independent of $InsP_3$ concentration if normalized to the half-time ($t_{1/2}$) (Fig. 4 legend). This prediction does not hold if there are compartments with different $InsP_3$ sensitivities. In Fig. 4 we compare the time courses of Ca^{2+} release at 30, 100 nM and 10 μM $InsP_3$. When normalized with $t_{1/2}$, they become superimposable, indicating that the results are consistent with the present model. We found that two exponential components were sufficient to fit the time course of Ca^{2+} release reasonably well, and our results are consistent with the faster component having $65.2 \pm 9.7\%$ of the total capacity and 6.76 ± 2.60 times greater channel density (mean±s.d., $n = 52$). Our results may also be consistent with the compartments having different types of $InsP_3$ receptors which have the same affinity for $InsP_3$ but have a different open probability or single-channel conductance. The Ca^{2+} stores may be structurally continuous[22,23] but they can be functionally divided if diffusion of Ca^{2+} within the Ca^{2+} stores is restricted as a result of a tortuous diffusion path and/or high-density intraluminal Ca^{2+} binding sites.

It has been suggested that there is regional heterogeneity of $InsP_3$ sensitivity in pancreatic acini cells[24] and in cultured smooth muscle cells[25]. Although diverse $InsP_3$ sensitivity of Ca^{2+} stores has been attributed to the different types of $InsP_3$ receptors or to their different degrees of modification[1,6], this remains to be proved. On the other hand, heterogeneous density of $InsP_3$ receptors on Ca^{2+} stores may explain apparent variations in $InsP_3$ sensitivity, which may be amplified through regulation of $InsP_3$ receptors by cytoplasmic Ca^{2+} (refs 16–20) as Ca^{2+}-mediated sensitization of Ca^{2+} release may occur more easily in Ca^{2+} stores with higher densities of $InsP_3$ receptors. Thus stores with different densities of $InsP_3$ receptors may play different roles in the generation of Ca^{2+} oscillations and Ca^{2+} waves. □

Received 22 March; accepted 18 November 1994.

1. Berridge, M. J. *Nature* **361,** 315–325 (1993).
2. Meyer, T. & Stryer, L. *Proc. natn. Acad. Sci. U.S.A.* **87,** 3841–3845 (1990).
3. Muallem, S., Pandol, S. J. & Beeker, T. G. *J. biol. Chem.* **264,** 205–212 (1989).
4. Parker, I. & Ivorra, I. *Science* **250,** 977–979 (1990).
5. Oldershaw, K. A., Nunn, D. L. & Taylor, C. W. *Biochem. J.* **278,** 705–708 (1991).
6. Ferris, C. D., Cameron, A. M., Huganir, R. L. & Snyder, S. H. *Nature* **356,** 350–352 (1992).
7. Bootman, M. D., Berridge, M. J. & Taylor, C. W. *J. Physiol., Lond.* **450,** 163–178 (1992).
8. Irvine, R. F. *FEBS Lett.* **263,** 5–9 (1990).
9. Missiaen, L., Taylor, C. W. & Berridge, M. J. *Nature* **352,** 241–244 (1991).
10. Missiaen, L., De-Smedt, H., Droogmans, G. & Casteels, R. *Nature* **357,** 599–602 (1992).
11. Loomis-Husselbee, J. W. & Dawson, A. P. *Biochem. J.* **289,** 861–866 (1993).
12. Parys, J. B., Missiaen, L., De Smedt, H. & Casteels, R. *J. biol. Chem.* **268,** 25206–25212 (1993).
13. Raju, B., Murphy, E., Levy, L. A., Hall, R. D. & London, R. E. *Am. J. Physiol.* **256,** C540–C548 (1989).
14. Uyama, Y., Imaizumi, Y. & Watanabe, M. *Br. J. Pharmac.* **106,** 208–214 (1992).
15. Kobayashi, S., Somlyo, A. V. & Somlyo, A. P. *Biochem. biophys. Res. Commun.* **153,** 625–631 (1988).
16. Iino, M. *J. gen. Physiol.* **95,** 1103–1122 (1990).
17. Bezprozvanny, I., Watras, J. & Ehrlich, B. E. *Nature* **351,** 751–754 (1991).
18. Finch, E. A., Turner, T. J. & Goldin, S. M. *Science* **252,** 443–446 (1991).
19. Iino, M. & Endo, M. *Nature* **360,** 76–78 (1992).
20. Hirose, K., Iino, M. & Endo, M. *Biochem. biophys. Res. Commun.* **194,** 726–732 (1993).
21. Swillens, S. *Molec. Pharmac.* **41,** 110–114 (1992).
22. Reonard-Rooney, D. C., Hajnóczky, G., Seitz, M. B., Schneider, T. G. & Thomas, A. P. *J. biol. Chem.* **268,** 23601–23610 (1993).
23. Somlyo, A. V. in *Handbook of Physiology* Section 2: Vol. II (eds Bohr, D. F., Somlyo, A. P. & Sparks, H. V.) 33–67 (American Physiological Society, Bethesda, 1980).
24. Kasai, H. & Petersen, O. H. *Trends Neurosci.* **17,** 95–101 (1994).
25. Short, A. D., Klein, M. G., Schneider, M. F. & Gill, D. L. *J. biol. Chem.* **268,** 25887–25893 (1993).
26. Hofer, A. M. & Machen, T. E. *Proc. natn. Acad. Sci. U.S.A.* **90,** 2598–2602 (1993).
27. Iino, M. *J. gen. Physiol.* **94,** 363–383 (1989).
28. Ahnert-Hilger, G. & Gratzl, M. *Trends pharmacol. Sci.* **9,** 195–197 (1988).
29. Ghosh, T. K., Mullaney, J. M., Tarazi, F. I. & Gill, D. L. *Nature* **340,** 236–239 (1989).

