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A human homolog of the yeast Ssk2/Ssk22 MAP kinase kinase kinases, MTK1, mediates stress-induced activation of the p38 and JNK pathways

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A human homolog of the yeast Ssk2 and Ssk22 mitogen-activated protein kinase kinase kinases (MAPKKK) was cloned by functional complementation of the osmosensitivity of the yeast *ssk2*Δ *ssk22*Δ *sho1*Δ triple mutant. This kinase, termed MTK1 (MAP Three Kinase 1), is 1607 amino acids long and is structurally highly similar to the yeast Ssk2 and Ssk22 MAPKKKs. In mammalian cells (COS-7 and HeLa), MTK1 overexpression stimulated both the p38 and JNK MAP kinase pathways, but not the ERK pathway. MTK1 overexpression also activated the MKK3, MKK6 and SEK1 MAPKKs, but not the MEK1 MAPKK. Furthermore, MTK1 phosphorylated and activated MKK6 and SEK1 *in vitro*. Overexpression of a dominant-negative MTK1 mutant [MTK1(K/R)] strongly inhibited the activation of the p38 pathway by environmental stresses (osmotic shock, UV and anisomycin), but not the p38 activation by the cytokine TNF-α. The dominant-negative MTK1(K/R) had no effect on the activation of the JNK pathway or the ERK pathway. These results indicate that MTK1 is a major mediator of environmental stresses that activate the p38 MAPK pathway, and is also a minor mediator of the JNK pathway.

Keywords: MAP kinase/phosphorylation/protein kinase/signal transduction/stress response

Introduction

In mammalian cells, a variety of extracellular stimuli generate intracellular signals that converge on a limited number of the so-called mitogen-activated protein (MAP) kinase pathways. The central core of each MAP kinase pathway is a conserved cascade of three protein kinases, which are commonly referred to as MAP kinase (MAPK), MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK) (Marshall, 1994). An activated MAPKKK phosphorylates and activates a specific MAPKK, which then activates a specific MAPK. While the prototypic MAPKs (ERK1 and ERK2) are indeed activated by mitogenic stimulation, the two other types of MAPKs [jun-N-terminal kinase (JNK; also known as SAPK) and p38 (also known as RK, p40, CSBP, SAPK2, Mix2 and HOG1)] are activated by environmental stresses such as osmotic shock, UV irradiation, heat shock, wound stress and inflammatory factors (Dérijard *et al.*, 1994; Freshney *et al.*, 1994; Han *et al.*, 1994; Kyriakis *et al.*, 1994; Lee *et al.*, 1994; Rouse *et al.*, 1994; Sluss *et al.*, 1994; Waskiewicz and Cooper, 1995; Zervos *et al.*, 1995; Kyriakis and Avruch, 1996; Mertens *et al.*, 1996). A signature motif that distinguishes the three subfamilies of MAPKs is found at the activating phosphorylation site: TEY for the ERK family enzymes; TPY for JNKs; and TGY for p38 (Davis, 1994). Both JNK and p38 exist in multiple forms which are encoded by several genes and are further diversified by alternative pre-mRNA splicing (Kyriakis *et al.*, 1994; Lee *et al.*, 1994; Sluss *et al.*, 1994; Zervos *et al.*, 1995; Mertens *et al.*, 1996). In general, the specificity of MAPKKs for MAPKs is high: ERK1 and ERK2 are activated by either MEK1 or MEK2 (Cobb and Goldsmith, 1996); JNK is activated by SEK1 (also known as MKK4) (Sánchez *et al.*, 1994; Dérijard *et al.*, 1995; Lin *et al.*, 1995); and p38 is activated by either MKK3 or MKK6 (Dérijard *et al.*, 1995; Raingeaud *et al.*, 1996; Cuenda *et al.*, 1997) (Figure 1). However, while a number of putative kinases, including MEKKs, TAK1, MLK, Tpl2 and ASK1, have been reported that activate MKK3, MKK6 and/or SEK1 (Yan *et al.*, 1994; Lin *et al.*, 1995; Yamaguchi *et al.*, 1995; Blank *et al.*, 1996; Hirai *et al.*, 1996; Rana *et al.*, 1996; Salmeron *et al.*, 1996; Ichijo *et al.*, 1997), the function of these MAPKKK candidates is largely unclear. In contrast, the MAP kinase pathways in yeast are much more clearly defined, partly because of the organism's intrinsic simplicity, but also because of the availability of sophisticated genetic methodology (Cooper, 1994).

Yeast (*Saccharomyces cerevisiae*) has several distinct MAP kinase cascades that transduce distinct extracellular stimuli (e.g. mating pheromone, high osmolarity and low osmolarity) (Herskowitz, 1995). Extracellular hyperosmolarity is detected by either of two yeast transmembrane osmosensors, Sho1 and Sln1 (Figure 1) (Ota and Varshavsky, 1993; Maeda *et al.*, 1994, 1995). No structural similarity exists between the two osmosensors and they seem to function independently of each other. Sho1 has four predicted transmembrane segments and a C-terminal cytoplasmic region containing an SH3 domain. Sho1 interacts with, and activates, the Pbs2 MAPKK through the Ste11 MAPKKK (Posas and Saito, 1997). The second osmosensor, Sln1, is a homolog of prokaryotic two-component signal transducers and transmits signals to the redundant Ssk2 and Ssk22 MAPKKKs, via the Sln1-Ypd1-Ssk1 multi-step phospho-relay system (Maeda *et al.*, 1994, 1995; Posas *et al.*, 1996). Activated Ssk2 and Ssk22 MAPKKKs independently phosphorylate and activate the Pbs2 MAPKK (Maeda *et al.*, 1995; Posas and Saito, 1997). Thus, signals emanating from the two transmembrane osmosensors converge at the Pbs2 MAPKK, which then activates the Hog1 MAPK (Brewster *et al.*, 1993). Because a major outcome of the activation of this osmoregulatory

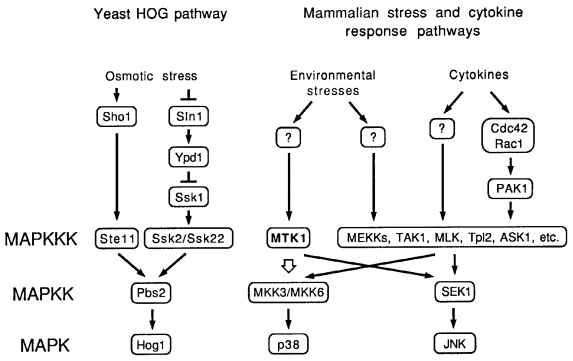

Fig. 1. Schematic diagram comparing the yeast HOG (high osmolarity glycerol response) pathway and mammalian stress response MAP kinase cascades. Question marks indicate that critical signaling components remain to be identified. The arrows do not necessarily indicate a direct interaction. The roles of some human MAPKKK candidates may not be as precisely defined as this diagram might suggest.

MAPK pathway is the elevated synthesis of glycerol, this pathway is referred to as the HOG (high osmolarity glycerol response) pathway (Brewster *et al.*, 1993; Albertyn *et al.*, 1994). The HOG pathway is essential for the survival of yeast in high osmolarity environments (Boguslawski, 1992; Brewster *et al.*, 1993).

The HOG osmoregulatory signal transduction pathway is homologous to the mammalian p38 pathway. The MAPKKs in the HOG and p38 pathways (yeast Pbs2 and mammalian MKK3 and MKK6) are very similar, as are the respective MAPKs (yeast Hog1 and mammalian p38) (Boguslawski and Polazzi, 1987; Brewster *et al.*, 1993; Han *et al.*, 1994; Dérijard *et al.*, 1995). Moreover, the mammalian p38 can functionally complement the osmosensitivity of yeast *hog1*Δ mutations (Han *et al.*, 1994). The similarity between the yeast HOG pathway and the mammalian p38 pathway suggests the existence of a mammalian homolog of the Ssk2/Ssk22 MAPKKKs, that would activate the Pbs2 MAPKK. In this paper, we report the functional cloning of the mammalian Ssk2/Ssk22 homolog which we named MTK1 and the role of MTK1 in the mammalian stress signal transduction.

Results

Cloning of a human homolog of the yeast Ssk2/Ssk22 MAPKKKs by functional complementation

The *ssk2*Δ *ssk22*Δ *sho1*Δ triple mutant yeast cells do not grow on high osmolarity media, because they cannot activate the Pbs2 MAPKK and the Hog1 MAPK, and consequently cannot initiate proper adaptive responses (Figure 1). Expression of any one of the functional *SSK2*, *SSK22* and *SHO1* genes is sufficient to suppress the osmosensitivity of the triple mutants (Maeda *et al.*, 1995). In order to isolate a human homolog of either the *SSK2*, *SSK22* or *SHO1* gene, the yeast strain MY007 (*ssk2*Δ *ssk22*Δ *sho1*Δ) was transformed with human cDNA libraries made in the yeast expression vector pNV7 (Ninomiya-Tsuji *et al.*, 1991) and osmoresistant (Osm^R) transformants were selected. Four Osm^R yeast cells were identified among the 2×10^6 transformants generated with a HeLa (human cervical carcinoma) cDNA library. All

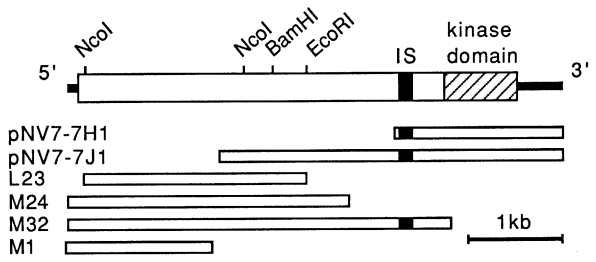

Fig. 2. Restriction map of human MTK1 cDNA and schematic model of the coding region. The protein kinase catalytic domain is shown by striping, and an insertion sequence (IS) that is probably generated by alternative splicing is indicated by the solid box. Below the restriction map, thinner boxes indicate the sizes and extents of representative MTK1 cDNA clones.

