of a germinal center B cell, and HS Sultan, a mature B cell line (data not shown).

Transfection of COS7 cells with pMT3-SOK-1, which encoded SOK-1 with a nine amino acid hemagglutinin (HA) epitope tag on the N-terminus, resulted in expression of a protein with an approximate $M_r$ of 50 kDa (Figure 4A). The kinase displayed a high degree of constitutive activity toward myelin basic protein (MBP) in immune complex kinase assays (Figure 4B). Phosphoamino acid analysis demonstrated that the kinase phosphorylated MBP on serine and threonine residues, but not on tyrosine (data not shown).

To determine the subcellular localization of SOK-1, pMT3-SOK-1, encoding HA-SOK-1, was microinjected into NIH 3T3 fibroblasts. HA-SOK-1 was localized almost exclusively in the cytoplasm (Figure 5).

### *Regulation of SOK-1*

We explored potential modes of regulation of the kinase. SOK-1 undergoes marked autophosphorylation when SOK-1 immune complexes are incubated with [$\gamma$-$^{32}$P]ATP (Figure 6A). This results in significantly retarded mobility on SDS–polyacrylamide gels and a 2.1-fold increase in kinase activity (Figure 6A). To examine further the effects of autophosphorylation on kinase activity, we exposed SOK-1, which had been allowed to undergo autophosphorylation for 20 min in the presence of [$\gamma$-$^{32}$P]ATP, to protein serine phosphatase 2A (PP2A), and determined the kinase activity and the extent of dephosphorylation of SOK-1 (Figure 6B). PP2A markedly decreased the $^{32}PO_4$ content of SOK-1, and dephosphorylation was associated with a 35% reduction in kinase activity.

We also found that autophosphorylation markedly reactivated SOK-1 which had been partially inactivated by PP2A (Figure 6C). Exposure to PP2A reduced SOK-1 kinase activity by 45%. After only 10 min of incubation with ATP to allow autophosphorylation, kinase activity increased 2.2-fold and was greater than the activity of SOK-1 which had been exposed to okadaic acid-inactivated PP2A. An additional 10 min of reactivation time led to additional phosphorylation of SOK-1, but minimal further increase in kinase activity (to 2.6-fold). This suggests that sites important for kinase activity were phosphorylated early, and sites phosphorylated between 10 and 20 min were largely irrelevant to kinase activation. While we cannot rule out the presence of a kinase in the immune complexes other than SOK-1 which phosphorylates and activates SOK-1, these data, taken together, suggest that phosphorylation, probably autophosphorylation, is an important mechanism of activation of SOK-1. Since SOK-1 is markedly activated by autophosphorylation in immune complex kinase assays, incubations for kinase assays of >5 min can be expected to mask any differences between control and stimulated cells. Consequently, all subsequent kinase assays were performed for ≤5 min.

Although phosphorylation clearly activates SOK-1 and dephosphorylation partially inactivates it, Figure 6B suggests that mechanisms other than phosphorylation might play a role in regulation, since near-complete dephos-