ACKNOWLEDGEMENTS. Supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and a grant from the Ichiro Kanehara Foundation.

Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun

Irma Sánchez*, Rowland T. Hughes†, Bruce J. Mayer‡§, Karen Yee†, James R. Woodgett‖, Joseph Avruch*, John M. Kyriakis* & Leonard I. Zon†§#¶

* Diabetes Research Laboratory, Medical Services, Massachusetts General Hospital East, and Department of Medicine Harvard Medical School, 149 13th Street, Charlestown, Massachusetts 02129, USA
† Division of Hematology/Oncology, ‡ Department of Microbiology and Molecular Genetics, § Howard Hughes Medical Institute, Children's Hospital and ¶ Dana-Farber Cancer Institute, Harvard Medical School, 300 Longwood Avenue, Enders 650, Boston, Massachusetts 02115, USA
‖ Ontario Cancer Institute, Princess Margaret Hospital, 500 Sherbourne Street, Toronto, Ontario M4X 1K9, Canada

THE stress-activated protein kinases (SAPKs), which are distantly related to the MAP kinases, are the dominant c-Jun amino-terminal protein kinases activated in response to a variety of cellular stresses, including treatment with tumour-necrosis factor-α and interleukin-β (refs 1, 2). SAPK phosphorylation of c-Jun probably activates the c-Jun transactivation function[3]. SAPKs are part of a signal transduction cascade related to, but distinct from, the MAPK pathway[1]. We have now identified a novel protein kinase, called SAPK/ERK kinase-1 (SEK1), which is structurally related to the MAP kinase kinases (MEKs)[4]. SEK1 is a potent activator of the SAPKs *in vitro* and *in vivo*. An inactive SEK1 mutant blocks SAPK activation by extracellular stimuli without interfering with the MAPK pathway. Although alternative mechanisms of SAPK activation may exist, as an immediate upstream activator of the SAPKs, SEK1 further defines a signalling cascade that couples cellular stress agonists to the c-Jun transcription factor.

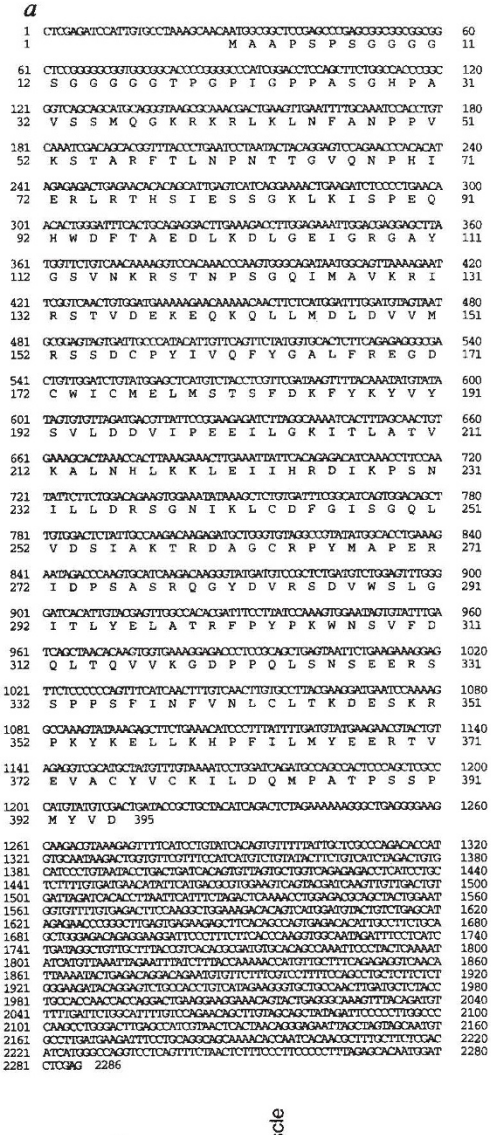
We previously isolated two *Xenopus* complementary DNAs, XMEK2 and XMEK3, that encode potentially novel MEKs[5]. XMEK2 is sufficiently different from MEK1 in structure to suggest it has a distinct role in signal transduction[5]. To define its function in mammalian cells, we isolated a murine homologue of XMEK2, which we refer to as SEK1 (Fig. 1*a*). A comparison of SEK1 with other mammalian and *Drosophila* MEKs and related yeast kinases demonstrates the extensive homology of the family (Fig. 1*b*) (reviewed in ref. 6). Specifically, subdomain VIII of the catalytic domain[7] contains two sites of potential phosphorylation (at Ser 220 and Thr 224) which align with those in MEK1, whose phosphorylation by Raf-1 and MEK kinase results in MEK1 activation[8–10].