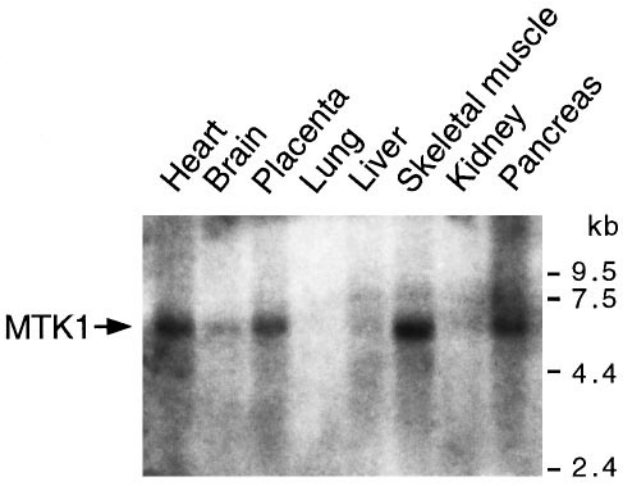

Fig. 3. Distribution of MTK1 mRNA in various human tissues. A membrane with 2 μg of poly(A)$^+$ RNA from various human tissues (Clontech) was probed with a ^{32}P-labeled MTK1 cDNA corresponding to nucleotides 1557–3605. Positions of molecular size standards are indicated on the right in kilobases (kb).

four Osm^R transformants contained an apparently identical plasmid with a 1.8 kb insert. Nucleotide sequence determination of the representative clone, pNV7-7H1 (Figure 2), indicated that it encodes a protein kinase domain that is very similar to the catalytic domains of Ssk2 and Ssk22. We also obtained three Osm^R cells from the 1.9×10^6 transformants generated using a Jurkat (human T lymphoma) cDNA library. Two of these contained an apparently identical plasmid represented by pNV7-7J1, which contains a 3.8 kb insert that overlaps with the pNV7-7H1 cDNA insert (Figure 2). The other Osm^R transformant contained a plasmid that is similar to pNV7-7J1 but with a longer 3′ non-coding sequence (not shown). The protein kinase encoded by these cDNA clones was named MTK1 (MAP Three Kinase 1).

Northern blot analysis of various human tissues demonstrated that an ~6 kb MTK1 mRNA is expressed at high levels in heart, placenta, skeletal muscle and pancreas, and at lower levels in other tissues (Figure 3). Because the 7H1 and 7J1 cDNA inserts are significantly shorter than the estimated size of the MTK1 mRNA, we isolated additional MTK1 cDNA clones by DNA–DNA hybridization. One MTK1 cDNA clone (L23) was isolated from a fetal liver library and three additional clones (M1, M24 and M32) were obtained from an adult skeletal muscle

A

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MREAAAALVP PPAFAVTPAA AMEEPPPPPP PPPPPPEPET ESEPECCLAA    50
RQEGTLGDSA CKSPESDLED FSDETNTENL YGTSPPSTPR QMKRMSTKHQ   100
RNNVGRPASR SNLKEKMNAP NQPPHKDTGK TVENVEEYSY KQEKKIRAAL   150
RTTERDHKKN VQCSFMLDSV GGSLPKKSIP DVDLNKPYLS LGCSNAKLPV   200
SVPMPIARPA RQTSRTDCPA DRLKFFETLR LLLKLTSVSK KKDREQRGQE   250
NTSGFWLNRS NELIWLELQA WHAGRTINDQ DFFLYTARQA IPDIINEILT   300
FKVDYGSFAF VRDRAGFNGT SVEGQCKATP GTKIVGYSTH HEHLQRQRVS   350
FEQVKRIMEL LEYIEALYPS LQALQKDYEK YAAKDFQDRV QALCLWLNIT   400
KDLNQKLRIM GTVLGIKNLS DIGWPVFEIP SPRPSKGNEP EYEGDDTEGE   450
LKELESSTDE SEEEQISDPR VPEIRQPIDN SFDIQSRDCI SKKLERLESE   500
DDSLGWGAPD WSTEAGFSRH CLTSIYRPFV DKALKQMGLR KLILRLHKLM   550
DGSLQRARIA LVKNDRPVEF SEFPDPMWGS DYVQLSRTPP SSEEKCSAVS   600
WEELKAMDLP SFEPAFLVLC RVLLNVIHEC LKLRLEQRPA GEPSLLSIKQ   650
LVRECKEVLK GGLLMKQYYQ FMLQEVLEDL EKPDCNIDAF EEDLHKMLMV   700
YFDYMRSWIQ MLQQLPQASH SLKNLLEEEW NFTKEITHYI RGGEAQAGKL   750
FCDIAGMLLK STGSFLEFGL QESCAEFWTS ADDSSASDEI IRSVIEISRA   800
LKELFHEARE RASKALGFAK MLRKDLEIAA EFRLSAPVRD LLDVLKSKQY   850
VKVQIPGLEN LQMFVPDTLA EEKSIILQLL NAAAGKDCSK DSDDVLIDAY   900
LLLTKHGDRA RDSEDSWGTW EAQPVKVVPQ VETVDTLRSM QVDNLLLVVM   950
QSAHLTIQRK AFQQSIEGLM TLCQEQTSSQ PVIAKALQQL KNDALELCNR  1000
ISNAIDRVDH MFTSEFDAEV DESESVTLQQ YYREAMIQGY NFGFEYHKEV  1050
VRLMSGEFRQ KIGDKYISFA RKWMNYVLTK CESGRGTRPR WATQGFDFLQ  1100
AIEPAFISAL PEDDFLSLQA LMNECIGHVI GKPHSPVTGL YLAIHRNSPR  1150
PMKVPRCHSD PPNPHLIIPT PEGFSTRSMP SDARSHGSPA AAAAAAAAVA  1200
ASRPSPSGGD SVLPKSISSA HDTRGSSVPE NDRLASIAAE LQFRSLSRHS  1250
SPTEERDEPA YPRGDSSGST RRSWELRTLI SQSKDTASKL GPIEAIQKSV  1300
RLFEEKRYRE MRRKNIIGQV CDTPKSYDNV MHVGLRKVTF KWQRGNKIGE  1350
GQYGKVYTCI SVDTGELMAM KEIRFQPNDH KTIKETADEL KIFEGIKHPN  1400
LVRYFGVELH REEMYIFMEY CDEGTLEEVS RLGLQEHVIR LYSKQITIAI  1450
NVLHEHGIVH RDIKGANIFL TSSGLIKLGD FGCSVKLKNN AQTMPGEVNS  1500
TLGTAAYMAP EVITRAKGEG HGRAADIWSL GCVVIEMVTG KRPWHEYEHN  1550
FQIMYKVGMG HKPPIPERLS PEGKDFLSHC LESDPKMRWT ASQLLDHSFV  1600
KVCTDEE                                                 1607
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B

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             I                    II                   III
MTK1   WQRGNKIGEGQYGKVYTCISVDTGELMAMKEIRFQPND  HKTIKETADELKIF
MEKK4  ------------------------------------  --------------
Ssk2   --KR-F--G-TF-R--SAVDL-N--IL-V---NI-DSKSMQ-IFPLIKE-MSVL
Ssk22  --KRSF--G-TF-Q--SA-NLEN--IL-V---KIHDTTTMK-IFPLIKE-MTVL
Wik1   --Q-HFVRS-MF-D---GVNME--D-L-V---KL-DSRTFRS-VDQIHN-MTVL

         IV                   V
MTK1   EGIKHPNLVRYFGVELHREEMYIFMEYCDEGTLEEVSRLGLQ  EHVIRLYSKQ
MEKK4  ------------------------------------------  -------T--
Ssk2   -ILN---I-S-Y---V--DKVN------EG-S-AALLEH-RIED-M-TQV-TL-
Ssk22  -MLN---I-Q-Y---V--DKVN------EG-S-ASLLDH-RIED-M-TQV-TFE
Wik1   -RLN---V-T-Y---V--EKV-----F-QG-S-ADLLAH-RIED-N-LKV-VV-

       VIa           VIb                VII
MTK1   ITIAINVLHEHGIVHRDIKGANIFLTSSGLIKLGDFGCSVKLKNNAQTMPGE
MEKK4  --V-------------------------------------------------
Ssk2   LLEGLAY---S------V-PE--L-DFN-V--YV---AAK-IA--GTRLASMNK
Ssk22  LLEGLAY--QS-V------PE--L-DFN-I--YV---TARTVVGSRTRTVRNAA
Wis4   LLEGLAYI-SQH-L-----P---L-DHR-M--YS---SALYVSPPTDPEVRYED

                                        VIII
MTK1                              VNSTLGTAAYMAPEVITRAKGEGHGRAA
MEKK4                             ----------------------------
Ssk2   IENADGEHEDVTHVSDSKAVKNNENALLDMM--PM-----S--GSTTK-KLG-D
Ssk22  VQDFGVETKS                L-EMM--PM-----T-SGSAVK-KLG-D
Wik1   IQPE                      LQHLA--PM-----I-LGT- K-DFG-M