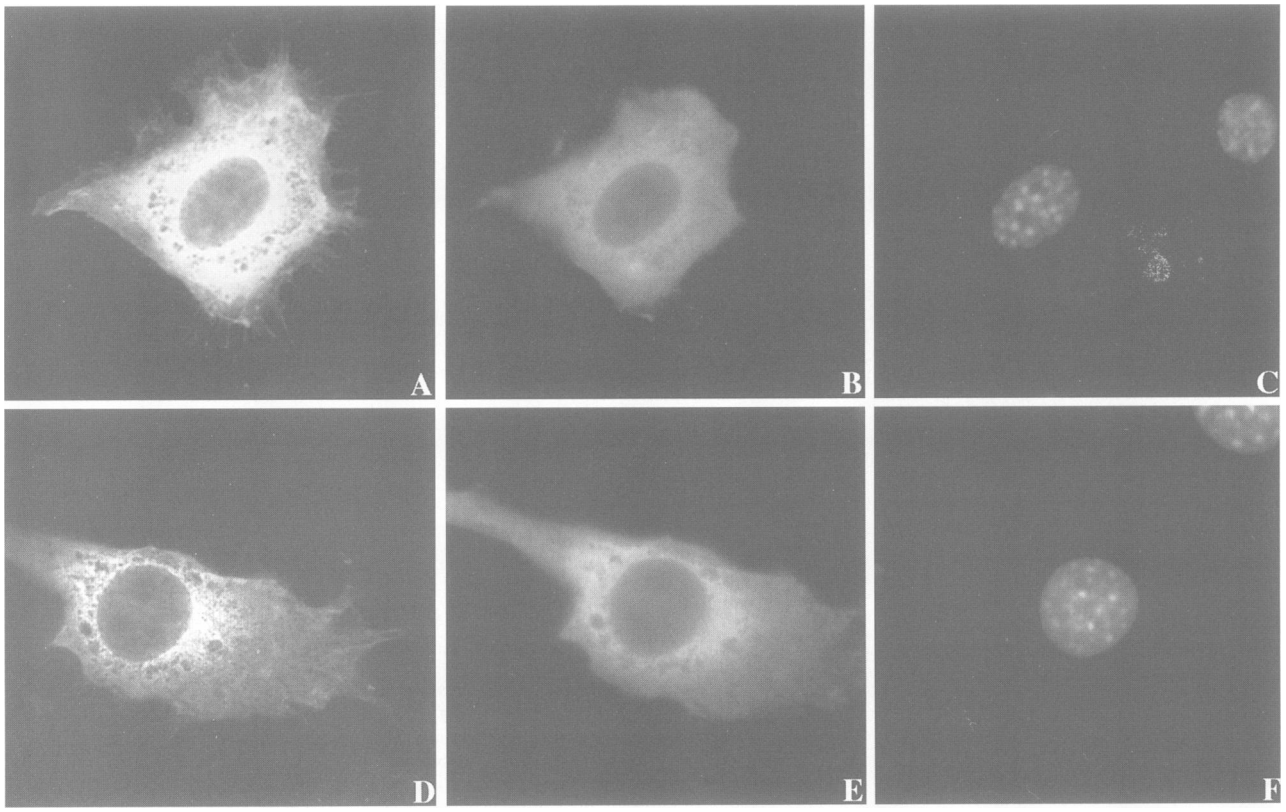


**Fig. 5.** Subcellular localization of SOK-1. pMT3 encoding HA epitope-tagged SOK-1 was microinjected into NIH 3T3 fibroblasts at a concentration of 100 μg/ml. HA-SOK-1 was detected by staining with the anti-HA antibody as described in Materials and methods. HA-SOK-1 was localized primarily in the cytoplasm (**B** and **E**). A representative slice of 0.2 μm of the same cells using a confocal laser scanning microscope is also shown (**A** and **D**). These cells were also counterstained with Hoechst 33258 to visualize the nuclei (**C** and **F**).

phorylation of SOK-1 was associated with only a modest reduction in kinase activity. SOK-1 has an N-terminal catalytic domain, placing it, on the basis of organization, in the group of Ste20s including Sps1, GC kinase and MST1. These kinases lack the Rac/Cdc42Hs binding domain present in the regulatory domains of Ste20 and the PAK family of kinases, and the role of their C-terminal non-catalytic regions is unclear. We explored whether the C-terminal region of SOK-1 might play a role in regulation of its kinase activity. We compared the kinase activity of SOK-1, expressed from pCMV5-SOK-1, which encoded SOK-1 with a nine amino acid M2 epitope tag at the N-terminus, with that of M2-SOK-1ΔC, a deletion mutant containing the catalytic domain but missing the C-terminal 93 amino acids of the non-catalytic region. Although cell extracts were matched for total protein prior to immunoprecipitation with anti-M2 antibody, immunoblots of the extracts revealed that M2-SOK-1ΔC was expressed at a much lower level than full-length M2-SOK-1 (Figure 7, bottom). Despite the lower expression of M2-SOK-1ΔC, and the presence of much less M2-SOK-1ΔC compared with full-length M2-SOK-1 in the immunoprecipitates, kinase activity, measured as phosphorylation of MBP, was equivalent, consistent with significantly greater specific activity of M2-SOK-1ΔC (Figure 7). These data suggest that the C-terminal non-catalytic region inhibits kinase activity of SOK-1 and is the first demonstration of a role for the non-catalytic region of the GC kinase/MST1/Sps1 group of Ste20-like kinases.

We also examined the effect of autophosphorylation on the activity of SOK-1ΔC. When SOK-1ΔC immune complexes were incubated with ATP (100 μM for 20 min), kinase activity increased only 1.4-fold (3.9 to 5.5 U). Exposure of SOK-1ΔC to PP2A, however, significantly reduced kinase activity (from 5.5 to 2.9 U), suggesting phosphorylation is also an important regulatory mechanism in SOK-1ΔC, and that the critical phosphorylation site(s) is within the catalytic domain. The minimal (1.4-fold) activation of SOK-1ΔC we observed in the autophosphorylation experiment is probably due to the marked constitutive activity of SOK-1ΔC, which may be near maximally active when immunoprecipitated from cells.

### *Activation of SOK-1*

Incubation of Ramos B cells with okadaic acid (1 μM, 20 min) activated SOK-1 (Table I), compatible with regulation of SOK-1 (and/or an upstream activator) by phosphorylation. We also tested numerous agonists which were representative of multiple different classes of stimuli for their ability to activate SOK-1. Only inducers of oxidant stress consistently activated SOK-1 when native kinase was assayed after immunoprecipitation from Ramos B cells or MDCK renal tubular epithelial cells, or when HA-tagged SOK-1 was assayed after immunoprecipitation from transfected COS7 cells (Table I). $H_2O_2$ (0.5 mM) activated SOK-1 ~3-fold ($P < 0.01$). Activation of SOK-1 was evident as early as 10 min following exposure of Ramos B cells to $H_2O_2$, peaked at 20 min, and remained elevated at 60 min (Figure 8). Activation was evident at 0.1 mM, the lowest concentration tested (2.1-fold increase

