independent of InsP<sub>3</sub> concentration if normalized to the halftime  $(t_{1/2})$  (Fig. 4 legend). This prediction does not hold if there are compartments with different InsP<sub>3</sub> sensitivities. In Fig. 4 we compare the time courses of Ca2+ release at 30, 100 nM and 10  $\mu$ M InsP<sub>3</sub>. 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<sup>2+</sup> release reasonably well, and our results are consistent with the faster component having  $65.2 \pm 9.7\%$  of the total capacity and  $6.76 \pm 2.60$  times greater channel density (mean $\pm$ s.d., n = 52). Our results may also be consistent with the compartments having different types of InsP<sub>3</sub> receptors which have the same affinity for InsP3 but have a different open probability or single-channel conductance. The Ca<sup>2+</sup> stores may be structurally continuous<sup>22,23</sup> but they can be functionally divided if diffusion of Ca<sup>2+</sup> within the Ca<sup>2+</sup> stores is restricted as a result of a tortuous diffusion path and/or highdensity intraluminal Ca<sup>2+</sup> binding sites.

It has been suggested that there is regional heterogeneity of InsP<sub>3</sub> sensitivity in pancreatic acini cells<sup>24</sup> and in cultured smooth muscle cells<sup>25</sup>. Although diverse InsP<sub>3</sub> sensitivity of Ca<sup>2+</sup> stores has been attributed to the different types of InsP3 receptors or to their different degrees of modification<sup>1,6</sup>, this remains to be proved. On the other hand, heterogeneous density of InsP<sub>3</sub> receptors on Ca<sup>2+</sup> stores may explain apparent variations in InsP<sub>3</sub> sensitivity, which may be amplified through regulation of InsP<sub>3</sub> receptors by cytoplasmic Ca<sup>2+</sup> (refs 16-20) as Ca<sup>2+</sup>-mediated sensitization of  $Ca^{2+}$  release may occur more easily in  $Ca^{2+}$ stores with higher densities of InsP<sub>3</sub> receptors. Thus stores with different densities of InsP<sub>3</sub> receptors may play different roles in

### the generation of Ca<sup>2+</sup> oscillations and Ca<sup>2+</sup> waves.

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

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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-a and interleukin-ß (refs 1, 2). SAPK phosphorylation of c-Jun probably activates the c-Jun transactivation function<sup>3</sup>. SAPKs are part of a signal transduction cascade related to, but distinct from, the MAPK pathway<sup>1</sup>. We have now identified a novel protein kinase, called SAPK/ERK kinase-1 (SEK1), which is structurally related to the MAP kinase kinases (MEKs)<sup>4</sup>. 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<sup>3</sup>. XMEK2 is sufficiently different from MEK1 in structure to suggest it has a distinct role in signal transduction<sup>5</sup>. To define its function in mammalian cells, we isolated a murine homologue of XMEK2, which we refer to as SEK1 (Fig. 1a). A comparison of SEK1 with other mammalian and Drosophila MEKs and related yeast kinases demonstrates the extensive homology of the family (Fig. 1b) (reviewed in ref. 6). Specifically, subdomain VIII of the catalytic domain<sup>7</sup> 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<sup>8-10</sup>.

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

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 tumournecrosis factor- $\alpha$  (TNF- $\alpha$ ) or anisomycin, agonists known to activate the SAPKs in vivo<sup>1</sup>, or epidermal growth factor (EGF), an agonist that does not activate SAPKs in most cell types<sup>1</sup>. GST-SEK1, immobilized on glutathione-agarose, was assayed for its ability to activate recombinant prokaryotically expressed