        IX                        X
MTK1   DIWSLGCVVIEMVTGKRPWHEYEHNFQIMYKVGMGHKP  PIPERLSPEGKDFL
MEKK4  ------------------------------------  -----------A--
Ssk2   -V-------L--I--R---ANLDNEWA---H-AA--T-QF-TKDEV-SA-MK--
Ssk22  -V-A-----L--A--R---SNLDNEWA---H-AA-RI-QL-NRDEMTAA-RAL-
Wik1   --------IL--M--ST--S-MDNEWA---H-AAM-T-SI-QN-KI-SLAR--I

           XI
MTK1   SHCLESDPKMRWTASQLLDHSFVKVCTDEE
MEKK4  ---------I----------A---------
Ssk2   ER--IQN-SK-AS-VE--MDPWIVQIREIAFGDDSSSTDTEERE
Ssk22  GKVFG-R-HYEGYCCGTT-RPLDDTNP
Wik1   EQ-F-R--EQ-PR-VD--T-PWITDFRKKTIITMPPATITKKTSLS....
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C

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MTK1   (260)  SNELIWLELQAWHAGRTINDQDFFLYTARQAIPDIINEILTFKVDYG
MEKK4  (253)  ----------------------L---------------------N--
Ssk2   (422)  FSDD--I--K--MN---VE--NKS-RIF-DSTDSVFQ--MA--LEDN
Ssk22  (188)  LSDE-----K--LN---MQEMEQS-TYL-DSSDSVFE--MK-QIPQ-
Wik1   (273)  YISEV--G----LH--LNA--AEVIRKS-EGVEPVLR-VID-QIQDE
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Fig. 4. Sequence analysis of human MTK1. (**A**) Predicted protein sequence of human MTK1. The double-underlined sequence (1175–1223) indicates a putative alternatively spliced segment that is not included in some MTK1 mRNA. (**B**) Homology between the kinase domains of human MTK1 (amino acids 1342–1607) and other related MAPKKKs (mouse MEKK4, *S.cerevisiae* Ssk2 and Ssk22 and *S.pombe* Wis1) (Maeda *et al.*, 1995; Gerwins *et al.*, 1997; Shiozaki *et al.*, 1997). MEKK4 is probably the mouse homolog of the human MTK1. Roman numerals above the sequences refer to subdomains conserved across the protein kinase family (Hanks and Quinn, 1991). Dashes indicate identical amino acids with MTK1. (**C**) Sequence comparison between the N-terminal non-kinase domain of MTK1 and related MAPKKKs. Numbers in parentheses are the positions of the first amino acids. Dashes indicate identical amino acids with MTK1.

cDNA library (Figure 2). The combined length of the MTK1 cDNA sequences, 5445 nucleotides excluding the poly(A) tail, accounts for most of the estimated size of the MTK1 mRNA, and contains an open reading frame encoding a protein of 1607 amino acids (Figure 4A). The MTK1 protein kinase catalytic domain is located in the C-terminal 266 amino acids (1342–1607) and is most closely related to the MAPKKK family (Figures 4B and 5). Indeed, the human MTK1 appears to be the homolog of the mouse MEKK4, which has 98% identity with MTK1 in the kinase domain (Gerwins *et al.*, 1997). In addition to MEKK4, high degrees of identity were found with Ssk2 (42% identify), Ssk22 (36%) and the *Schizosaccharomyces pombe* Wik1 (41%) in the kinase domain (Figures 4B and 5). Wik1 is an *S.pombe* homolog of the Ssk2/Ssk22 MAPKKKs and mediates the signals generated by high osmolarity and other environmental stresses (Shiozaki *et al.*, 1997).

The structural similarity of Ssk2, Ssk22 and Wik1 to MTK1 is not limited to their kinase domains. When the N-terminal non-kinase domain of MTK1 (amino acids 1–1340) was compared with the proteins in the GenBank database using the BLAST program, these three kinases

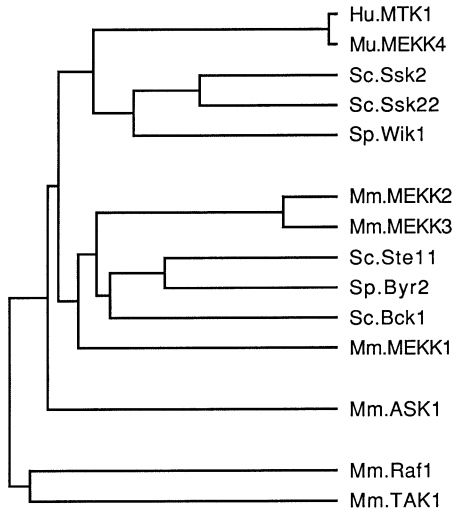

Fig. 5. Phylogram comparing sequences of protein kinase domains of representative MAPKKKs. This diagram was generated using the PILE-UP program. Sc, *S.cerevisiae*; Sp, *S.pombe*; Hu, human; Mu, mouse; and Mm, mammals.

(in addition to MEKK4) were the only proteins that had statistically significant similarity ($P < 0.001$). The similarity is scattered throughout the N-terminal non-kinase domain. Figure 4C shows a stretch of MTK1 (260–306) that has particularly high similarity to the corresponding sequences in Ssk2, Ssk22 and Wik1 (Figure 4C). Although a region in mouse MEKK4 (225–398) was proposed to be a pleckstrin homology (PH) domain (Gerwins *et al.*, 1997), we did not find any similarity between MTK1 and PH domain proteins.

A variant form of mouse MEKK4 exists that lacks a 52-amino acid sequence, probably a product of alternative splicing (Gerwins *et al.*, 1997). In MTK1, this sequence corresponds to amino acids 1175–1223 (underlined in Figure 4A). The three MTK1 cDNA clones we isolated that spanned this region (7H1, 7J1 and M32) contained the corresponding 49 amino acids. However, sequencing analyses of RT–PCR products derived from HeLa and Jurkat mRNA preparations indicated that a shorter form of MTK1 mRNA that lacks the 49 codons also exists in these cells (data not shown).

MTK1 can functionally replace the Ssk2 and Ssk22 MAPKKKs in the yeast HOG MAP kinase pathway

To gain insight into the role of MTK1 in human cells, we initially investigated the mechanism by which MTK1 suppresses the osmosensitivity of *ssk2*Δ *ssk22*Δ *sho1*Δ mutant yeast. Expression of the MTK1 cDNA sequences, either 7J1 or 7H1, suppressed the osmosensitivity of the triple mutant, but not the osmosensitivity of *pbs2*Δ or *hog1*Δ mutants (Figure 6A). These results suggest that the suppressive action of MTK1 occurs upstream of the Pbs2 and Hog1 kinases, in yeast. To confirm further that MTK1 acts upstream of Pbs2 and Hog1, we generated a constitutively active MTK1 mutant, MTK1ΔN, which contains only the kinase domain (Val1333 to the C-terminus). Previously, we demonstrated that hyper-activation of the Pbs2 or Hog1 kinases by the expression of a constitutively active Ssk2 mutant (Ssk2ΔN) is lethal (Maeda *et al.*, 1995). If MTK1 acts upstream of Pbs2, the MTK1ΔN expression should also be lethal to wild-type yeast cells, and either a *pbs2*Δ or *hog1*Δ mutation should suppress this lethality. This is indeed the case (Figure 6B). The lethal effect of MTK1ΔN is dependent upon MTK1 kinase activity, because expression of a catalytically inactive mutant, MTK1(K/R)ΔN, is not toxic. Expression of either Ssk2ΔN or MTK1ΔN led to the activation of the Pbs2 MAPKK as indicated by the tyrosine phosphorylation of its specific substrate, Hog1 (Figure 6C). No Hog1 phosphorylation was observed when MTK1ΔN was expressed in the *pbs2*Δ cells, or when MTK1(K/R)ΔN was expressed in wild-type cells (Figure 6C). These results demonstrate that, when expressed in yeast, MTK1 acts upstream of the Pbs2 MAPKK.

It was possible that MTK1 conferred osmoresistance to the *ssk2*Δ *ssk22*Δ *sho1*Δ triple mutant cells by activating the Ste11 MAPKKK (see Figure 1). However, MTK1 cDNA clones could also suppress the osmosensitivity of the FP50 (*ssk2*Δ *ssk22*Δ *ste11*Δ) triple mutant cells, demonstrating that the suppression by MTK1 does not require Ste11 MAPKKK (data not shown). Because Ssk2, Ssk22 and Ste11 are the only known kinases that activate the Pbs2 MAPKK (Posas and Saito, 1997), it is concluded that MTK1 suppresses the osmosensitivity by directly activating the Pbs2 MAPKK. Thus, MTK1 can functionally replace the Ssk2 and Ssk22 MAPKKKs in the yeast HOG osmoregulatory MAP kinase pathway. The structural similarity between MTK1 and Ssk2/Ssk22 is also consistent with this conclusion.