Northern blot analysis demonstrates that SEK1 messenger RNA is ubiquitously expressed at very high levels in brain and muscle (Fig. 1*c*), consistent with our previous findings of the XMEK2 pattern of expression during *Xenopus* development[5].

To analyse SEK1 function, SEK1 was cloned into the mammalian expression vector pEBG, which drives the expression of a glutathione-*S*-transferase (GST) fusion protein, including the entire coding sequence of SEK1. pEBG-SEK1 was transfected into 293 cells and the cells were then treated with tumour-necrosis factor-α (TNF-α) or anisomycin, agonists known to activate the SAPKs *in vivo*[1], or epidermal growth factor (EGF), an agonist that does not activate SAPKs in most cell types[1]. GST–SEK1, immobilized on glutathione–agarose, was assayed for its ability to activate recombinant prokaryotically expressed

To whom correspondence should be addressed.

SAPKs *in vitro* (Fig. 2*a*, *c*). GST–SEK1 isolated from serum-deprived 293 cells phosphorylates recombinant SAPK-α and β molecules *in vitro* (Fig. 2*c*) and activates their c-Jun kinase activity (Fig. 2*a*, *c*). Moreover, agonists that activate SAPKs also activate SEK1. SEK1 isolated from untreated cells activates SAPK-α and β molecules threefold; that from TNFα-treated cells activates SAPKs sixfold, and GST–SEK1 from anisomycin-treated cells activates SAPKs nearly ninefold (Fig. 2*a*). Pretreatment of 293 cells with anisomycin also enhances the ability of SEK1 to phosphorylate the SAPK polypeptide (Fig. 2*c*). By



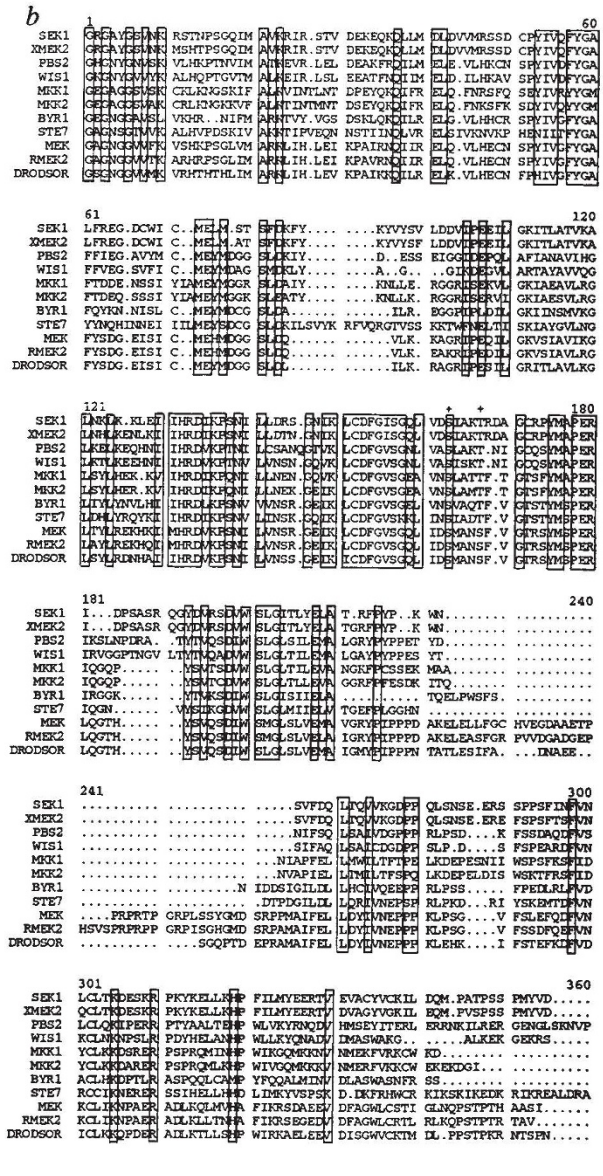
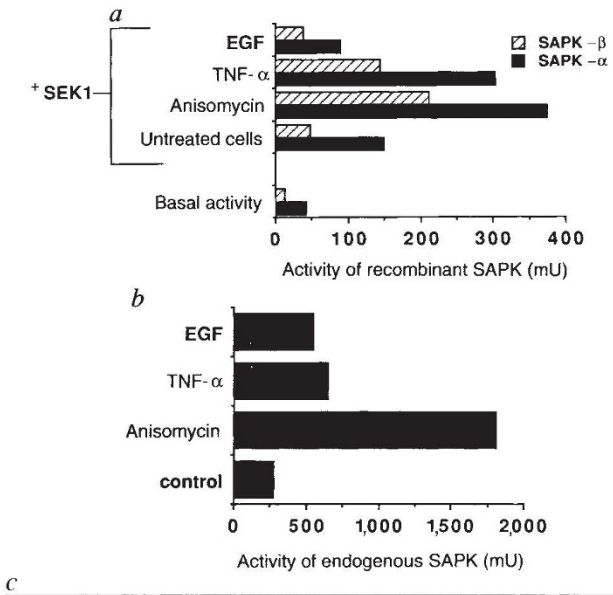


FIG. 1 Sequence of SEK1 cDNA. *a*, Nucleotide and amino-acid sequence of SEK1. Amino-acid sequence is listed underneath the cDNA sequence with numbers starting at the initiation codon. *b*, Comparative alignment of the catalytic domains of SEK1 and other MEK family members. *c*, Northern analysis of relative expression of SEK1 mRNA.