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SAPKs *in vitro* (Fig. 2*a*, *c*). GST–SEK1 isolated from serumdeprived 293 cells phosphorylates recombinant SAPK- $\alpha$  and  $\beta$ 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

a		
1	CTCGAGATCCATTIGIGCCTAAAGCAACAATGGCGGCTCCGAGCCCGAGCGGCGGCGGCGGCGG	60
1	MAAPSPSGGGG	11
61		120
12	SGGGGGGFGFIGFFASGAFA	21
121	GTICAGCAGCATGCAGGTAAGCGCAAACGACTGAACFTIGAATTTTGCAAATCCACCTGT	180
32	VSSMQGKRKRLKLNFANPPV	51
181	CAAATCGACAGGGTTTACCCTGAATCCTAATACTACAGGAGTCCAGAACCCACACAT	240
52	K S T A R F T L N P N T T G V Q N P H I	71
241	NUMBER OF THE ACTION OF THE AC	300
72	ERLRTHSIESSGKLKISPEO	91
301	ACACTGGGATTTCACTGCAGAGGACTTGAAAGACCTTGGAGAAATTGGAGGAGGAGGAGCTTA	360
92	HWDFTAEDLKDLGEIGRGAY	111
361	TAKE A A PROVIDE A PROPERTY AND A CONTRACT OF A CARAGE A CARACTERISTIC OF A CARACTERISTICO OF A CARACTERISTICO OF A CARACTERISTICO OF A CARACTERISTIC OF A CARACTERIS	420
112	G S V N K R S T N P S G O I M A V K R I	131
421	TOGUTCAACTOTOGATGAAAAAAAAAAAAAAAAACTUCICATOGATUTOGATUTAATAAT	480
132	RSTVDEKEQKQLLMDLDVVM	151
491	427777784236730787107767177871707707707707707707777777777	540
152	RSSDCPYIVOFYGALFREGD	171
541	CIGITOGATCIGIATOGAOCICAIGICIACCICOTICGATAAGTITIACAAATAIGIATA	600
172	CWICMELMSTSFDKFYKYVY	191
601	TELEVISION CONTRACTOR & CONTRAC	660
192	SVLDDVIPEEILGKITLATV	211
661	GAAAGCACTAAACCACTTAAAGAAACTTGAAATTATTCACAGAGACATCAAACCTTCCAA	720
212	KALNHLKKLEIIHRDIKPSN	231
721	100 000 000 000 000 000 0000 0000 0000	780
232	ILLDRSGNIKLCDFGISGOL	251
781	TUTOGACTUTATTOCCAAGACAAGAGATOCTOOGTGTAGOCCOTATATGOCACCTGAAAG	840
252	V D S I A K T R D A G C R P Y M A P E R	271
941	בכבודייוידאביבורייזיגיוייזיאניויידייבורבא אייאביויאנייא אייזיא אייא אייא אייא אייא א	900
272	I D P S A S R O G Y D V R S D V W S L G	291
901	GATCACATIGIAGAGTTOGCCACACGATITCCTIATCCAAAGTGGAATAGTGIATTTGA	960
292	ITLYELATRFPYPKWNSVFD	311
961	THE STAR ACTION AND AND AND AND AND AND AND AND AND AN	102
312	OLTOVVKGDPPOLSNSEERS	331
1021	TTCTCCCCCCAGTTTCATCAACTTTGTCAACTTGTGCCCTTACGAAGGATGAATCCAAAAG	108
332	SPPSFINFVNLCLTKDESKK	221
1081	GCCAAAGTATBAAGAGCTTCTGAAACATCCCTTTATTTTGATGIATGAAGAAGJACIGT	114
352	PKYKELLKHPFILMYEERTV	371
1141	AGAGTCGCATGCTATGTTTGTAAAATCCTGGATCAGATGCCAGCCA	120
312	EVACIVCKILDQAFAIF55F	222
1201	CATGTATGTCGACTGATACCGCTGCTACATCAGACTCTAGAAAAAAGGGCTGAGGGGAAG	126
392	MYVD 395	
1992		
1261	CANGACGTAAAGAGTTTTCATCCIGTATCACAGTGTTTTTTATIGCTCGCCCAGACACCAT	132
1321	CARLY AND AND CONTRACT OF A CARLEY AND AND CHARTER AND CARLY	144
1441	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	150
1501	GATTAGATCACACCTTAATTCATTTCTAGACTCAAAACCTOGAGACOCAOCTACTOGAAT	156
1561	OFIGTTTTGTGAGACTTCCAAGGCTGGAAAGACACAGTCAIGGATGTACTGTCTGAGCAT	162
1621	AGAGAAOCCOOOCTIGAGIGAGAAGAOCTICACAGCCAGIGAGACACATIOCCTICIGCA	168
1741	GCIGGGPGPCPCPCCCCCCCCCCCCCCCCCCCCCCCCCC	180
1801	ATCATOTTAAATTAGAATTTATCTTTACCAAAAACCATGTTCCTTTCAGAGAGGTCAACA	186
1861	TTAAAATACTGAGACAGGACAGAATGTGTTCTTTOGTCCTTTTOCAGCCTGCTCTTCTCT	192
1921	GGGAAGATACAGGAGTCTGOCACCTGTCATAGAAGGGTGCTGOCAACTTGATGCTCTACC	198
1981	TGOCACCAACCACCAGGACTGAAGGAAGGAAACAGTACTGAGGGCAAAGTTTTACAGATGT	204
2041	CANCELET CONCELETATION CONCELETATION CONCELERING TO CONCELETATION CONCEL	216
2161	GOOTTGATGAAGATTTTCCTGCAGGCAGCAAAACACCAATCACAACGCTTTCCTTCTCCAC	222
2221	ATCATOGGCCAGGTCCTCAGTTTCTAACTCTTTCCCTTCCC	228
2281	CTOGAG 2286	