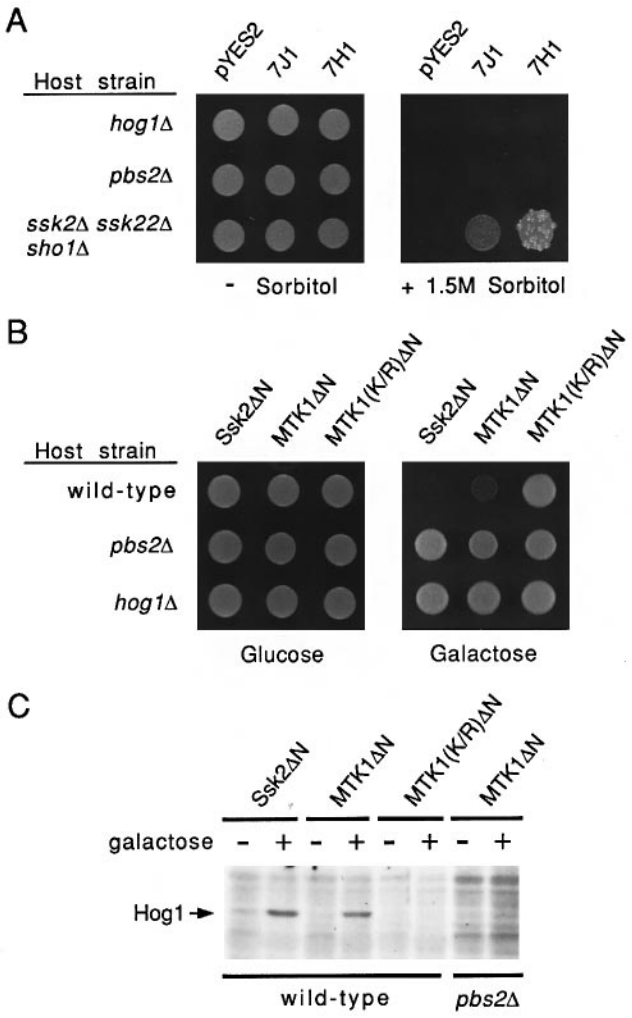

Fig. 6. MTK1 functionally complements *ssk2 ssk22 sho1* deficiency and specifically activates the HOG pathway in *S.cerevisiae*. (**A**) Expression of MTK1 restores osmoresistance to the osmosensitive *ssk2*Δ *ssk22*Δ *sho1*Δ triple disruptants. Yeast strains TM232 (*hog1*Δ), TM261 (*pbs2*Δ) and MY007 (*ssk2*Δ *ssk22*Δ *sho1*Δ) were transformed with either an empty expression vector (pYES2) or plasmids containing MTK1 cDNA fragments, pNV7-7J1 (7J1) or pNV7-7H1 (7H1). Approximately 10^3 cells were spotted on SC-Ura plus galactose plates in the presence or absence of 1.5 M sorbitol. Cells were grown at 30°C. (**B**) Overexpression of the MTK1 catalytic domain is lethal in wild-type yeast cells but not in *pbs2*Δ or *hog1*Δ cells. Strains TM141 (wild-type), TM261 (*pbs2*Δ) and TM232 (*hog1*Δ) were transformed with pGal-SSK2ΔN, p426GAG-MTK1ΔN or p426GAG-MTK1(K/R)ΔN. Transformants were spread on SC-Ura plates containing glucose or galactose as indicated and incubated at 30°C. (**C**) Hog1 tyrosine phosphorylation induced by the expression of the MTK1 catalytic domain. TM141 (wild-type) cells containing pGal-SSK2ΔN, p426GAG-MTK1ΔN or p426GAG-MTK1(K/R)ΔN, or TM261 (*pbs2*Δ) cells containing p426GAG-MTK1ΔN were grown in SC-Ura plus 2% raffinose. Gene expression was induced by the addition of galactose to a final concentration of 2.5%. Samples were taken before (–) the addition of galactose or 1 h later (+). Cell pellets were boiled in SDS loading buffer and analyzed by Western blotting using anti-phosphotyrosine antibody 4G10.

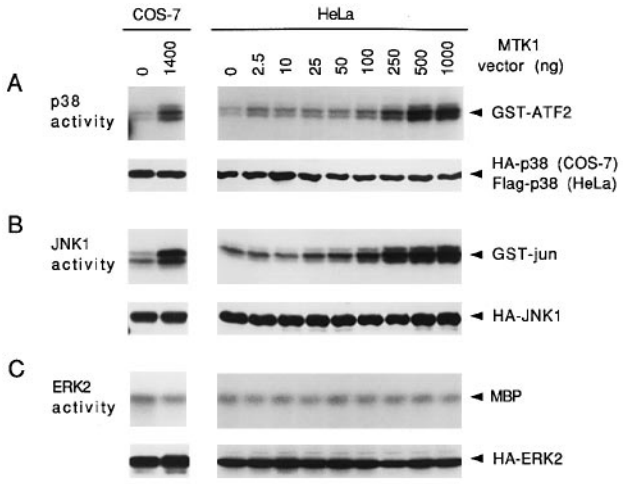

Fig. 7. MTK1 overexpression activates p38 and JNK1, but not ERK2, *in vivo*. COS-7 cells (left panel) were transiently transfected with 0.7 μg of expression vector encoding HA-p38, HA-JNK1 or HA-ERK2, together with 1.4 μg of either pcDNAI-MTK1 expression vector carrying full-length MTK1 or control vector pcDNAI-Amp. HeLa cells (right panel) were co-transfected with a fixed amount (1 μg) of individual MAPK expression plasmids and increasing amounts of pcDNAI-MTK1 expression vector as indicated. The total amount of transfected DNA was kept constant by including appropriate amounts of the empty vector pcDNAI-Amp. After 48 h, cells were lysed and the epitope-tagged MAPKs were immunoprecipitated. The kinase activity was measured in an immunocomplex kinase assay using exogenous substrates. The expression level of each MAPK in cell lysates was monitored by Western blotting using appropriate antibodies. These experiments were repeated at least three times with similar results.

MTK1 activates both the p38 and JNK pathways, but not the ERK pathway, in vivo

To study the role of MTK1 in mammalian cells, we determined whether any of the known MAP kinase pathways are activated by MTK1. Initially, COS-7 cells were transiently transfected with full-length MTK1 together with one of the three HA-tagged MAPK constructs (HA-p38, HA-JNK1 and HA-ERK2). The HA-tagged MAPKs were immunoprecipitated from cell extracts and their kinase activities were measured *in vitro* using specific substrates [GST-ATF2, GST-jun and myelin basic protein (MBP) respectively]. As shown in Figure 7 (left panel), the activities of p38 and JNK1 kinases, but not ERK2 kinase, were stimulated by co-transfection of MTK1. Consistent detection of the activated p38 required the inclusion of deoxycholate in the lysis buffer, suggesting that the majority of the activated p38 is translocated to nuclei. In this and following experiments, the amount of tagged protein in the various immunoprecipitates was determined by immunoblotting and found to be the same for each set of experiments.

To compare, more quantitatively, the capacity of MTK1 to activate various MAP kinase pathways, HeLa cells were transfected with increasing amounts of MTK1 vector together with a constant amount of the epitope-tagged MAPK constructs (either Flag-p38, HA-JNK1 or HA-ERK2). The activities of the tagged MAPK were then assayed *in vitro*. MTK1 activated both p38 and JNK1 equally well and, consistent with the results from COS-7 cells, MTK1 did not stimulate the ERK2 kinase in HeLa cells (Figure 7, right panel). Therefore, MTK1 mediates signal transduction of both the p38 and JNK pathways, but not the ERK pathway.

MTK1 phosphorylates and activates MKK3, MKK6 and SEK1, both in vivo and in vitro

While the overexpression of MTK1 leads to the activation of p38 and JNK1 MAPKs, the direct substrates of MTK1 are likely to be members of the MAPKK family. Therefore, we tested whether MTK1 stimulates the activities of any of the following four MAPKKs: MKK3, MKK6, SEK1 and MEK1. Together, these four MAPKKs represent the three major MAP kinase pathways (i.e. p38, JNK and ERK pathways) (Marshall, 1994; Waskiewicz and Cooper, 1995; Kyriakis and Avruch, 1996). In the following experiments, COS-7 cells were co-transfected with various amounts of full-length MTK1 vector together with one of the GST-tagged MAPKK constructs (GST-MKK3, GST-MKK6, GST-SEK1 and GST-MEK1). The GST-tagged MAPKKs were precipitated from cell extracts using glutathione–Sepharose beads, and their kinase activities were assayed *in vitro* using specific substrates [GST-p38(K/N) for the MKK3, MKK6 and SEK1 activities; and GST-ERK1(K/N) for the MEK1 activity]. As shown in Figure 8 (top row), the catalytic activities of MKK3, MKK6 and SEK1 were stimulated by MTK1 in a dose-dependent manner. In contrast, MEK1 was not stimulated by MTK1 co-transfection.

Activation of MAPKKs is dependent on the phosphorylation of specific sites in their catalytic domain (Lange-Carter *et al.*, 1993). To examine if MTK1 induces the phosphorylation of the activation sites of MKK3, MKK6 and SEK1, precipitated GST-MAPKKs were probed with antibodies specific to their phosphorylated forms. Figure 8 (middle row) shows that MTK1 co-transfection indeed results in increased levels of phosphorylated MKK3, MKK6 and SEK1 (MEK1 was not tested, because there was no increase in its catalytic activity). These results, therefore, suggest that MTK1 directly phosphorylates and activates MKK3, MKK6 and SEK1. However, the alternative possibility that MTK1 activates another kinase that phosphorylates the MAPKKs cannot be excluded by these experiments alone.