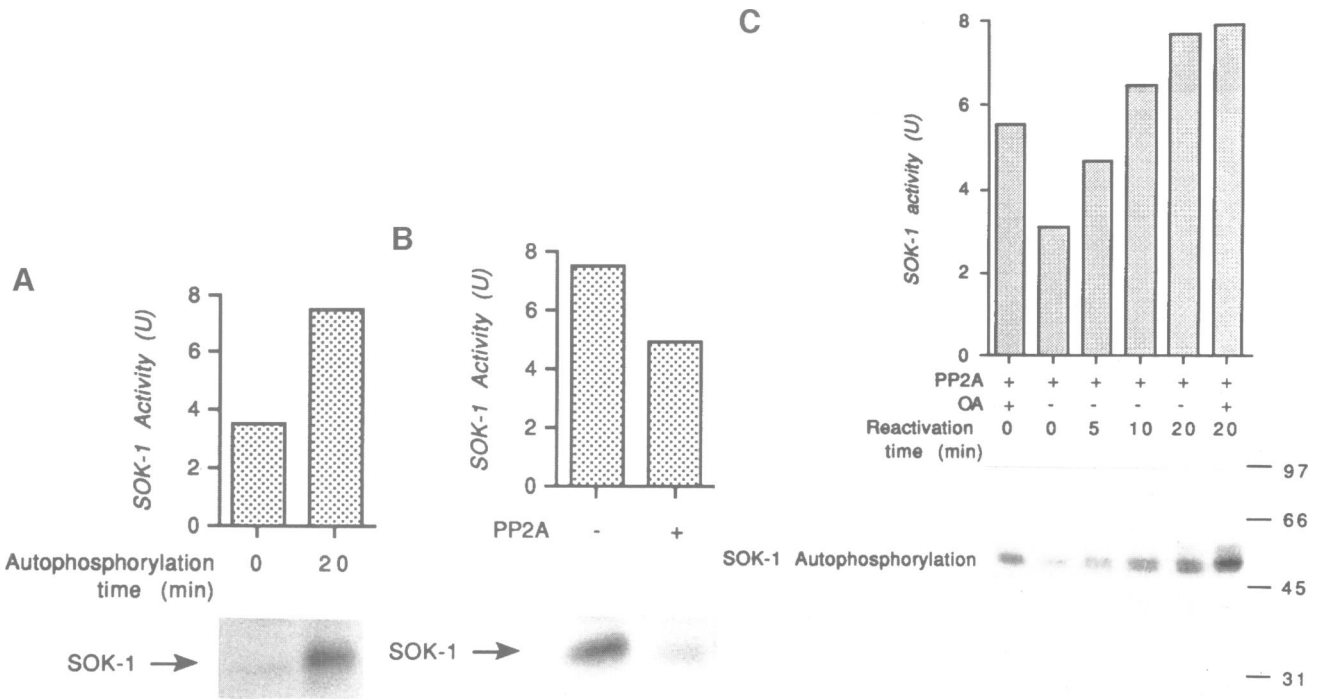


**Fig. 6.** Role of autophosphorylation in the activation of SOK-1. (**A**) Activation of SOK-1 by autophosphorylation. COS7 cells were transfected with pCMV5-SOK-1. Forty-eight hours later, cell lysates were subjected to immunoprecipitation with anti-M2 antibody. Immune complexes were incubated with [$\gamma$-$^{32}$P]ATP (100 $\mu$M) for 0 or 20 min, followed by addition of MBP and kinase assay for 3 min at 30°C. Autophosphorylation of SOK-1 is shown below the graph. (**B**) Phosphatase-induced inactivation of SOK-1. SOK-1 immune complexes from pCMV5-SOK-1-transfected cells were incubated with [$\gamma$-$^{32}$P]ATP for 20 min, washed twice in assay buffer and then exposed to vehicle (–) or PP2A (+) for 20 min. Immune complexes were then either quenched with Laemmli sample buffer and run on an SDS gel to assess dephosphorylation of SOK-1 (autoradiogram of SOK-1 is shown below the graph) or were washed twice in assay buffer and then subjected to kinase assay after addition of MBP and [$\gamma$-$^{32}$P]ATP. (**C**) Reactivation of SOK-1 after phosphatase inactivation. SOK-1 immune complexes from COS7 cells transfected with pMT3-SOK-1 were exposed to PP2A, either with or without okadaic acid present (100 nM), for 20 min. Okadaic acid was then added to bring the final concentration to 100 nM in all tubes. Immune complexes were washed twice and then exposed to [$\gamma$-$^{32}$P]ATP (100 $\mu$M) for 0, 5, 10 or 20 min prior to the addition of MBP and subsequent kinase assay for 5 min at 30°C. Autophosphorylation of SOK-1 at the various time points is shown below the graph.

in kinase activity). Menadione is a quinone which is a potent intracellular generator of reactive oxygen intermediates. In the cell, menadione undergoes one-electron reduction to a semiquinone radical. This radical rapidly reduces $O_2$ to form superoxide anion radical and, subsequently, $H_2O_2$, hydroxyl radical and singlet oxygen (Thor *et al.*, 1982; Hockenbery *et al.*, 1993). Menadione (30 $\mu$M for 30 min) activated SOK-1 7-fold in MDCK cells. This is the first clear demonstration of activation of a member of this group of Ste20-like kinases by any stimulus. The activation of SOK-1 by $H_2O_2$ and menadione not only identifies a new oxidant stress response signal transduction pathway, but also suggests that one role of this and possibly other Ste20-like kinases of this group is to respond to environmental stresses just as Ste20 and related kinases do in the simplest eukaryotes.