METHODS. The entire XMEK2 cDNA was used as a probe to screen a 6.5-day murine embryo cDNA library (5×10^5 λgt11 plaques) at reduced stringency (final wash, 0.15 M NaCl/15 mM sodium citrate, 42 °C). A partial cDNA was isolated from this screen and used to screen a size-selected (>2 kb), random-primed murine erythroleukaemia cell cDNA library (5×10^5 clones) at high stringency. Sequence analysis confirmed three full-length clones. The SEK1 cDNA encoded two in-frame methionines at the 5′ end (nucleotides (nt) 29–31, nt 131–133). As is the case with p70 S6 kinase[23], these may represent alternative translational initiation sites. Western blotting with an antiserum that can detect the expressed SEK1 cDNA indicates a polypeptide of 41K–43K (not shown), suggesting that alternative translational initiation may occur. Sequences of SEK1 and the other MEK family members were compared using the PILEUP program and was maximized by eye. Conserved residues are boxed. Northern analysis of 10 μg poly(A)$^+$ mRNA was done using standard methods.



c

GST	-	-	-	-	+	+
GST-SEK1	+	+	+	+	-	-
SAPK	+	-	+	-	+	-
Anisomycin	+	+	-	-	+	+

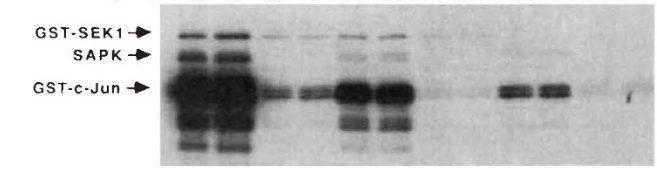
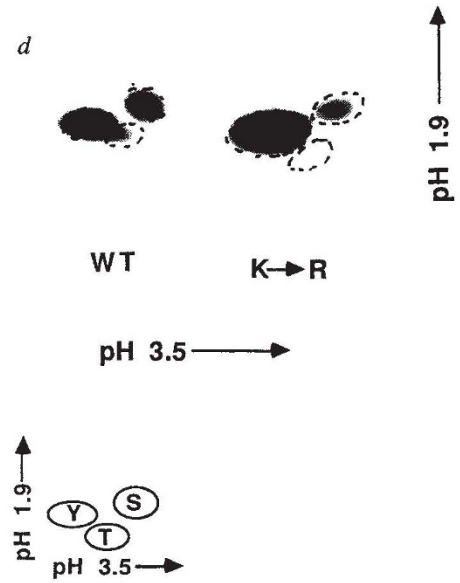


FIG. 2 Characterization of SEK1 function. *a*, SEK1 activates SAPK-αI and SAPK-p54β. GST–SEK1 was recovered from transfected 293 cells after treatment of the cells with the ligands shown and assayed for activation of recombinant SAPK isoforms. Values shown are for activated SAPK activity. *b*, Activity of endogenous SAPKs immunoprecipitated from non-transfected cells treated as in *a*; SAPK activation parallels activation of transfected SEK1. *c*, SEK1-catalysed phosphorylation of the SAPK polypeptide accompanies SAPK activation by SEK1. Recombinant SAPK-αI or vehicle was incubated with GST only, GST–SEK1 from control cells, or GST–SEK1 from anisomycin-treated cells as indicated. GST–c-Jun was then added to measure SAPK activity. GST–c-Jun, SAPK and GST–SEK1 polypeptides are indicated. *d*, Phosphoamino-acid analysis of SEK1 phosphorylation of SAPK. Left, top, SEK1 phosphorylation of wild-type SAPK-αI. Right panel phosphorylation of SAPK-αI(K→R) mutant. Bottom left, relative positions of phosphoamino acids: Y, tyrosine; T, threonine; S, serine.