To test directly whether MTK1 can phosphorylate and activate MAPKKs, an *in vitro* kinase assay was performed using purified enzymes and substrates. The kinase domain of wild-type MTK1 or a catalytic-site mutant MTK1(K/R) were fused to the GST domain [GST-MTK1ΔN and GST-MTK1(K/R)ΔN respectively], expressed in yeast and purified using glutathione–Sepharose beads. In the first series of *in vitro* experiments, we tested if the recombinant MTK1 kinase can phosphorylate various MAPKKs (Figure 9A). For this purpose, we prepared GST-tagged MAPKKs that are catalytically inactive [GST-MKK6(K/A), GST-SEK1(K/R) and GST-MEK1(K/M)]. Because of the catalytic site mutations, these recombinant MAPKKs have no kinase activities and will not autophosphorylate. When purified GST-MAPKKs were used as substrates in the *in vitro* kinase assay, GST-MTK1ΔN phosphorylated both GST-MKK6(K/A) and GST-SEK1(K/R), but not GST-MEK1(K/M). As anticipated, the catalytic site mutant GST-MTK1(K/R)ΔN did not phosphorylate any substrate in these assays. We then tested if the *in vitro* phosphorylation of MKK6 and SEK1 is functionally relevant using

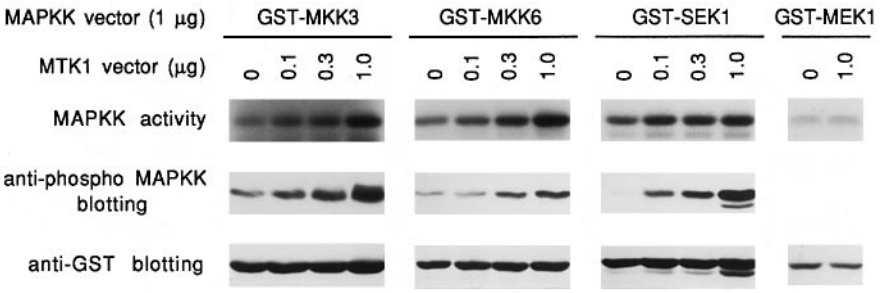

Fig. 8. MTK1 overexpression activates MKK3, MKK6 and SEK1, but not MEK1, *in vivo*. COS-7 cells were transiently transfected with 1 μg per plate of a mammalian expression vector encoding GST-MKK3, GST-MKK6, GST-SEK1 or GST-MEK1, together with various concentrations of the pcDNAI-MTK1 expression vector. The total amount of DNA was kept constant by adding appropriate amounts of the empty vector pcDNAI-Amp. After 48 h, GST-tagged MAPKKs were purified using glutathione–Sepharose beads and the activity of individual GST-tagged MAPKK was assessed by an *in vitro* kinase assay using GST-p38(K/N) (for MKK3, MKK6 and SEK1) or GST-ERK1(K/N) (for MEK1) as substrates (upper panel). Phosphorylation of the precipitated GST-tagged MAPKK was analyzed by immunoblotting using antibodies specific to phosphorylated MKK3/6 or SEK1 (middle panel). The expression level of each GST-MAPKK in total cell lysates was monitored by anti-GST blot (lower panel).

a coupled kinase assay (Figure 9B). In this kinase assay, the catalytically active version of the GST-MAPKKs was pre-incubated with GST-MTK1ΔN [or the control GST-MTK1(K/R)ΔN] in the presence of non-radioactive ATP. Subsequently the kinase activities of the GST-MAPKKs were assayed in the presence of [γ-^{32}P]ATP and specific substrates [catalytically inactive MAPKs: GST-p38(K/N) for MKK6 and SEK1; GST-ERK1(K/N) for MEK1]. As shown in Figure 9B, both MKK6 and SEK1 were catalytically active after they were pre-incubated with GST-MTK1ΔN, but not with GST-MTK1(K/R)ΔN. Taken together, the *in vitro* kinase studies indicate that recombinant MTK1 kinase can specifically phosphorylate and activate both MKK6 and SEK1. The inability of MTK1 to phosphorylate MEK1 is consistent with our *in vivo* results.

Inhibition of the activation of the p38 pathway by a dominant-negative MTK1 mutant

To define further the role of MTK1 in mammalian signal transduction, the effect of overexpressing a kinase-inactive MTK1 mutant [MKT1(K/R)] on the activation of the MAP kinase pathways was studied. Because MTK1(K/R) does not have any detectable kinase activity (Figure 9), overexpression of MTK1(K/R) might inhibit signal transduction pathways in which MTK1 is intimately involved by abortively interacting with its upstream activator(s) and/or its downstream elements. Full-length MTK1(K/R), or an N-terminal deletion construct that lacks the first 606 amino acids [MTK1(K/R)ΔN], was expressed in COS-7 cells, and the kinase activities of the co-transfected, epitope-tagged MAPKs (Flag-p38, HA-JNK or HA-ERK2) were determined. Treatment of transfected COS-7 cells with sorbitol (300 mM, for 20 min) strongly stimulated p38 and JNK activities. The p38 activation induced by osmotic shock was inhibited by the full-length MTK1(K/R), but not by the N-terminal deletion mutant MTK1(K/R)ΔN (Figure 10A). In contrast, the osmotic shock-induced JNK activity was not affected by overexpression of either MTK1(K/R) or MTK1(K/R)ΔN (Figure 10B). The activation of ERK2 by phorbol 12-myristate 13-acetate (PMA) treatment (100 nM for 20 min) was also unaffected by the co-expression of either MTK1(K/R) or MTK1(K/R)ΔN (Figure 10C).

We then examined whether the activation of the p38 pathway by other stress stimuli is also inhibited by

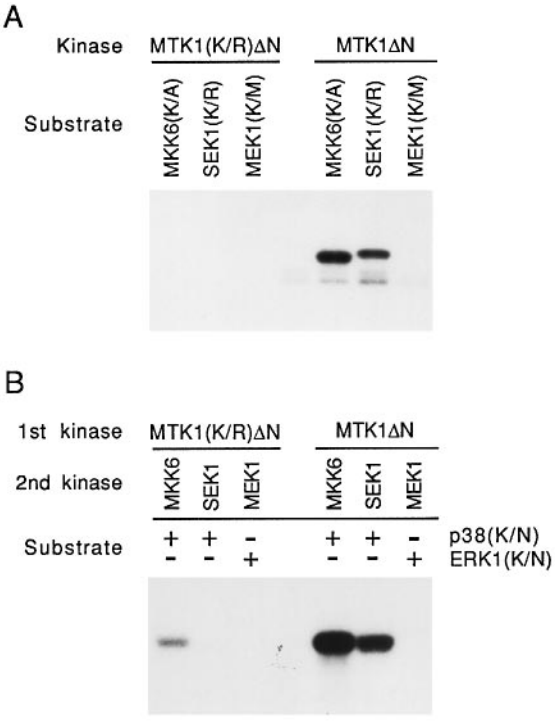

Fig. 9. Phosphorylation and activation of MKK6 and SEK1 by MTK1 *in vitro*. (**A**) *In vitro* phosphorylation of MAPKKs by MTK1. Purified GST-MTK1ΔN or catalytically inactive GST-MTK1(K/R)ΔN was incubated with the catalytically inactive substrates [GST-MKK6(K/A), GST-SEK1(K/R) or GST-MEK1(K/M)], in the presence of [γ-^{32}P]ATP. The kinase reaction was terminated by addition of sample buffer and phosphorylated substrate proteins were detected by autoradiography following SDS–PAGE. (**B**) *In vitro* activation of MAPKKs by MTK1. Purified GST-MTK1ΔN or GST-MTK1(K/R)ΔN was first incubated with GST-MKK6, GST-SEK1 or GST-MEK1, in the presence of non-radioactive ATP. The kinase activity of the pre-incubated MAPKKs was then measured using the specific substrates, GST-p38(K/N) (for MKK6 and SEK1) or GST-ERK1(K/N) (for MEK1), in the presence of [γ-^{32}P]ATP.

MTK1(K/R). As summarized in Figure 11, expression of MTK1(K/R) inhibited the stimulation of the p38 pathway by sorbitol, anisomycin and UV-C irradiation. In contrast, the stimulation of the p38 pathway by TNF-α was not affected by MTK1(K/R). The activation of the JNK pathway, either by sorbitol, anisomycin, UV-C irradiation or TNF-α, was not inhibited by MTK1(K/R). Thus, the

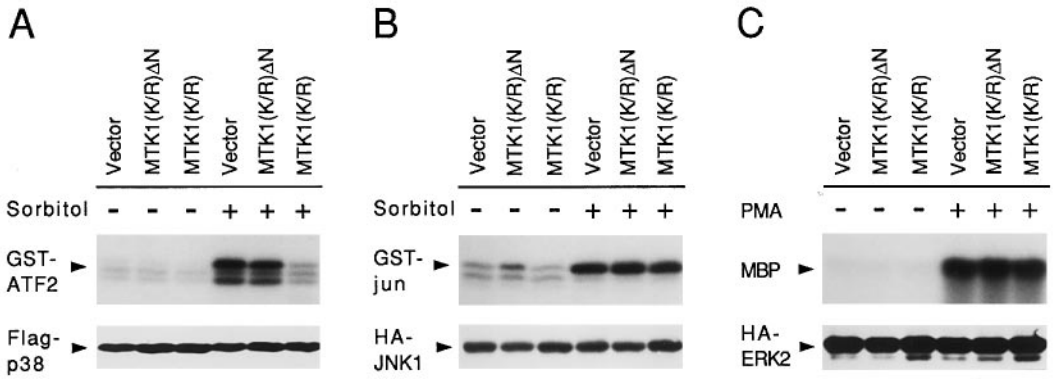

Fig. 10. Inhibition of the osmotic stress-induced p38 activation by catalytically inactive MTK1(K/R). COS-7 cells were transfected with 1.5 μg per plate of the empty vector pcDNAI-Amp (Vector) or expression plasmid carrying the full-length or the N-terminal truncated (ΔN) forms of catalytically inactive MTK1(K/R), together with 0.5 μg of expression plasmid encoding either Flag-p38 (**A**), HA-JNK1 (**B**) or HA-ERK2 (**C**). Transfected cells were serum-starved and stimulated with either sorbitol (0.3 M for 20 min) or PMA (100 nM for 20 min), as indicated. Kinase activity of the epitope-tagged MAPK was assayed as in Figure 7 (upper panel). Total cell lysates were analyzed for the amounts of individual epitope-tagged MAPKs by immunoblotting using appropriate antibodies (lower panel). Experiments were repeated at least three times with similar results.

dominant inhibitory effect of MTK1(K/R) appears specific only to the stress-induced activation of the p38 pathway.