In contrast to activation of SOK-1 by $H_2O_2$, the growth factors, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), and the phorbol ester, phorbol myristate acetate (PMA), combined with the calcium ionophore, ionomycin, which are potent activators of the ERK-1/-2 cascade, did not activate SOK-1 (Table I). In the same COS7 cells, these agonists activated ERK-1, expressed in pEBG, 5- to 7-fold (not shown). Oxidant stress appeared to be a specific activator among the several cellular stresses tested. Specifically, high and low osmolar stress, heat shock, tumor necrosis factor $\alpha$ (TNF$\alpha$) and anisomycin, which potently activate the

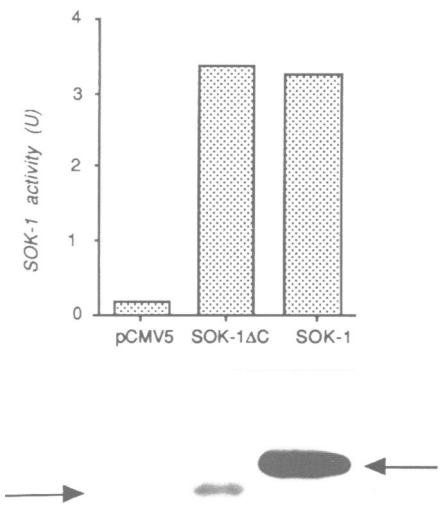


**Fig. 7.** Role of the C-terminal non-catalytic region in the regulation of SOK-1. COS7 cells were transfected with either pCMV5-SOK-1 (SOK-1) or pCMV5-SOK-1$\Delta$C (SOK-1$\Delta$C), encoding a truncated SOK-1 deleted for the C-terminal 93 amino acids of the non-catalytic domain, or empty vector, encoding only the M2 epitope tag (pCMV5). Extracts were matched for protein content prior to immunoprecipitation with anti-M2 antibody. Kinase activity was determined with MBP as substrate. Below the graph is an immunoblot demonstrating the lower levels of expression of SOK-1$\Delta$C (SOK-1 and SOK-1$\Delta$C are identified by arrows).

**Table I.** Fold activation of native SOK-1 in Ramos B cells and HA-SOK-1 in COS7 cells

| Agonist | Ramos | COS7 |
|---|---|---|
| $H_2O_2$ (0.5 mM, 20 min) | 2.9 | 2.8 |
| Okadaic acid (1 μM, 30 min) | 2.3 | – |
| Interferon-γ (50 ng/ml, 20 min) | 1.0 | – |
| TNFα (50 ng/ml, 20 min) | 1.5 | 0.9 |
| Anti-Ig (20 min) | 0.8 | – |
| Platelet activating factor (1 μM, 20 min) | 1.4 | – |
| PMA/ionomycin (300 nM/1 μM, 20 min) | 1.4 | 1.4 |
| Nitrogen mustard (10 μM, 30 min) | 1.2 | 1.4 |
| Cyclophosphamide (10 μM, 30 min) | 0.9 | 1.5 |
| *cis*-platin (10 μM, 30 min) | 1.0 | 1.1 |
| Heat shock (42°C, 5 min) | – | 0.9 |
| Anisomycin (50 μg/ml, 20 min) | – | 1.1 |
| Hyperosmolarity (NaCl 700 mM, 15 min) | – | 0.9 |
| Hypoosmolarity (150 mOsm, 15 min) | – | 0.9 |
| EGF (100 ng/ml, 10 min) | – | 1.3 |
| PDGF (20 ng/ml, 10 min) | 1.0 | 1.2 |

Native SOK-1 in Ramos B cells was assayed with MBP as substrate after immunoprecipitation with rabbit polyclonal anti-SOK-1. HA-SOK-1 was assayed after immunoprecipitation with anti-HA antibody from extracts of COS7 cells which had been transfected with pMT3-SOK-1 (5 μg per 10 cm dish). Hypoosmolar stress was induced by placing cells in Krebs–Henseleit buffer without NaCl (Pombo *et al.*, 1994).
–, not determined.

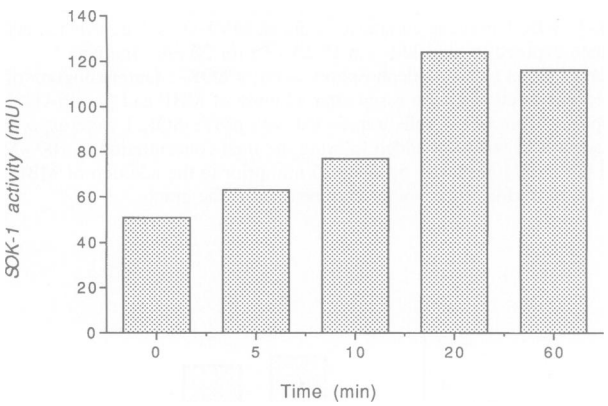


**Fig. 8.** Time course of activation of SOK-1 by $H_2O_2$. Ramos B cells were exposed to $H_2O_2$ (0.5 mM) for the times indicated in the figure. Extracts were subjected to immunoprecipitation with anti-SOK-1 antibody followed by kinase assay. There was no $H_2O_2$-induced increase in MBP kinase activity when immunoprecipitation was performed with pre-immune serum (not shown).

SAPK and/or p38 cascades in these and other cells (see figures and Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994; Kyriakis *et al.*, 1994; Rouse *et al.*, 1994; Pombo *et al.*, 1995), did not activate SOK-1. Platelet activating factor, which signals via a heterotrimeric G protein-coupled receptor and is a potent activator of intracellular $Ca^{2+}$ transients in Ramos cells, was also ineffective. Exposure of transfected COS7 cells to the alkylating agents cyclophosphamide, nitrogen mustard and *cis*-platin also did not activate SOK-1. Cross-linking surface IgM on Ramos B cells with anti-Ig antibody coupled to beads, which induces apoptosis in these cells, did not activate SOK-1 but did markedly enhance tyrosine phosphorylation of several proteins in these cells (data not shown).