SAPK- $\alpha$  and  $\beta$  molecules threefold; that from TNF $\alpha$ -treated cells activates SAPKs sixfold, and GST–SEK1 from anisomycintreated cells activates SAPKs nearly ninefold (Fig. 2a). Pretreatment of 293 cells with anisomycin also enhances the ability of SEK1 to phosphorylate the SAPK polypeptide (Fig. 2c). By

<i>b</i>	1	<b>-</b>				60
SEK1 XMEK2 PBS2 WIS1 MKK1 MKK2 BYR1	CRCAYDSIMI CRCAYDSIMI CHCNYDNIAS CRCAYDWMY CRCACOSIAS CRCACOSIAS CRCACOSIAS	KSTNPSGQI MSHTPSGQI VLHKPTNVI ALHQPTGVN CKLKNGSKI CKLKNGKKV VKHRNIFN	4 AVERIR.ST 4 AVERIR.ST 4 ADERIR.ST 4 ALEEIR.LEI 4 ALEEIR.LEI 5 ALEVINTLM 7 ALEVINTLM 4 ARETVY.VG	/ DEKEQKOLLM / DEKEQKOLLM DEAKFROILM EEATFNOILM DEYQKOIFR DSEYQKOIFR DSEYQKOIFR	I DLDVVMRSSE I TLDVVMRSSE I ELE. VLHKCN I ELD. ILHKAV ELQ. FNRSFQ ELQ. FNRSFK ELQ. VLHHCR	CPYIV2FYGA CPYIV2FYGA SPYIVDFYGA SPYIVDFYGA SEYIVRYYGM SEYIV2FYGA
MEK RMEK2 DRODSOR	GACNOGANE CACNOGANE CACNOGANE CACNOGANE	C VSHKPSGLVA C ARHRPSGLIN C ARHRPSGLIN C VRHTHTHLIN	ARRLIN.LEI ARRLIN.LEI ARRLIN.LEI ARRLIN.LEI	KPAIRNOIIR KPAIRNOIIR KPAIRNOIIR	ELS IVENVEP ELQ . VLHECN ELQ . VLHECN ELK . VLHECN	HENIIIFYGA SPYIVGFYGA SPYIVGFYGA FPHIVGFYGA
SEK1 XMEK2 PBS2 WIS1 MKK1 MKK2 BYR1 STE7 MEK RMEK2 DRODSOR	61 LFREG.DCWJ FFIEG.DCWJ FFIEG.AVYM FFVEG.SVFJ FTDEQ.SSSJ FTDEQ.SSSJ FTDEQ.SSSJ FYZKN.NISL FYSDG.EISJ FYSDG.EISJ FYSDG.EISJ	C. MEM.ST C. MEM.AT C. MEMADOC C. MEMADOC VIANEMAGOR VIANEMAGOR C. MEMADOC C. MEMADOC C. MEMADOC C. MEMADOC	SFOKFY. SPOKFY. SLOKIY. SLOKIY. SLORIY. SLOA. SLOA. SLOQ. SLOQ. SLOQ.		LDDVIPESIL LDDVIPESIL EIGGIDSPOL GIKUSSYL RGGRISSEKVI BGGRISSEKVI KSTMPKLTI KAGRIPESIL RAGRIPESIL	120 GKITLATVKA AFLANAVING ARTAYAVVQG GKIAESVLRG GKIAESVLRG GKISLAVURG GKVSIAVING GKVSIAVING GRVSIAVLRG GRITLAVLKG
SEK1 XMEK2 PBS2 WIS1 MKK1 MKK2 BYR1 STE7 MEK RMEK2 DRODSOR	121 INRIK KLET LARLKEDAN LKELKEDAN LKELKEDAN LSYLHER KU LSYLHER KU LSYLHER KU LSYLRER KU LDHLYRQYKI LAYLREKKU LAYLREKKU LSYLRINHAL	IHRDINSM IHRDINGSM IHRDMRTM IHRDINGTM IHRDINGM IHRDINGM IHRDINGSM IHRDINGSM MHRDMRSM IHRDMRSM IHRDMRSM	LLDRS . ENER LCSANGUYK