Discussion

We isolated the human homolog of the yeast Ssk2 and Ssk22 MAPKKKs by functional complementation in yeast. The seven cDNA clones isolated in two independent screenings using the *ssk2*Δ *ssk22*Δ *sho1*Δ host cells all encoded MTK1, and in a similar screening using *ssk2*Δ *ssk22*Δ *ste11*Δ host cells, only MTK1 clones were obtained. Thus, although the two human cDNA libraries contain clones of other MAPKKKs, none were selected, demonstrating that our screening procedure was highly specific for the functional homolog of the Ssk2 and Ssk22 MAPKKKs. The identification of MTK1 as an activator of MKK3 and MKK6 completes the parallel between the yeast HOG pathway and the mammalian p38 pathway (see Figure 1).

An interesting observation was that the homology between MTK1 and Ssk2/Ssk22 was also found in their N-terminal non-catalytic domains. The similarity between the N-terminal regions of the yeast and mammalian MAPKKKs may indicate a common regulatory mechanism. The deletion of the N-terminal sequences from Ssk2, Ssk22 and MTK1 constitutively activates their kinase domains, suggesting that the N-terminal sequence contains a negative regulatory domain that interacts with the kinase catalytic domain. Alternatively, the similarity in the N-terminal domain might be a reflection of a common activation mechanism. The N-terminal region of Ssk2 and Ssk22 interacts with the two-component regulator molecule Ssk1, which is an activator of the Ssk2/Ssk22 MAPKKKs (Maeda *et al.*, 1995). Because both the yeast HOG pathway and the mammalian p38 pathway are activated by osmotic stress, it is possible that they share a common upstream activation mechanism. Although the presence of two-component regulators in mammalian cells has not been demonstrated, similar molecules were identified in diverse eukaryotes (Wurgler-Murphy and Saito, 1997). Obviously, it is also possible that the mammalian and yeast cells use completely different osmosensing mechanisms (Rosette and Karin, 1996).

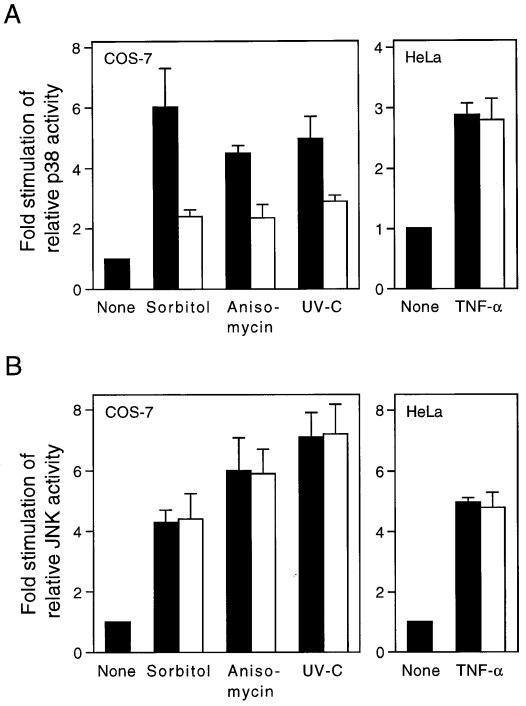

Fig. 11. Effects of dominant-negative MTK1(K/R) on the activation of the p38 and JNK pathways by various stimuli. COS-7 cells (left panel) or HeLa cells (right panel) were transfected with 1.8 μg/plate of either empty vector pcDNAI-Amp (black bars) or expression plasmid for dominant-negative MTK1(K/R) (white bars), together with 0.3 μg of expression vector encoding either Flag-p38 (**A**) or HA-JNK1 (**B**). After 48 h, cells were treated with sorbitol (0.3 M for 20 min), anisomycin (10 μg/ml for 30 min), UV-C (80 J/m^2 followed by a 1 h incubation at 37°C), TNF-α (100 ng/ml for 15 min) or without any stimulus (none). The kinase activity of immunoprecipitated epitope-tagged p38 or JNK1 was measured by an *in vitro* kinase assay as described in Figure 10. Data represent the averages and standard deviations of three independent determinations, expressed as fold increase with respect to empty vector-transfected cells without any stimulation.

Both the p38 and JNK MAP kinase pathways are activated by a variety of extracellular stresses (osmotic shock, the protein synthesis inhibitor anisomycin, UV-C irradiation, etc.) as well as cytokines such as TNF-α,

IL-1 and TGF-β. Although MTK1 can activate, when overexpressed, both the p38 and JNK pathways, the dominant-inhibitory effect of MTK1(K/R) was observed only for the p38 pathway. Furthermore, MTK1(K/R) inhibited the p38 pathway only when it was activated by extracellular stresses: if the p38 pathway was activated by TNF-α, the overexpression of MTK1(K/R) had no significant inhibitory effect. Signaling from the TNF-α receptor is believed to be mediated by small GTPases including Cdc42 and Rac1: overexpression of the active forms of Cdc42 ($Cdc42^{Q61L}$) or Rac1 ($Rac1^{Q61L}$) efficiently activates the JNK pathway (Coso *et al.*, 1995; Minden *et al.*, 1995). However, in our preliminary experiments, co-expression of MTK1(K/R) did not inhibit the activation of JNK by either $Cdc42^{Q61L}$ or $Rac1^{Q61L}$ (data not shown). Together with our observation that the overexpression of MTK1(K/R) does not inhibit the TNF-α-induced activation of the p38 and JNK pathways, we tentatively conclude that the activation of MTK1 is not mediated by the cytokine-Cdc42-Rac1 pathway.

While our data alone do not prove any specific mechanism about the MTK1 activation, the following model explains many of our observations. The stress activation of the JNK pathway is likely to be mediated by multiple MAPKKKs, including MTK1. If MTK1(K/R) inhibits the signaling mediated by MTK1 but not by other MAPKKKs, then the stress activation of the JNK pathway will not be significantly affected by overexpression of MTK1(K/R). In contrast, the stress activation of p38 may be mediated by MTK1 alone (or mainly by MTK1). If so, then MTK1(K/R) expression will have a severe inhibitory effect on the stress activation of the p38 pathway, as we observed. The cytokine activation of the p38 pathway is probably mediated by MAPKKK(s) distinct from MTK1, because MTK1(K/R) had no effect on the TNF-α induction of the p38 activity. Figure 1 schematically shows a possible role for MTK1 in the p38 and JNK MAP kinase pathways.

The dominant-inhibitory action of MTK1(K/R) required the expression of the full-length construct, suggesting that the N-terminal non-catalytic domain (1–606) contains an essential domain for the dominant-inhibitory effect. Thus, it is likely that the dominant-inhibitory effect of MTK1(K/R) involves an abortive interaction of its N-terminal non-catalytic domain with an upstream factor. As speculated before, it is an attractive hypothesis that the N-terminal region of MTK1 interacts with a two-component activator that is homologous to yeast Ssk1.

MEKK4, which is a putative mouse homolog of the human MTK1, was proposed to be a specific regulator of the JNK pathway, because MEKK4 overexpression stimulates only the JNK pathway, but not the p38 pathway (Gerwins *et al.*, 1997). Our observation, however, demonstrated that while MTK1 can activate both the p38 and JNK pathways, MTK1 may be more important in the p38 pathway than in the JNK pathway. The observed disparity between mouse MEKK4 and human MTK1 might be due to the differences between the two kinases, or between the experimental systems such as the use of different lysis buffers. A more direct comparison of the two kinases will be needed to resolve this question.

In summary, MTK1 is the closest mammalian homolog of the yeast Ssk2/Ssk22 MAPKKKs. MTK1 is a major mediator of the environmental stresses to the p38 MAPK and is also a minor mediator for the JNK pathway. The N-terminal non-kinase domain may contain a regulatory domain whose function is analogous to the corresponding region of Ssk2 and Ssk22.

Materials and methods

Yeast strains

The following yeast strains were used: MY007 (*MAT***a** *ura3 leu2 his3 ssk2::LEU2 ssk22::LEU2 sho1::HIS3*); FP50 (*MAT***a** *ura3 leu2 his3 ssk2::LEU2 ssk22::LEU2 ste11::HIS3*); TM141 (*MAT***a** *ura3 leu2 his3 trp1*); TM232 (*MAT*α *ura3 leu2 his3 hog1::LEU2*); and TM261 (*MAT*α *ura3 leu2 his3 pbs2::LEU2*).