Although these data clearly place SOK-1 on an oxidant stress response pathway, SOK-1 does not appear to activate

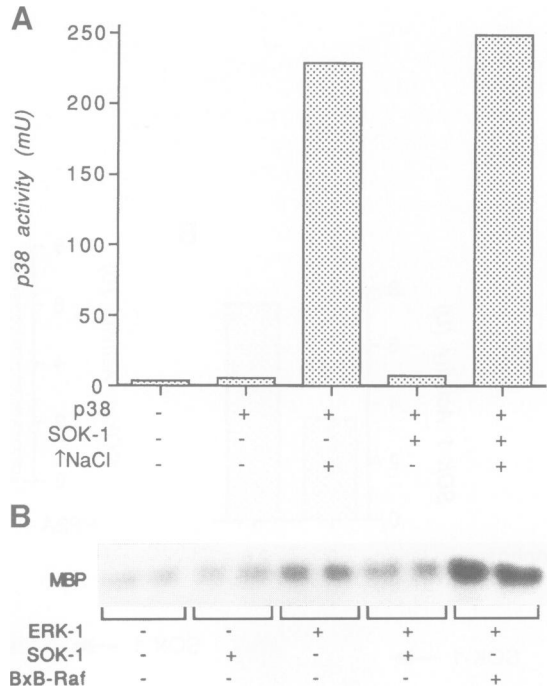


**Fig. 9.** SOK-1 does not activate the p38 or Erk-1 cascades. (**A**) p38. COS7 cells were transfected with pEBG vector (p38 –) or pEBG encoding p38 as a GST fusion protein (p38 +), and either pMT3 vector (SOK-1 –) or pMT3 encoding HA-tagged SOK-1 (SOK-1 +). To confirm that p38 could be activated, cells were exposed to NaCl (500 mM) for 10 min (NaCl +). p38 kinase activity was assayed with ATF-2(8–94) as substrate (Morooka *et al.*, 1995). (**B**) Erk-1. COS7 cells were transfected with pEBG vector (Erk-1 –) or pEBG encoding Erk-1 as a GST fusion protein (Erk-1 +), and either pMT3 vector (SOK-1 –), pMT3 encoding HA-tagged SOK-1 (SOK-1 +) or, as a positive control, pMT3 encoding BxB-Raf (+), a constitutively active c-Raf-1 which is missing the N-terminal regulatory domain. Erk-1 assays were performed in duplicate with MBP as substrate.

the known stress-activated MAP kinase pathways. We recently reported that SOK-1 (previously called UK-1 but renamed SOK-1 to reflect the fact that the kinase was activated by oxidant stress), unlike the closely related GC kinase, did not activate the SAPKs in co-transfection experiments (Pombo *et al.*, 1995). Co-transfection of HA-SOK-1 with the other MAP kinases, p38 (Figure 9A) and ERK-1 (Figure 9B), both expressed in pEBG, did not result in the activation of the MAP kinases. In these same cells, p38 was markedly activated by exposure of cells to osmolar stress, and ERK-1 was activated by co-transfection of pRSV-BXB-Raf-1 [encoding a constitutively active variant of c-Raf-1 (Bruder *et al.*, 1992)].

## Discussion

In *S.cerevisiae*, mating factors activate a serpentine receptor which triggers activation of a MAP kinase cascade via βγ subunits of a heterotrimeric G protein. This cascade consists of an MEKK (Ste11), an MEK (Ste7) and one or more MAP kinases (Kss1 and Fus3) (reviewed in Elion, 1995; Herskowitz, 1995; Levin and Errede, 1995). The MAP kinases then activate a complex genetic program probably, in part, by phosphorylating the transcription factor, Ste12. In addition, they induce cell cycle arrest in $G_1$, possibly by phosphorylating Far1 which then binds to and inhibits cyclin-dependent kinases. These events set

the stage for conjugation. Epistasis analyses indicate that another protein kinase, Ste20, functions between $G_{\beta\gamma}$ and the MEKK, Ste11 (Leberer *et al.*, 1993). Although Ste20 phosphorylates Ste11 in cell-free systems (Wu *et al.*, 1995), it is not clear as yet whether Ste11 is regulated directly by Ste20 in the cell. The pheromone response pathway is the best characterized of the MAP kinase pathways in *S.cerevisiae*, but there are at least four others which are activated predominantly in response to stresses such as starvation, heat shock and high or low osmolar stress. An Ste20, Sps1, has been identified for one of these, the formation of spores in response to nutritional starvation (Friesen *et al.*, 1994).

Three mammalian Ste20-like kinases have been reported to date: PAK1 and isoforms (Manser *et al.*, 1994; Martin *et al.*, 1995), GC kinase (Katz *et al.*, 1994) and MST1 (Creasy and Chernoff, 1995). The roles of these kinases are just beginning to be explored, but mounting evidence suggests that mammalian Ste20-like kinases also function upstream of MEKK/MEK/MAP kinase pathways. PAK1 or GC kinase activate the SAPK cascade when the Ste20-like kinase and the MAP kinase are co-expressed (Pombo *et al.*, 1995; Zhang *et al.*, 1995). In addition, PAK1 activates p38 (Zhang *et al.*, 1995).

PAK1 is 70% identical to Ste20 within the kinase domain, and both PAK1 and Ste20 activate SAPK in cell-free extracts of *Xenopus* oocytes (Polverino *et al.*, 1995). PAK1 shares other similarities with Ste20 and the related yeast kinase, Cla4 (Cvrckova *et al.*, 1995). Most importantly, each contains a motif ($ISxPx_{4-6}HxxH$) in their non-catalytic region which is conserved in kinases that bind to members of the Rho family of small GTPases (Burbelo *et al.*, 1995; Cvrckova *et al.*, 1995). These kinases bind the GTP-bound form of Rac1 or Cdc42 which allows autophosphorylation and activation to occur (Manser *et al.*, 1994; Cvrckova *et al.*, 1995).