LCSANGUYK LLNSN . GOVK LLNEN . GOVK LLNEK . GEIK VVNSR . GEIK LLNSK . GEIK LVNSR . GEIK LVNSR . GEIK	LCDFGTSGL LCDFGVSGN LCDFGVSGN LCDFGVSGN LCDFGVSGPA LCDFGVSGPA LCDFGVSGPA LCDFGVSGQL LCDFGVSGQL LCDFGVSGQL	VDSLAKTRDA VDSLAKTRDA VASLAKT.NI VASLAKT.NI VNSLATTF.T VNSLAMTF.T VNSLAMTF.V IDSMANSF.V IDSMANSF.V IDSMANSF.V	180 GIRPANAPER COSSMAPER COSSMAPER COSSMAPER CISPMAPER CISTMAPER CISTMAPER CISSMAPER CIRSMAPER CIRSMAPER
SEK1 IMEK2 PBS2 WI51 MKK1 BYR1 STE7 MEK RMEK2 DRODSOR	181 IDPSASR I.SLAPDRA. IRVGGPINGV IQGQP IRGGK IQGP LQGTH LQGTH	CONDERSIDE QUIDERSIDE INTERSIDE INTERSIDE STATES ST	SLGITLYEA SLGITLYEA SLGITLEYA SLGITLEYA SLGITLEYA SLGITLEYA SLGISIIELA SLGISIIELA SMGLSLVEYA SMGLSLVEYA SLGLSLVEYA	T.RFPYP.K TGRPPYP.K LGRYMYPPET LGRYMYPPES NGKPCSSEK GGRPFCSDK TGEFFLGGHN VGRYFLPPPD IGRYFLPPPD IGRYFLPPPD	WN. WN. YD. YT. TQELPWSFS. ARELELLFGC ARELEASFGR TATLESIFA.	240  HVEGDAAETP PVVDGADGEP INAEE .
SEK1 XMEK2 PB52 WIS1 MKK1 MKK2 BYR1 STE7 MEK RMEK2 DRODSOR	241	CRPLSSYOND GRPISCHOMD SCOPTD	SVFDQ SVFDQ SVFDQ SIFAQ NIFSQ NIFSQ NVAPIEL IDDSIGILIL SRPPMAIFEL SRPPMAIFEL	LICOWKGDPF LICOWKGDPP LISALCDGDPP LISALCDGDPP LISALCTGDPF LICULTFSPQ LICULTFSPQ LORIVNEPPF LOVIVNEPPF LOVIVNEPPF LOVIVNEPPF	QLSNSE.ERS QLSNSE.ERE RLPSDK SLP.DS LKDEPESNII LKDEPELDIS RLPSSV KLPSGV KLPSGV KLPSGI	300 SPPSFINFUN FSPSFTSTUN FSSDAQUFUS FSPEARDFUN WSPSFKSFID WSRTFKSFID VSKENTDFUN FSLEFQIFUN FSLEFQIFUN FSLEFQIFUN FSLEFQIFUN FSTEFKLEVD
SEK1 XMEK2 PBS2 WIS1 MKK1 MKK2 BYR1 STE7 MEK2 DRODSOR	301 LCLTRDESKR LCLORUPERR KCLNKNPSLR YCLKROSRER ACLHROPTLR RCCIKNERER KCLIKNPAER KCLIKNPAER ICLKEPPER	PKYKELLKIP PKYKELLKIP PTYAALTER PDYHELANIP PSPROMINEP SSIHELLHO ADLKQLMVA ADLKLLINNA ADLKLLINNA	FILMYEERT FILMYEERT WLVKYRNDJ WLLKYQNADJ WIVGQMKKV YFQQALMIN LIMKYVSPSK FIKRSDAED FIKRSDAED WIRKAELEEY	EVACYVCKIL DVAGYVGKIL HMSEYITERL DMASWAKG NMEKFVRKCW DLASWASNFR D.IKFRHWCR DFAGWLCSTI DFAGWLCSTI DFAGWLCRTL DFAGWLCRTL	DQM. PATPSS BQM. PVSPSS ERRIKILRER ALKEK KD EKEKDGI SS KIKSK IKEDK GLNQPSTPTH RLKQPSTPTR DL. PPSTPKR	360 PMYVD GENGLSKNVP GEKRS RIKREALDRA AASI TAV NTSEN