Buffers

Buffer A is 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$ and 2 mM dithiothreitol. Lysis buffer is 20 mM Tris–HCl (pH7.5), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM dithiothreitol, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin. Kinase buffer is 25 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 0.5 mM sodium vanadate and 2 mM dithiothreitol. SDS loading buffer is 50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% Bromophenol Blue and 10% glycerol.

cDNA cloning by functional complementation

An osmosensitive yeast strain, MY007 (*ssk2*Δ *ssk22*Δ *sho1*Δ), was transformed with either a HeLa or a Jurkat cDNA library constructed in the yeast expression vector pNV7 (Ninomiya-Tsuji *et al.*, 1991), in which expression of the cDNA is under the control of the inducible *GAL1* promoter. Osmoresistant transformants were selected by growing on SC-Uracil plates containing 2% galactose and 1.5 M sorbitol. Plasmid DNA was recovered from the transformed yeast cells as described (Rose and Broach, 1991).

Cloning by DNA–DNA hybridization and sequence analysis

Lambda phage cDNA libraries made from human fetal liver or skeletal muscle mRNA were screened using the 5′-terminal *Xho*I–*Bam*HI fragment derived from the pNV7-7J1 insert as a hybridization probe, as described previously (Streuli *et al.*, 1987). The cDNA inserts in positive clones were subcloned into the *Eco*RI site of the pBluescript vector. In all cases, both strands of DNA were sequenced by the dideoxyribonucleotide method using modified T7 DNA polymerase (Tabor and Richardson, 1989). The sequence data of human MTK1 cDNA have been submitted to the DDBJ/EMBL/GenBank databases under accession number AF002715.

Northern blot analysis

The RNA tissue blot (Clontech) contains 2 μg each of poly(A)$^+$ RNA isolated from various human tissues, fractionated by denaturing agarose gel and transferred onto a nylon membrane. The blot was hybridized to a probe corresponding to nucleotides 1557 to 3605 within the non-catalytic region of MTK1. DNA was radioactively labeled by *Escherichia coli* DNA polymerase (Klenow fragment) using random hexamers and [α-^{32}P]dATP.

Plasmids

The full-length *MTK1* cDNA sequence was cloned into the expression vector pcDNAI-Amp (Invitrogen) between the unique *Bam*HI and *Xho*I restriction sites to create pcDNAI-MTK1. The kinase-deficient mutant, MTK1(K/R) was generated by replacing Lys1371 with an arginine codon using polymerase chain reaction-directed mutagenesis (Maeda *et al.*, 1995). MTK1(K/R)ΔN contains Met607 to the C-terminus of MTK1(K/R). The complete open reading frames of mammalian MKK3 and MEK1 were cloned in-frame at the C-terminus of the glutathione S-transferase (GST) gene in the eukaryotic expression vector pEBG (Lin *et al.*, 1995) to construct pEBG-MKK3 and pEBG-MEK1. The HA-JNK1, HA-ERK2 and GST-c-jun (1–79) expression vectors were provided by M.Karin (Minden *et al.*, 1995). The Flag-tagged mouse p38 expression vector (Flag-p38) and a bacterial GST-MKK6 expression vector were gifts from R.J.Davis (Raingeaud *et al.*, 1996). pMT3-HA-human p38 (HA-p38), pEBG-SEK1 (GST-SEK1) and pEBG-MKK6 (GST-MKK6) were kindly provided by J.M.Kyriakis (Rana *et al.*, 1996). A bacterial expression vector GST-ATF2 (1–109) was a gift from M.Kaneki (Pandey *et al.*, 1996). The yeast expression vector p426GAG (P_{GAL1}-GST, *URA3*,

2 micron) is based on the high-copy number yeast vector pRS426 (Christianson *et al.*, 1992) and contains the GST domain under the control of the yeast *GAL1* promoter. p426GAG-MTK1ΔN was constructed by subcloning a DNA segment of MTK1 (Val1333 to the C-terminus) between the unique *Bam*HI and *Eco*RI sites in p426GAG.

Tissue culture and transient transfection

COS-7 and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, L-glutamine, penicillin and streptomycin. For transient transfection assays, cells grown in 35 mm dishes were transfected with the appropriate combinations of expression plasmids using lipofectamine (GIBCO BRL), according to the manufacturer's protocol. Total amount of plasmid DNA was adjusted to 2 μg per plate with vector DNA (pcDNAI-Amp) and transfected COS-7 cells were cultured overnight in serum-free medium. The cells were harvested 48 h after transfection and where indicated the cells were treated with sorbitol (300 mM for 20 min), anisomycin (10 μg/ml for 30 min), UV-C (80 J/m^2 followed by a 1 h incubation at 37°C), TNF-α (100 ng/ml for 15 min) or phorbol 12-myristate 13-acetate (100 nM for 20 min).

Immunoprecipitation and protein kinase assays

To assess the kinase activity of epitope-tagged MAPKs, transfected cells were lysed in the Lysis buffer containing 0.5% deoxycholate. Cell lysates were incubated with the appropriate antibody for 2 h at 4°C. Immunocomplexes were recovered with the aid of Gamma-Bind-Sepharose beads (Pharmacia), washed twice with Lysis buffer, once in 100 mM Tris–HCl (pH 7.5), 0.5 M LiCl and twice with Kinase buffer, as described previously (Coso *et al.*, 1995). Immunoprecipitates were resuspended in 30 μl of the Kinase buffer containing 3 μg of specific substrate (GST-c-jun for JNK1; GST-ATF2 for p38; or MBP for ERK2). The kinase reaction was initiated by addition of 10 μCi [γ-^{32}P]ATP and 50 μM cold ATP, as previously described (Minden *et al.*, 1995). After 30 min incubation at 30°C, reactions were terminated by adding 15 μl of 3× SDS loading buffer. Samples were boiled for 5 min, separated by SDS–PAGE on 12.5% polyacrylamide gels, dried and visualized either by autoradiography or PhosphorImager analysis. GST-tagged versions of MAPKKs were purified by incubation of cell lysates with glutathione–Sepharose beads (Pharmacia) at 4°C for 1 h. The kinase activities of GST-MAPKKs in the precipitates were measured as described above except that the kinase reaction was done at 30°C for 5 min using 3 μg of specific substrate [GST-p38(K/N) for SEK1, MKK3 and MKK6; or GST-ERK1(K/N) for the MEK1].

Immunoblotting

Thirty micrograms of whole-cell lysates or appropriate amounts of glutathione–Sepharose affinity purified proteins were resolved by SDS–PAGE and transferred onto nitrocellulose membranes. After blocking with 3.5% skimmed milk, membranes were probed with appropriate antibodies and visualized using enhanced chemiluminescence detection (Amersham). The following antibodies were used: mouse monoclonal antibodies (mAb) 12CA5 specific to the HA epitope (Boehringer Mannheim); mouse mAb M2 specific to the Flag epitope (IBI-Kodak); rabbit polyclonal antisera specific to phosphorylated SEK1 and MKK3/6 respectively (New England Biolabs); and goat anti-GST antibody (Pharmacia). Western blot analyses for Hog1 tyrosine phosphorylation in yeast cells were done using mouse mAb 4G10 as described previously (Maeda *et al.*, 1995).

Preparation of recombinant fusion proteins

The GST fusion proteins for MEK1, MEK1(K/M), MKK6, MKK6(K/A), SEK1, SEK1(K/R), p38 (K/N) and ERK1(K/N) were expressed in *E.coli* and purified by affinity chromatography using glutathione–Sepharose (Pharmacia) essentially as described previously (Posas *et al.*, 1996). The catalytic domain of MTK1 (MTK1ΔN) and its catalytically inactive mutant MTK1(K/R)ΔN, were tagged at their N-terminus with GST and expressed in yeast under the *GAL1* promoter (MTK1 produced in *E.coli* had no catalytic activity). Yeast cells were grown in a selective medium containing 2% raffinose. When the OD_{600} reached 0.3, protein expression was induced by addition of galactose. After 5 h, following the addition of galactose, yeast cells were harvested and extracts were prepared by the glass beads method (Posas *et al.*, 1996). Protein concentration was determined according to Bradford (1976).

In vitro phosphorylation of MAPKKs by MTK1

GST-MTK1ΔN and GST-MTK1(K/R)ΔN were purified from yeast extracts using glutathione–Sepharose beads. One and a half micrograms of the catalytically inactive GST-MAPKKs, purified from *E.coli*, were incubated with 1.25 μg of purified GST-MTK1ΔN or GST-MTK1(K/R)ΔN in buffer A containing 50 μM [γ-^{32}P]ATP (0.2 μCi/μl). After 10 min incubation at 30°C, reactions were stopped by the addition of 2× SDS loading buffer. Labeled proteins were resolved by SDS–PAGE and detected by autoradiography.

In vitro activation of MAPK kinases by MTK1

In vitro activation of MAPKKs was monitored by a coupled kinase assay. Three quarters of a microgram of the recombinant GST-MAPKKs, purified from *E.coli*, were incubated with 1.25 μg of purified MTK1ΔN or MTK1ΔN(K/M) in buffer A containing 50 μM ATP. After 10 min incubation at 30°C, 2.5 μg of catalytically inactive MAPK substrate, purified from *E.coli*, was added in the presence of [γ-^{32}P]ATP (0.2 μCi/μl). The mixture was incubated for an additional 5 min at 30°C and the reactions were stopped by addition of 2× SDS loading buffer. GST-p38(K/N) was used as a substrate for MKK6 and SEK1 whereas GST-ERK1(K/N) was used to measure the activation of MEK1. Labeled proteins were resolved by SDS–PAGE and detected by autoradiography.

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References

Albertyn,J., Hohmann,S., Thevelein,J.M. and Prior,B.A. (1994) *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. *Mol. Cell. Biol.*, **14**, 4135–4144.