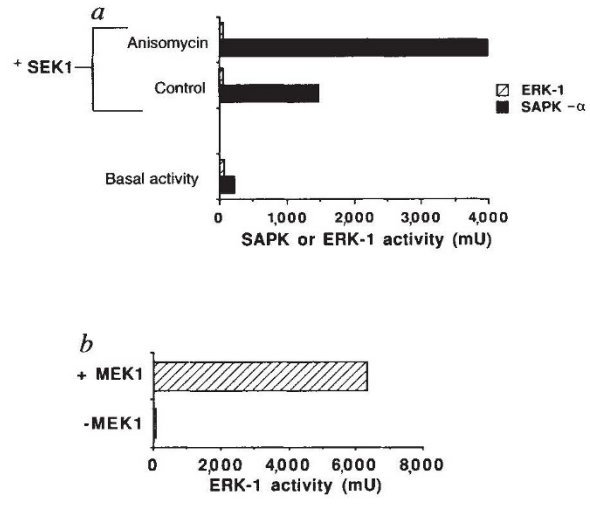


METHODS. pEBG is a mammalian expression vector derived from pEF-BOS (ref. 24) by insertion of a polylinker containing *Bam*HI and *Cla*I sites. Expression of a GST-insert fusion protein is driven by the EF-1α promoter. SEK1 K→R was produced by an overlapping PCR method[25] using a mutant PCR primer (5′-CAGATAATGGCAGTTAGAAGAATTCGG-TCAACT-3′) to produce an A-to-G transpostion at nt 414, resulting in a Lys-to-Arg substitution at Lys 129. Procaryotically expressed SAPK-αI, p54β and ERK1 were expressed from the pGEX-KG vector, purified by glutathione–agarose chromatography[26]. Transfection into 293 cells was by the calcium phosphate method; 5 μg of the relevant plasmids were transfected. GST–SEK1, MEK1 or SAPK-β were isolated as follows. Cells were serum-starved for 16 h and then treated with anisomycin (50 μg ml^{-1} for 40 min, TNF-α (50 ng ml^{-1}, 15 min), EGF (100 ng ml^{-1}, 15 min) or PMA (1 μM for 20 min to activate MEK1), at which time cells were lysed in 1 ml ice-cold lysis bufer[1]. Lysates were cleared by centrifugation and incubated with glutathione–agarose for 30 min and washed as described[1]. SAPKs were immunoprecipitated and assayed as before[1]. For GST–SEK1 and GST–MEK1 assays, 30 μl 1:1 beads (GST–SEK1 or MEK1) in kinase assay buffer[1] were incubated at 30 °C for 30 min with 15 μl 0.15 mg ml^{-1} SAPK or MAPK preparation and [γ^{32}P]ATP (100 μM, 15 μCi). At this time, 1 μg GST–c-Jun (SAPK assays) or MBP (ERK1 assays) and an additional 100 μM [γ^{32}P]ATP were added and the reaction continued for 15 min. Proteins were separated by SDS–PAGE and the appropriate bands excised and counted. Background activity in the absence of SAPK/ERK1 was subtracted from the values in *a*: 25 mU (control), 75 mU (TNF-α) 75 mU (anisomycin) and 20 mU (EGF). Experiments were performed twice and assayed in duplicate; a representative experiment is shown. A unit of SAPK activity is defined in ref. 1. Phosphoamino-acid in SAPK were analysed as described[27].

contrast, EGF fails to stimulate SEK1 activity beyond the three-fold levels seen in unstimulated cells. Parallel assays of endogenous SAPK activity in 293 cells (Fig. 2*b*) demonstrates that the degree of activation of the SAPKs in response to TNF-α and anisomycin parallels closely that of the recombinant of GST-SEK1. There is, however, a modest activation of SAPK by EGF in 293 cells which is not reflected by SEK1 activation. This result implies that SEK-independent mechanisms of SAPK activation might exist.

MEKs are dual-specificity protein kinases which phosphorylate MAPK polypeptides at the tyrosine and threonine residues whose phosphorylation is required for activation[11]. SAPKs also require Tyr and Thr phosphorylation for activity[2,12]. Under conditions that result in *in vitro* SAPK activation by SEK1 (Fig. 2*c*), wild-type SAPK is phosphorylated at Tyr, Ser and Thr (Fig. 2*d*, top left), whereas a kinase-inactive SAPK mutant is phosphorylated predominantly at Tyr, with no detectable Thr phosphorylation (Fig. 2*d*, top right). Thus SEK1 does not show prominent dual specificity and appears to phosphorylate SAPKs preferentially at Tyr. Insofar as SAPKs are proline-directed[13] and the site of SAPK activating phosphorylation is TPY[1,2], a significant portion of the regulatory Thr phosphorylation could arise as a result of autophosphorylation, occurring after SEK1-catalysed Tyr phosphorylation. Indeed, the MAPK-specific MEKs show a similar preference for Tyr when presented with, as substrates, MAPK mutants which are incapable of phosphotransferase activity[14].

To determine the specificity of SEK1, we compared the ability of SEK1 to activate SAPK-αI and ERK1. 293 cells were transfected with either pEBG-SEK1 or pEBG-MEK1. Cells were treated with either anisomycin to activate SEK1, or with phorbol myristate acetate (PMA) to activate MEK1. As shown in Fig. 3*a*, SEK1 dramatically activates SAPKs under conditions that catalyse little or no activation of ERK1. GST–SEK1 activates ERK1 1.1-fold, compared with an 18-fold activation of SAPK-αI. By contrast, incubation of ERK1 with GST–MEK1 beads