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  $\times$  10<sup>5</sup>  $\lambda$ gt11 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 sizeselected (>2 kb), random-primed murine erythroleukaemia cell cDNA library (5  $\times$  10<sup>5</sup> 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<sup>23</sup>, 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)<sup>+</sup> mRNA was done using standard methods.

## **LETTERS TO NATURE**



FIG. 2 Characterization of SEK1 function. a, SEK1 activates SAPK-al and SAPK-p54*β*. 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-al 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- $\alpha$ I. Right panel phosphorylation of SAPK- $\alpha$ I(K $\rightarrow$ R) mutant. Bottom left, relative positions of phosphoamino acids: Y, tyrosine; T, threonine; S, serine.

contrast, EGF fails to stimulate SEK1 activity beyond the threefold levels seen in unstimulated cells. Parallel assays of endogenous SAPK activity in 293 cells (Fig. 2b) demonstrates that the degree of activation of the SAPKs in response to TNF- $\alpha$  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<sup>11</sup>. SAPKs also require Tyr and Thr phosphorylation for activity<sup>2,12</sup>. Under conditions that result in *in vitro* SAPK activation by SEK1 (Fig. 2c), wild-type SAPK is phosphorylated at Tyr, Ser and Thr (Fig. 2d, top left), whereas a kinase-inactive SAPK mutant is phosphorylated predominantly at Tyr, with no detectable Thr phosphorylation (Fig. 2d, top right). Thus SEK1 does not show



METHODS. pEBG is a mammalian expression vector derived from pEF-BOS (ref. 24) by insertion of a polylinker containing BamHI and Clal sites. Expression of a GST-insert fusion protein is driven by the EF-1 $\alpha$ promoter. SEK1 K→R was produced by an overlapping PCR method<sup>25</sup> 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-al, p54 $\beta$  and ERK1 were expressed from the pGEX-KG vector, purified by glutathione-agarose chromatography<sup>26</sup>. Transfection into 293 cells was by the calcium phosphate method; 5 µg of the relevant plasmids were transfected. GST–SEK1, MEK1 or SAPK- $\beta$  were isolated as follows. Cells were serum-starved for 16 h and then treated with anisomycin (50 µg  $mI^{-1}$  for 40 min, TNF- $\alpha$  (50 ng ml<sup>-1</sup>, 15 min), EGF (100 ng ml<sup>-1</sup>, 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<sup>1</sup>. Lysates were cleared by centrifugation and incubated with glutathione-agarose for 30 min and washed as described<sup>1</sup>. SAPKs were immunoprecipitated and assayed as before<sup>1</sup>. For GST-SEK1 and GST-MEK1 assays, 30 µl 1:1 beads (GST-SEK1 or MEK1) in kinase assay buffer<sup>1</sup> were incubated at 30 °C for 30 min with 15  $\mu l$  0.15 mg ml  $^{-1}$  SAPK or MAPK preparation and [ $\gamma^{32}$ P]ATP (100  $\mu M,$ 15 µCi). At this time, 1 µg GST–c-Jun (SAPK assays) or MBP (ERK1 assays) and an additional 100 µM [ $\gamma^{32}$ 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 described27.

prominent dual specificity and appears to phosphorylate SAPKs preferentially at Tyr. Insofar as SAPKs are proline-directed<sup>13</sup> and the site of SAPK activating phosphorylation is TPY<sup>1,2</sup>, 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 phospho-transferase activity<sup>14</sup>.