The other group of Ste20-like kinases includes Sps1 in *S.cerevisiae*, and GC kinase and MST1 in mammals. SOK-1 belongs to this group based on its organization (N-terminal catalytic domain) and greater sequence similarity within the kinase domain. SOK-1 is more similar in sequence to Sps1 (50% identical) than it is to Ste20 (42% identical). Furthermore, Sps1 is more similar to SOK-1 than Sps1 is to Ste20 (44% identical).

Mechanisms of regulation of this group of Ste20-like kinases were not known. None of these kinases contain an identifiable Rac/Cdc42Hs binding domain in their non-catalytic regions, and the role of this region is uncertain. Our data suggest that the non-catalytic region markedly suppresses activity of the kinase, since the specific activity of a truncated mutant of SOK-1 (SOK-1ΔC), missing most of the non-catalytic region, was markedly higher than that of full-length SOK-1. This is the first identification of a regulatory mechanism controlling activity of this group of Ste20-like kinases. We suspect inhibition of activity is due to binding of the C-terminal region to the catalytic domain since the C-terminal region (missing the kinase domain) co-immunoprecipitates with SOK-1ΔC when the two are co-expressed (data not shown). The C-terminal region may exert its inhibitory effect by preventing access of an activator, possibly SOK-1 itself (see below), to a critical site within the catalytic domain, or by inhibiting interaction of the kinase domain with substrates.

Phosphorylation, probably autophosphorylation, is the second major regulatory mechanism controlling kinase activity. Incubation of SOK-1 immune complexes with ATP enhanced both phosphorylation of SOK-1 and kinase activity, and treatment with PP2A reduced phosphorylation and kinase activity. Exposure of SOK-1ΔC to PP2A also reduced kinase activity, suggesting that one important regulatory phosphorylation site is within the kinase domain. A candidate regulatory phosphorylation site is threonine 178. This residue lies within a region of subdomain VIII that is highly conserved in Ste20-like kinases (GTPF/YWMAPEV) and is critical for kinase activity of Ste20 (Wu *et al.*, 1995).

Identification of these two regulatory mechanisms suggests that the regulation of this group of Ste20-like kinases may be similar to that of PAK1. Binding of the inhibitory regulatory region of PAK1 to the small GTP binding proteins appears to allow the kinase to undergo autophosphorylation, which activates the kinase. For SOK-1, binding of the inhibitory regulatory region to an as yet unidentified activator may partially activate SOK-1 and allow autophosphorylation, which further activates the kinase. Thus, autophosphorylation activates both PAK1 and SOK-1 (and possibly other kinases from this group), but the activators to which the regulatory domains bind, allowing autophosphorylation to occur, would differ. Specificity in the activation of Ste20-like kinases (and, subsequently, MAP kinase cascades) in response to a stimulus could be determined by protein or lipid interaction domains within the regulatory region.

Autophosphorylation and autoactivation of a kinase in immune complexes, if unrecognized, greatly complicate the identification of activators. After a 20 min incubation in the presence of ATP, MBP kinase activity of SOK-1 previously inactivated by PP2A was equal to that of SOK-1 which had not been inactivated by PP2A (Figure 6C). Autophosphorylation and autoactivation may explain some of the difficulty in identifying activators of this group of Ste20-like kinases when standard immune complex kinase assays of 20–30 min are performed. Under these conditions, no activators of MST1 were identified (Creasy and Chernoff, 1995) and, for GC kinase, TNFα only weakly stimulated kinase activity (Pombo *et al.*, 1995).

Difficulty in identifying activators of this group of kinases might also reflect a high degree of specificity. Even when assay conditions were optimized, oxidant stress was the only stimulus that activated SOK-1. Stimuli which were representative of a number of different classes of agonists, including growth factors, phorbol ester, $Ca^{2+}$ ionophore and agonists with G protein-linked receptors, did not activate SOK-1. In addition, inflammatory cytokines and various environmental stresses did not increase SOK-1 activity, indicating that activation of SOK-1 by oxidant stress is not part of a generalized response to cellular stress.

Activation by oxidant stress suggests that this kinase, and possibly other members of the group, may, like Ste20 and related kinases identified thus far in yeast, play an important role in the responses of the cell to environmental stress. The survival of aerobic organisms depends upon their mounting an effective response to oxidant stress. Thus, it is not surprising that a protein kinase which

triggers part of that response is highly conserved in evolution.

Reactive oxygen radicals, via damage to many cellular components including DNA, can cause cell death or, if less severe, cell cycle arrest at either the $G_1$ or $G_2$ checkpoints (Russo *et al.*, 1995). DNA damage not only activates checkpoint controls but may also activate protein kinases, including the SAPKs, c-Raf-1 and ERKs, which are integral components of cytoplasmic signal transduction cascades, and the non-receptor tyrosine kinase, c-Abl (Hibi *et al.*, 1993; Radler-Pohl *et al.*, 1993; Kharbanda *et al.*, 1995a,b; Livingstone *et al.*, 1995; Russo *et al.*, 1995; van Dam *et al.*, 1995). We wanted to determine whether activation of SOK-1 was likely to be triggered by DNA damage or by oxidant stress acting via a DNA damage-independent mechanism. To explore this, we determined whether any of three alkylating agents, *cis*-platin, cyclophosphamide or nitrogen mustard, activated SOK-1. Alkylating agents activate the DNA damage-induced checkpoint controls and protein kinases (Kharbanda *et al.*, 1995a), but do not produce oxidant stress. Our data suggest that oxidant stress-induced activation of SOK-1 is not mediated by DNA damage response pathways, since none of the three agents activated the kinase. Thus, activation of SOK-1 by oxidant stress is not part of a generalized response to either cellular or genotoxic stress.