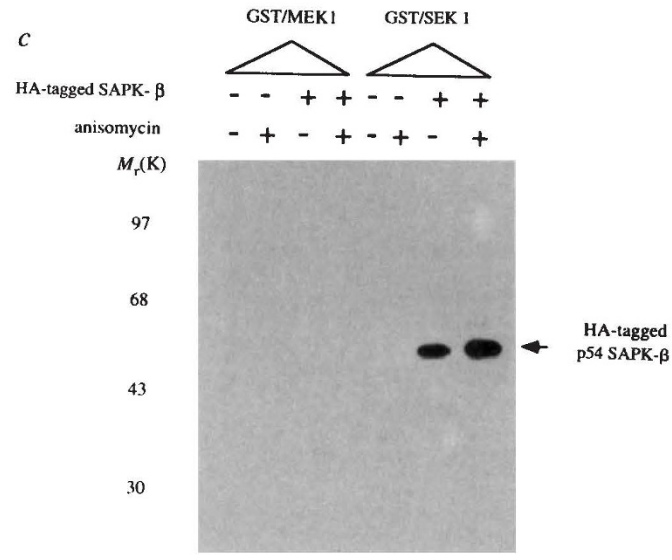


FIG. 3 Specificity of SEK1 for SAPKs. *a*, Comparison of SEK1 activity towards SAPK-αI and MAPK (ERK1). *b*, The ERK1 preparation can be activated by MEK1. *c*, Physical association between SAPK and SEK1. COS7 cells were transfected with pEBG-MEK1 (lanes 1, 2, 5 and 6) or SEK1 (lanes 3, 4, 7 and 8) and HA-SAPK-p54β (lanes 5–8). Odd-number lanes, control cells; even-number lanes, anisomycin-treated cells. GST–MEK1 and SEK1 were recovered on glutathione–agarose and probed by immunoblotting with anit-HA antiserum.

METHODS. GST–SEK1 and MEK1 were assayed as described in Fig. 2 legend. COS7 cells were transfected by the DEAE–dextran method[28]. Immunoblotting was with the ECL system (Amersham). 1U ERK1 will transfer 1 pmol min^{-1} PO_4 from ATP to MBP. As for Fig. 2, activity of contaminating c-Jun kinase or MBP kinase in the preparations of GST–SEK1 and MEK1 was subtracted from the values shown. These activities were 14 mU (control SEK1 c-Jun kinase), 135 mU (anisomycin SEK1 c-Jun kinase), and 82 mU (MEK1 MBP kinase).

gives >20-fold activation of the ERK1 MBP-kinase activity (Fig. 3*b*). Thus SEK1 shows at least 15-fold more activity towards SAPK then towards ERK1. On the other hand, MEK1 gives no detectable activation of SAPKs *in vitro* (refs 1, 2 and data not shown).

If SAPKs were specific SEK1 substrates, an association of these two polypeptides *in vivo* might be detectable. COS7 cells were cotransfected with haemagglutinin (HA)-tagged SAPK-p54β and either pEBG-SEK1 or pEBG-MEK1. GST–SEK1 or GST–MEK1 were then purified from cell lysates by glutathione–agarose chromatography and immunoblotted with an antibody against the HA tag. As seen in Fig. 3*c*, HA-tagged SAPK complexes and copurifies specifically with GST–SEK1 and not GST–MEK1. We do not know if the association between SAPK and SEK1 is direct or is through a third, unidentified molecule.

Further evidence of the physiological significance of SEK1 as an upstream activator of the SAPKs is provided by the properties of a mutant kinase-inactive pEBG-SEK1 construct in which the critical lysine (Lys 129) in the ATP-binding site has been mutated to arginine. This mutant, pEBG-SEK1K-R, was cotransfected with pEBG-SAPK-p54β into 293 cells and the ability of anisomycin to activate GST–SAPK activity in these cells assayed. Anisomycin activates the GST-SAPKβ (Fig. 4*a*, right; compare lanes 1 and 2). But coexpression of the GST–SAPKβ with the mutant SEK1K-R supresses this activation completely (Fig. 4*a*, right; compare lanes 2 and 3). Thus GST–SEK1K-R can act as a dominant inhibitor of SAPK activation *in vivo*, indicating that anisomycin activation of the SAPKs requires elements that interact with SEK1 and that these elements (for example, SEK kinases) can be completely sequestered by overexpression of recombinant inactive SEK1.

Evidence for the specificity of the SEK1 K→R mutant for the SAPK pathway is shown in Fig. 4*b*. Expression of the transforming *abl* gene leads to activation of p42/p44 MAPKs, presumably through the Ras pathway (refs 15 and 16, and Fig. 4*b*, right, lanes 1 and 2). The activation of the ERK1 by Abl is not interfered with by SEK1(K→R), whereas this mutant completely blocks SAPK activation (compare Fig. 4*a* and *b*, right panels).

Whereas several mechanisms of SAPK activation may exist, on the basis of the *in vitro* activation of SAPKs by SEK1, the