To determine the specificity of SEK1, we compared the ability of SEK1 to activate SAPK- $\alpha$ I 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- $\alpha$ I. By contrast, incubation of ERK1 with GST–MEK1 beads





FIG. 3 Specificity of SEK1 for SAPKs. *a*, Comparison of SEK1 activity towards SAPK- $\alpha$ I 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-p54 $\beta$  (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.

gives >20-fold activation of the ERK1 MBP-kinase activity (Fig. 3b). 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-p54 $\beta$  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. 3c, 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

FIG. 4 Expression of a kinase-inactive SEK1, SEK1( $K \rightarrow R$ ), inhibits 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-p54ß was expressed in 293 cells alone or together with pEBG-SEK1( $K \rightarrow R$ ). 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( $K \rightarrow R$ ). 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( $K \rightarrow R$ ) 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  $\mu$ g). Anisomycin treatment and assays are described in Fig. 2 legend.

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METHODS. GST–SEK1 and MEK1 were assayed as described in Fig. 2 legend. COS7 cells were transfected by the DEAE–dextran method<sup>28</sup>. Immunoblotting was with the ECL system (Amersham). 1U ERK1 will transfer 1 pmol min<sup>-1</sup> PO<sub>4</sub> 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).

cotransfected with pEBG-SAPK-p54 $\beta$  into 293 cells and the ability of anisomycin to activate GST-SAPK activity in these cells assayed. Anisomycin activates the GST-SAPK $\beta$  (Fig. 4*a*, right; compare lanes 1 and 2). But coexpression of the GST-SAPK $\beta$  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  $K \rightarrow R$  mutant for the SAPK pathway is shown in Fig. 4b. Expression of the transforming *abl* gene leads to activation of p42/p44 MAPKs, presumably through the Ras pathway (refs 15 and 16, and Fig. 4b, right, lanes 1 and 2). The activation of the ERK1 by Abl is not interfered with by SEK1( $K \rightarrow R$ ), whereas this mutant completely blocks SAPK activation (compare Fig. 4a 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



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<sup>17</sup><sup>19</sup> 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( $K \rightarrow R$ ) to block Ras activation of MAPKs and the inability of Raf-1 to activate the SAPKs<sup>20</sup>, indicate that SEK1 and the SAPKs lie on a signalling pathway that is largely distinct from the Ras/Raf/MEK/MAPK

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

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A KINASE distinct from the MEK activator Raf<sup>1-3</sup>, termed MEK kinase-1 (MEKK), was originally identified by virtue of its homology to kinases involved in yeast mating signal cascades<sup>4</sup>. Like Raf, MEKK is capable of activating MEK in vitro<sup>4.5</sup>. High-level expression of MEKK in COS-7 cells<sup>4</sup> or using vaccinia virus vectors<sup>5</sup> 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<sup>9,10</sup>. We find that MEKK regulates a new signalling cascade by phosphorylating an

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<sup>21</sup>. 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. 

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#### SAPK activator, SEK1 which in turn phosphorylates and activates SAPK.

Stably transfected NIH3T3 subclones express MEKK in response to isopropyl- $\beta$ -D-thiogalactosidase (IPTG) (Fig. 1a) but MAPK activity remains unchanged (Fig. 1b). In contrast, SAPK activity is increased six- to eightfold in MEKK-inducible cell lines but not in the parent NIH3T3 cells. These MEKKexpressing 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. 1c). Increased SAPK activity is evident by 3 hours and maximal after 12 hours of induction; MAPK activity is unchanged throughout the 23hour incubation with inducer (Fig. 1d). 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<sup>11,12</sup>. MEKK induced electrophoretic retardation of SAPK, which was suggestive of quantitative phosphorylation (Fig. 2a), 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. 2b). 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

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