Oxidant stress activates the ERKs, and may activate the SAPKs somewhat (Kyriakis *et al.*, 1994; Russo *et al.*, 1995), but this does not appear to be via activation of SOK-1. SOK-1 did not activate any of four MAP kinase cascades, SAPKs (Pombo *et al.*, 1995), p38 (Figure 9A), ERK-1 (Figure 9B) or MEK-5/ERK-5 (data not shown) (Zhou *et al.*, 1995), further suggesting that the stress response pathway regulated by SOK-1 is unique. At least five MAP kinase cascades have been identified in yeast, and there are likely to be many more identified in mammals in addition to the four we studied. Since evolutionary conservation of the activation of MEKK/MEK/MAPK cascades by Ste20-like kinases extends to mammals (Polverino *et al.*, 1995; Pombo *et al.*, 1995; Zhang *et al.*, 1995), and all Ste20-like kinases identified to date in yeast or mammals, with the exception of MST1 (Creasy and Chernoff, 1995), have been shown to activate one or more MAP kinase cascades, it is likely that SOK-1 controls a novel oxidant stress-activated MAP kinase cascade.

## Materials and methods

### *Isolation and sequence analysis of human SOK-1 cDNA clone*

Degenerate oligonucleotide primers were based on the DNA sequences from within highly conserved regions of subdomains II and VI of protein kinases (Lange-Carter *et al.*, 1993). Sense [GA(A/G)(C/T)TIATGGCIG-TIAA(A/G)CA] and antisense [TTIGCICC(T/C)TTIAT(A/G)TCIC(G/T)(A/G)TG] primers were used to amplify DNA from a human placenta cDNA library using *Taq* polymerase. The PCR products were ligated into the pCRII vector (Invitrogen). A 350 bp fragment was obtained which was not in the database but which had significant homology to the catalytic domain of protein serine/threonine kinases. This fragment was used to screen 500 000 plaques from a human B cell cDNA library in λYES (provided by Stephen J.Elledge, Deparment of Biochemistry, Baylor College of Medicine). Seven positive clones were isolated, and those containing the largest inserts were analyzed by DNA sequencing of both strands using the dideoxy chain termination method with Sequenase 2.0 (USB, Inc.). DNA and amino acid sequence comparisons were made using the University of Wisconsin Genetics Computer Group programs BLAST, Pileup and Bestfit, and the BEAUTY (BLAST Enhanced Allignment Utility) and BLASTPAT (BLAST PATtern database search tool) programs from the Human Genome Center, Baylor College of Medicine.

### *Northern blot analysis*

Total RNA was isolated from rat organs by the guanidinium thiocyanate–phenol–chloroform method (Witzgall *et al.*, 1993). Twenty micrograms of total RNA were size fractionated on a 1% formaldehyde–agarose gel and transferred to GeneScreen Plus (NEN) membrane as described (Witzgall *et al.*, 1993). Blots were hybridized with a 409 bp *Hin*dIII–*Bam*HI fragment from the 3′ half of SOK-1 (nucleotides 995–1403) which included 284 bp of open reading frame encoding part of the non-catalytic region, and 125 bp from the 3′-untranslated region. This probe was labeled with [α-$^{32}$P]dCTP by random priming. Hybridization was carried out for 18 h at 45°C in 5× SSPE (where 1× SSPE is 150 mM NaCl, 10 mM $NaH_2PO_4$, 0.7 mM EDTA), 44% formamide, 5× Denhart's solution, 1% SDS, 10% dextran sulfate and 100 μg/ml denatured salmon sperm DNA. The membranes were washed twice for 15 min at room temperature in 2× SSPE, twice for 30 min at 65°C in 2× SSPE with 2% SDS and once for 30 min at room temperature in 0.2× SSPE. Membranes were exposed for 5 days at –70°C with intensifying screens.

### *Plasmids, transfection protocols and kinase assays*

Plasmids used included pMT3 (pMT2 modified to encode the nine amino acid HA epitope N-terminal to the insert) (Pombo *et al.*, 1995), pCMV5 (a cytomegalovirus-based vector encoding the nine amino acid M2 epitope tag N-terminal to the insert), pEBG [a vector that is driven by the human EF-1α promoter which encodes glutathione *S*-transferase (GST) N-terminal to the insert] (Sanchez *et al.*, 1994; Pombo *et al.*, 1995) and pGEX-KG (the prokaryotic expression vector which encodes GST N-terminal to the insert) (Guan and Dixon, 1991).

To create pCMV5-SOK-1ΔC, pCMV5-SOK-1 was cut with *Hin*dIII and then religated. This construct encoded amino acids 1–333 and included the entire kinase domain but did not include the C-terminal 93 amino acids of the protein. pEBG-SAPKp54β, pEBG-p38 and pEBG-ERK-1 encoded the three MAP kinases, p54β (the β isoform of the SAPK, p54), p38 and ERK-1, as GST fusion proteins. pRSV-BXB-Raf-1 encoded a variant of c-Raf-1 without the regulatory domain. BXB-Raf-1 is constitutively active and transforming (Bruder *et al.*, 1992; Sanchez *et al.*, 1994; Pombo *et al.*, 1995).