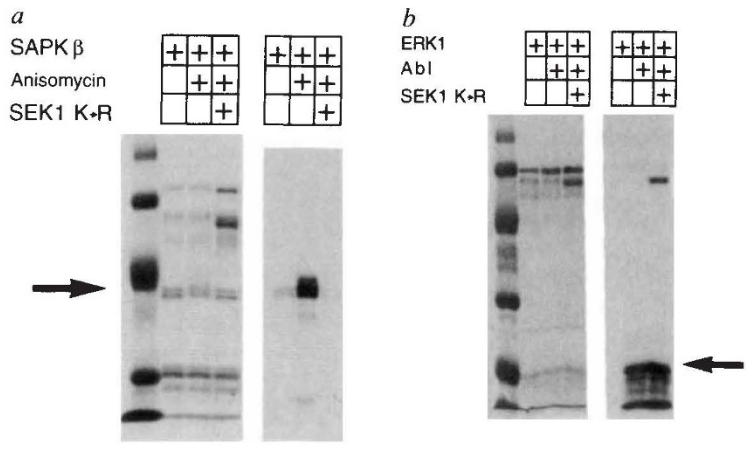


FIG. 4 Expression of a kinase-inactive SEK1, SEK1(K→R), inhibits its anisomycin activation of SAPKs but does not interfere with Abl activation of MAPKs. *a*, Expression of SEK1(K→R) inhibits activation of SAPK by anisomycin. pEBG-SAPK-p54β was expressed in 293 cells alone or together with pEBG-SEK1(K→R). Cells were treated with anisomycin and SAPK activity measured. Left, Coomassie-blue stained gel; right, autoradiogram. Arrow indicates GST–c-Jun. *b*, 293 cells were transfected with a retroviral vector encoding a SH3 deleted transforming Abl gene and pEBG-ERK1, or pEBG-ERK1 plus pEBG-SEK1(K→R). GST–ERK1 was isolated and assayed for MBP kinase activity. Left, Coomassie-blue stained gel; right, autoradiogram; arrow indicates MBP. Note that ERK1 can phosphorylate SEK1(K→R) on two consensus MAPK recognition sites at positions 385–391 (right panel, lane 3, and data not shown), although the function of this phosphorylation remains to be determined.
METHODS. 293 cells were transfected as described in Fig. 2 legend. For the Abl experiment, pGNG (SH3-deleted *abl* gene) was transfected (5 μg). Anisomycin treatment and assays are described in Fig. 2 legend.

in vivo activation of SEK1 by SAPK agonists and the ability of dominant-negative SEK1 to inhibit SAPK activation by extracellular agonists, we propose that SEK1 is a physiological activator of the SAPKs *in vivo*. We chose the name SEK1 (for SAPK/ERK kinase) because other extracellularly regulated kinases (ERKs) have been isolated[17–19] and we do not know yet if these are physiological substrates of SEK1. The inability of Ras and Ras-coupled agonists to activate SEK1 or the SAPKs strongly *in vivo*, coupled with the inability of SEK1(K→R) to block Ras activation of MAPKs and the inability of Raf-1 to activate the SAPKs[20], indicate that SEK1 and the SAPKs lie on a signalling pathway that is largely distinct from the Ras/Raf/MEK/MAPK pathway. In an accompanying paper, we demonstrate that SEK1 is phosphorylated and activated in intact cells not by Raf-1, but by MEK-kinase-1 (ref. 20), a mammalian homologue of the STE11 kinase from *Saccharomyces cerevisiae*[21]. These results support the contention that at least two signal transduction cascades exist in mammals with distinct functions: the MEKK/SEK/SAPK-mediated stress response and the Raf/MEK/MAPK-mediated mitogenic response. The specificity of SEK1 and the MEKs defines the segregation of the stress and mitogenic pathways. Thus, earlier work in yeast, demonstrating multiple independent but homologous signalling pathways (reviewed in ref. 22) can now be extended to mammalian systems. □

Received 29 September; accepted 15 November 1994.

1. Kyriakis, J. M. *et al. Nature* **369,** 156–160 (1994).
2. Dérijard, B. *et al. Cell* **76,** 1025–1037 (1994).
3. Pulverer, B. J. *et al. Nature* **353,** 670–674 (1991).
4. Crews, C. M., Alessandrini, A. & Erikson, R. L. *Science* **258,** 478–480 (1992).
5. Yashar, B. M. *et al. Molec. cell. Biol.* **13,** 5738–5748 (1993).
6. Ahn, N. G., Seger, R. & Krebs, E. G. *Curr. Opin. Cell Biol.* **4,** 992–999 (1992).
7. Hanks, S. K., Quinn, A. M. & Hunter, T. *Science* **241,** 42–52 (1988).
8. Zheng, C.-F. & Guan, K.-L. *EMBO J.* **13,** 1123–1131 (1994).
9. Alessi, D. R. *et al. EMBO J.* **13,** 1610–1619 (1994).
10. Yan, M. & Templeton, D. J. *J. biol. Chem.* **269,** 19067–19073 (1994).
11. Nakielny, S., Cohen, P., Wu, J. & Sturgill, T. W. *EMBO J.* **11,** 2123–2129 (1992).
12. Kyriakis, J. M., Brautigan, D. L., Ingebritsen, T. S. & Avruch, J. *J. biol. Chem.* **266,** 10043–10046 (1991).
13. Mukhopadhyay, N. K. *et al. J. biol. Chem.* **267,** 3325–3335 (1992).
14. Her, J.-H. *et al. Biochem. J.* **296,** 25–31 (1993).
15. Leevers, S. J. & Marshall, C. J. *EMBO J.* **11,** 569–574 (1992).
16. Wood, K. W., Sarnecki, C., Roberts, T. M. & Blenis, J. *Cell* **68,** 1041–1050 (1992).
17. Han, J., Lee, J.-D., Bibbs, L. & Ulevitch, R. J. *Science* **265,** 808–811 (1994).
18. Rouse, J. *et al. Cell* **78,** 1027–1037 (1994).
19. Freshney, N. W. *et al. Cell* **78,** 1039–1049 (1994).
20. Yan, M. *et al. Nature*, **372,** 788–800 (1994).
21. Lange-Carter, C. A. *et al. Science* **260,** 315–319 (1993).
22. Blumer, K. J. & Johnson, G. L. *Trends biochem. Sci.* **19,** 236–240 (1994).
23. Grove, J. R. *et al. Molec. cell. Biol.* **11,** 5541–5550 (1991).
24. Mizushima, S. & Nagata, S. *Nucleic Acids Res.* **18,** 5322 (1990).
25. Ho, S. N. *et al. Gene* **77,** 51–59 (1989).
26. Smith, D. B. & Johnson, D. S. *Gene* **67,** 31–40 (1988).
27. Boyle, W. J., van der Geer, T. & Hunter, T. *Meth. Enzym.* **201,** 110–152 (1991).
28. Aruffo, A. & Seed, B. *Proc. natn. Acad. Sci.* U.S.A. **84,** 8573–8477 (1987).