Blank,J.L., Gerwins,P., Elliot,E.M., Sather,S. and Johnson,G.L. (1996) Molecular cloning of mitogen-activated protein/ERK kinase kinases (MEKK) 2 and 3. *J. Biol. Chem.*, **271**, 5361–5368.

Boguslawski,G. (1992) *PBS2*, a yeast gene encoding a putative protein kinase, interacts with the *RAS2* pathway and affects osmotic sensitivity of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.*, **138**, 2425–2432.

Boguslawski,G. and Polazzi,J.O. (1987) Complete nucleotide sequence of a gene conferring polymyxin B resistance on yeast: similarity of the predicted polypeptide to protein kinases. *Proc. Natl Acad. Sci. USA*, **84**, 5848–5852.

Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

Brewster,J.L., de Valoir,T., Dwyer,N.D., Winter,E. and Gustin,M.C. (1993) An osmosensing signal transduction pathway in yeast. *Science*, **259**, 1760–1763.

Christianson,T.W., Sikorski,R.S., Dante,M., Shero,J.H. and Hieter,P. (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene*, **110**, 119–122.

Cobb,M.H. and Goldsmith,E.J. (1996) How MAP kinases are regulated. *J. Biol. Chem.*, **270**, 14843–14846.

Cooper,J.A. (1994) MAP kinase pathways: Straight and narrow or tortuous and intersecting? *Curr. Biol.*, **4**, 1118–1121.

Coso,O.A., Chiariello,M., Yu,J.-C., Teramoto,H., Crespo,P., Xu,N., Miki,T. and Gutkind,J.S. (1995) The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell*, **81**, 1137–1146.

Cuenda,A., Cohen,P., Buée-Scherrer,V. and Goedert,M. (1997) Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6); comparison of the specificities of SAPK3 and SAPK2 (RK/p38). *EMBO J.*, **16**, 295–305.

Davis,R.J. (1994) MAPKs: new JNK expands the group. *Trends Biochem. Sci.*, **19**, 470–473.

Dérijard,B., Hibi,M., Wu,I.H., Barrett,T., Su,B., Deng,T., Karin,M. and Davis,R.J. (1994) JNK1: A protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*, **76**, 1025–1037.

Dérijard,B., Raingeaud,J., Barrett,T., Wu,I.-H., Han,J., Ulevitch,R.J. and Davis,R.J. (1995) Independent human MAP kinase signal transduction pathways defined by MEK and MKK isoforms. *Science*, **267**, 682–685.

Freshney,N.W., Rawlinson,L., Guesdon,F., Jones,E., Cowley,S., Hsuan,J. and Saklatvala,J. (1994) Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell*, **78**, 1039–1049.

Gerwins,P., Blank,J.L. and Johnson,G.L. (1997) Cloning of a novel mitogen-activated protein kinase kinase kinase, MEKK4, that selectively regulates the c-Jun amino terminal kinase pathway. *J. Biol. Chem.*, **272**, 8288–8295.

Han,J., Lee,J.-D., Bibbs,L. and Ulevitch,R.J. (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science*, **265**, 808–811.

Hanks,S.K. and Quinn,A.M. (1991) Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. *Methods Enzymol.*, **200**, 38–62.

Herskowitz,I. (1995) MAP kinase pathways in yeast: for mating and more. *Cell*, **80**, 187–197.

Hirai,S., Izawa,M., Osada,S., Spyrou,G. and Ohno,S. (1996) Activation of the JNK pathway by distantly related protein kinases, MEKK and MUK. *Oncogene*, **12**, 641–650.

Ichijo,H. *et al*. (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science*, **275**, 90–94.

Kyriakis,J.M. and Avruch,J. (1996) Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J. Biol. Chem.*, **271**, 24313–24316.

Kyriakis,J.M., Banerjee,P., Nikolakaki,E., Dai,T., Rubie,E.A., Ahmad, M.F., Avruch,J. and Woodgett,J.R. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature*, **369**, 156–160.

Lange-Carter,C.A., Pleiman,C.M., Gardner,A.M., Blumer,K.J. and Johnson,G.L. (1993) A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science*, **260**, 315–319.

Lee,J.C. *et al*. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*, **372**, 739–746.

Lin,A., Minden,A., Martinetto,H., Claret,F.-X., Lange-Carter,C., Mercurio,F., Johnson,G.L. and Karin,M. (1995) Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. *Science*, **268**, 286–290.

Maeda,T., Wurgler-Murphy,S.M. and Saito,H. (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature*, **369**, 242–245.

Maeda,T., Takekawa,M. and Saito,H. (1995) Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. *Science*, **269**, 554–558.

Marshall,C.J. (1994) MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr. Opin. Genet. Dev.*, **4**, 82–89.

Mertens,S., Craxton,M. and Goedert,M. (1996) SAP kinase-3, a new member of the family of mammalian stress-activated protein kinases. *FEBS Lett.*, **383**, 796–805.

Minden,A., Lin,A., Claret,F.-X., Abo,A. and Karin,M. (1995) Selective activation of the JNK signaling cascade and c-jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell*, **81**, 1147–1157.

Ninomiya-Tsuji,J., Nomoto,S., Yasuda,H., Reed,S.I. and Matsumoto,K. (1991) Cloning of a human cDNA encoding a CDC2-related kinase by complementation of a budding yeast *cdc28* mutation. *Proc. Natl Acad. Sci. USA*, **88**, 9006–9010.

Ota,I.M. and Varshavsky,A. (1993) A yeast protein similar to bacterial two-component regulators. *Science*, **262**, 566–569.

Pandey,P., Raingeaud,J., Kaneki,M., Weichselbaum,R., Davis,R.J., Kufe,D. and Kharbanda,S. (1996) Activation of p38 mitogen-activated protein kinase by c-abl-dependent and -independent mechanisms. *J. Biol. Chem.*, **271**, 23775–23779.

Posas,F. and Saito,H. (1997) Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: Scaffold role of Pbs2p MAPKK. *Science*, **276**, 1702–1705.

Posas,F., Wurgler-Murphy,S.M., Maeda,T., Witten,E.A., Thai,T.C. and Saito,H. (1996) Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 'two-component' osmosensor. *Cell*, **86**, 865–875.

Raingeaud,J., Whitmarsh,A.J., Barrett,T., Dérijard,B. and Davis,R.J. (1996) MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell. Biol.*, **16**, 1247–1255.

Rana,A., Gallo,K., Godowski,P., Hirai,S., Ohno,S., Zon,L., Kyriakis,J.M. and Avruch,J. (1996) The mixed lineage kinase SPRK phosphorylates and activates the stress-activated protein kinase activator, SEK-1. *J. Biol. Chem.*, **271**, 19025–19028.

Rose,M.D. and Broach,J.R. (1991) Cloning genes by complementation in yeast. *Methods Enzymol.*, **194**, 195–230.

Rosette,C. and Karin,M. (1996) Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. *Science*, **274**, 1194–1197.

Rouse,J., Cohen,P., Trigon,S., Morange,M., Alonso-Llamazares,A., Zamanillo,D., Hunt,T. and Nebreda,A.R. (1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell*, **78**, 1027–1037.

Salmeron,A., Ahmad,T.B., Carlile,G.W., Pappin,D., Narsimhan,R.P. and Ley,S.C. (1996) Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase. *EMBO J.*, **15**, 817–826.

Sánchez,I., Hughes,R.T., Mayer,B.J., Yee,K., Woodgett,J.R., Avruch,J., Kyriakis,J.M. and Zon,L.I. (1994) Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. *Nature*, **372**, 794–798.

Shiozaki,K., Shiozaki,M. and Russell,P. (1997) Mcs4 mitotic catastrophe suppressor regulates the fission yeast cell cycle through the Wik1-Wis1-Spc1 kinase cascade. *Mol. Biol. Cell*, **8**, 409–419.

Sluss,H.K., Barrett,T., Dérijard,B. and Davis,R.J. (1994) Signal transduction by tumor necrosis factor mediated by JNK protein kinases. *Mol. Cell. Biol.*, **14**, 8376–8384.

Streuli,M., Hall,L.R., Saga,Y., Schlossman,S.F. and Saito,H. (1987) Differential usage of three exons generates at least five different mRNAs encoding human leukocyte common antigens. *J. Exp. Med.*, **166**, 1548–1566.

Tabor,S. and Richardson,C.C. (1989) Selective inactivation of the exonuclease activity of bacteriophage T7 DNA polymerase by *in vitro* mutagenesis. *J. Biol. Chem.*, **264**, 6447–6458.

Waskiewicz,A.J. and Cooper,J.A. (1995) Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. *Curr. Opin. Cell. Biol.*, **7**, 798–805.

Wurgler-Murphy,S.M. and Saito,H. (1997) Two-component signal transducers and MAPK cascades. *Trends Biochem. Sci.*, **22**, 172–176.

Yamaguchi,K., Shirakabe,K., Shibuya,H., Irie,K., Oishi,I., Ueno,N., Taniguchi,T., Nishida,E. and Matsumoto,K. (1995) Identification of a member of the MAPKKK family as a potential mediator of TFG-β signal transduction. *Science*, **270**, 2008–2011.

Yan,M., Dai,T., Deak,J.C., Kyriakis,J.M., Zon,L.I., Woodgett,J.R. and Templeton,D.J. (1994) Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. *Nature*, **372**, 798–800.

Zervos,A.S., Faccio,L., Gatto,J.P., Kyriakis,J.M. and Brent,R. (1995) Mxi2, a mitogen-activated protein kinase that recognizes and phosphorylates Max protein. *Proc. Natl Acad. Sci. USA*, **92**, 10531–10534.

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