Subconfluent COS7 cells were transfected using the DEAE-dextran technique as described (Pombo *et al.*, 1995). One to 10 μg of expression plasmid DNA were used per plate and adjusted to a total of 20 μg of DNA with the appropriate empty vector. Forty-eight hours after transfection, cells were exposed to various stimuli (see figure legends) or vehicle, and extracts were prepared as described (Pombo *et al.*, 1994, 1995). Extracts were exposed to anti-HA or anti-M2 (Kodak) monoclonal antibodies, or to an anti-SOK-1 rabbit polyclonal antibody (see below) for 3 h, and then immune complexes were collected with protein G–Sepharose beads. Beads were washed three times in lysis buffer, three times in LiCl buffer [500 mM LiCl , 2 mM dithiothreitol (DTT), 100 mM Tris–HCl, pH 7.6] and three times in assay buffer (Pombo *et al.*, 1994). Kinase assays were started by the addition of MBP (for SOK-1 and ERK-1), GST–c-Jun(1–135) containing the transactivation domain of c-Jun (for SAPK) (Kyriakis *et al.*, 1994; Pombo *et al.*, 1994, 1995) or ATF-2(8–94) containing the transactivation domain of ATF-2 (for p38) (Morooka *et al.*, 1995), [γ-$^{32}$P]ATP (100 μM, 3000–9000 c.p.m./pmol) and $MgCl_2$ (10 mM). After 5–20 min at 30°C, the kinase reactions were stopped with Laemmli sample buffer. Following SDS–PAGE and autoradiography, the bands corresponding to the substrate were cut out of the gel and radioactivity was determined by liquid scintillation counting. For all kinase assays, an aliquot of the cell lysate was run on an SDS–polyacrylamide gel, transferred to Immobilon, and subjected to immunoblotting with the appropriate antibody to ensure equivalent expression of the kinases (Morooka *et al.*, 1995). Detection of antibody binding was with the Enhanced Chemiluminescence system.

### *Phosphatase inactivation and reactivation experiments*

COS7 cells were transfected with pMT3-SOK-1, pCMV5-SOK-1 or pCMV5-SOK-1ΔC. Forty eight hours later, cell lysates were subjected to immunoprecipitation with anti-HA or anti-M2 antibody. To assess inactivation of SOK-1 by phosphatase, immune complexes were incubated for 20 min at 30°C with the catalytic subunit of PP2A, either with or without the PP2A inhibitor, okadaic acid (100 nM). The PP2A

had been purified from rabbit skeletal muscle (Chen *et al.*, 1992) and was generously provided by Dr David Brautigan (Center for Cell Signalling, University of Virginia Health Science Center). After the 20 min incubation, okadaic acid was added to bring the final concentration to 100 nM in all tubes. Immune complexes were washed twice with kinase assay buffer, and then exposed to [$\gamma$-$^{32}$P]ATP (100 μM) for 0, 5, 10 or 20 min prior to the addition of MBP and subsequent kinase assay for 5 min at 30°C.

### *Production of anti-SOK-1 polyclonal antibodies*

A peptide (amino acids 333–426) from the non-catalytic region of SOK-1 was used to generate a polyclonal antibody in rabbit. This peptide was expressed in bacteria from pGEX-KG as a GST fusion protein, purified and used to immunize rabbits according to standard protocols (Harlow and Lane, 1988). The antibodies from each of two rabbits recognized 1 ng of GST–SOK-1 on a Western blot when used at a 1:1000 dilution. In addition, at a 1:250 dilution, the antibody immunoprecipitated HA–SOK-1 from lysates of transfected cells.

### *Microinjection and immunofluorescence*

Mouse fibroblast NIH 3T3 cells were grown on glass coverslips and microinjected with the pMT3-SOK-1 expression vector, encoding SOK-1 with the HA epitope tag at its N-terminus. Plasmid DNA had been purified twice on a CsCl gradient and extracted three times with phenol and choroform. Cells were injected in a 3.5 cm dish with an automated microinjection system [AIS; Zeiss (Ansorge and Pepperkok, 1988)] at a pressure between 80 and 170 kPa. The computer settings were as follows: angle, 45°; speed, 10; and time, 0.0 s. Plasmid DNA was injected at 100 μg/ml concentration (Pagano, 1994). Twenty-four hours after injection, cells were fixed with 4% paraformaldehyde for 15 min, treated with 0.1% SDS in phosphate-buffered saline (PBS) for 5 min, and permeabilized with 0.5% Triton X-100 (in PBS) for 15 min. Cells were then processed for immunfluorescence (Brown *et al.*, 1996).

All antibodies were diluted in Dulbecco's modified Eagle's medium containing 10% calf serum. Coverslips were incubated with affinity-purified anti-HA antibody (Boehringer Mannheim) at a final concentration of 0.026 mg/ml for 1 h. Coverslips were then incubated for 40 min in biotinylated goat anti-mouse antibody (Jackson Laboratories) which was diluted to 1:50 and then incubated for 40 min in fluorescein isothiocyanate-conjugated streptavidin, diluted 1:100 (Jackson Laboratories). All incubations were at 37°C in a humidified chamber. Between each step, cells were washed three times with PBS. Nuclei were conterstained with bisbenzimide (Hoecht 33258) for 2 min at 1 mg/ml in PBS. Coversips were mounted in Crystal/Mount (Biomedia) and visualized on a Zeiss Axiovert 100 photomicroscope. Cells were imaged with a Bio-Rad Laser Scanning Confocal Microscope.

### *Accession number*

The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number X99325.

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