ACKNOWLEDGEMENTS. I.S. and R.T.H. contributed equally to this work. We thank B. Drucker, B. Yashar, M. Crossley, B. D. Dynlacht, E. Takeuchi-Suzuki, A. Katling and E. Neufeld for advice, A. M. Forte and E. Rubie for technical assistance, and R. Erikson for MEK1 cDNA. I.S. is supported by a USPHS training grant. R.T.H. was supported by an M.R.C. (U.K.) Travelling Fellowship. J.M.K. is supported by grants from the USPHS, and the US Army Breast Cancer Research Program. J.R.W. is supported by grants from the Canadian MRC and NCI. J.A. is supported by grants from the USPHS and the ACS. L.I.Z. is supported by grants from the USPHS. L.I.Z. and B.J.M. are Assistant Investigators with the Howard Hughes Medical Institute.

Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1

Minhong Yan*, Tianang Dai†, Joseph C. Deak*, John M. Kyriakis‡, Leonard I. Zon§, James R. Woodgett† & Dennis J. Templeton*‖

* Institute of Pathology and Program in Cell Biology, Case Western Reserve University School of Medicine, 10900 E. Euclid Avenue, Cleveland, Ohio 44106, USA
† Ontario Cancer Institute, Princess Margaret Hospital, 500 Sherbourne Street, Toronto, Ontario M4X 1K9, Canada
‡ Diabetes Research Laboratory, Medical Services Massachusetts General Hospital and The Department of Medicine, Harvard Medical School, MGH East, 149 13th Street, Charlestown, Massachusetts 02129, USA
§ Division of Hematology, Howard Hughes Medical Institute, Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA

A KINASE distinct from the MEK activator Raf[1–3], termed MEK kinase-1 (MEKK), was originally identified by virtue of its homology to kinases involved in yeast mating signal cascades[4]. Like Raf, MEKK is capable of activating MEK *in vitro*[4,5]. High-level expression of MEKK in COS-7 cells[4] or using vaccinia virus vectors[5] also activates MEK and MAPK, indicating that MEKK and Raf provide alternative means of activating the MAPK signalling pathway. We have derived NIH3T3 cell sublines that can be induced to express active MEKK. Here we show that induction of MEKK does not result in the activation of MAPK, but instead stimulates the stress-activated protein kinases (SAPKs)[6–8] which are identical to a Jun amino-terminal kinase[9,10]. We find that MEKK regulates a new signalling cascade by phosphorylating an SAPK activator, SEK1 which in turn phosphorylates and activates SAPK.

Stably transfected NIH3T3 subclones express MEKK in response to isopropyl-β-D-thiogalactosidase (IPTG) (Fig. 1*a*) but MAPK activity remains unchanged (Fig. 1*b*). In contrast, SAPK activity is increased six- to eightfold in MEKK-inducible cell lines but not in the parent NIH3T3 cells. These MEKK-expressing cells are able to activate MAPK in response to some mitogenic signals, because treatment with phorbol ester increases MAPK activity in each of these clones (as well as in NIH3T3 cells), whereas SAPK activity is unaffected (Fig. 1*c*). Increased SAPK activity is evident by 3 hours and maximal after 12 hours of induction; MAPK activity is unchanged throughout the 23-hour incubation with inducer (Fig. 1*d*). Together these results indicate that, in contrast to the presumed role of MEKK in activating MEK and MAPK, MEKK acts instead to activate SAPKs. Expression of truncated ΔMEKK in these clones resulted in six- to eightfold inhibition of growth rate compared with parental NIH3T3 cells.

We modelled activation of SAPK by MEKK using cloned genes and purified proteins expressed using vaccinia virus vectors[11,12]. MEKK induced electrophoretic retardation of SAPK, which was suggestive of quantitative phosphorylation (Fig. 2*a*), and also increased the amount of phosphotyrosine in SAPK and activated its Jun N-terminal kinase activity. Thus, in this overexpression model as well as in the inducible cell line, MEKK expression results in activation of the SAPK pathway.

We considered the possibility that activation of SAPK occurred as a consequence of activation of the MEK and MAPK cascade. To stimulate MAPK independently of MEKK, we used activated Raf and a constitutively active allele of MEK1 termed MEK 2E (ref. 5). Both Raf and MEK 2E were able to induce phosphorylation of coexpressed MAPK (Fig. 2*b*). Neither of these MAPK activators induced phosphorylation of SAPK, indicating that the SAPK activation pathway is effectively insulated from the MAPK pathway.

MEKK was unable to phosphorylate SAPK *in vitro* (below). We therefore tested whether MEKK activated the newly identified SAPK activator, SEK1 (ref. 13), whose sequence is similar to MEK1. Immunopurified MEKK (but not the inactive mutant

‖ To whom correspondence should be addressed.