Human mitogen-activated protein kinase kinase kinase mediates the stress-induced activation of mitogen-activated protein kinase cascades

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The mitogen-activated protein kinase (MAPK) cascades represent one of the important signalling mechanisms in response to environmental stimuli. We report the identification of a human MAPK kinase kinase, MAPKKK4, via sequence similarity with other MAPKKKs. When truncated MAPKKK4 (Δ MAPKKK4) was overexpressed in HEK293 cells, it was constitutively active and induced the activation of endogenous p38 α , c-Jun N-terminal kinase (JNK)1/2 and extracellular signal-regulated kinase (ERK)2 *in vivo*. Kinase-inactive Δ MAPKKK4 partly inhibited the activation of p38 α , JNK1/2 and ERK2 induced by stress, tumour necrosis factor α or epidermal growth factor, suggesting that MAPKKK4 might be physiologically involved in all three MAPK cascades. Co-expressed MAP kinase kinase (MKK)-1, MKK-4, MKK-3 and MKK-6 were activated *in vivo* by Δ MAPKKK4. All of the above MKKs purified from *Escherichia*

coli were phosphorylated and activated by Δ MAPKKK4 immunoprecipitates *in vitro*. When expressed by lower plasmid doses, Δ MAPKKK4 preferentially activated MKK-3 and p38 α *in vivo*. Overexpression of Δ MAPKKK4 did not activate the NF- κ B pathway. Immunoprecipitation of endogenous MAPKKK4 by specific antibodies showed that MAPKKK4 was activated after the treatment of K562 cells with various stress conditions. As a broadly distributed kinase, MAPKKK4 might serve as a stress responder. MAPKKK4 is 91 % identical with the recently described murine MEKK-4 β and might be its human homologue. It is also identical with the recently cloned human MAP three kinase 1 except for the lack of an internal sequence homologous to the murine MEKK-4 α isoform. Differences in the reported functional activities of the three kinases are discussed.

INTRODUCTION

Three mitogen-activated protein kinase (MAPK) cascades constitute the major mechanisms of relaying extracellular signals triggered by growth factors, cytokines and cellular stress to specific responses and gene induction in mammalian cells (reviewed in [1,2]). These signalling cascades have important roles in the regulation of cell growth, differentiation, cell survival, apoptosis, and stress and inflammatory responses. They are the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK; also known as SAPK for stress-activated protein kinase) and p38 (also known as CSBP for CSAID-binding protein) pathways. Each MAPK cascade consists of a downstream kinase (MAPK) activated after Thr/Tyr dual phosphorylation by a midstream MAPK kinase (MAPKK). MAPKK is in turn activated after Ser/Thr phosphorylation by an upstream MAPKK kinase (MAPKKK). All MAPKs and MAPKKs in the same cascade are highly similar in the catalytic domains and they share activator kinases and phosphorylate similar substrates. MAPKKs are in general very specific for downstream MAPKs and keep the three cascades from cross-talking, except for MKK-4, which activates both JNK and p38 [3]. In contrast, MAPKKKs include kinases with high to distant sequence similarity. They have less specific activity and transduce signals to more than one MAPK cascade. The three major MAPK cascades are: (1) ERK1/2 activated by MEK1/2 (MAP or ERK kinase, referred to here as MKK for MAP kinase kinase), which are in turn activated by Raf-1, Mos, Tpl-2 and MEKK-1/2/3 (MEK kinase) (reviewed in [2]); (2) JNK1/2/3

activated by MKK-4/7 [3–5], which are in turn activated by MEKK1/2/3, TAK-1, Tpl-2, MLK-2, MLK-3, DLK and MAPKKK5 (also known as ASK1) (reviewed in [2,6–11]); (3) p38 α , β , γ , δ activated by MKK-3/6 (reviewed in [2]), which are in turn activated by TAK-1, MLK-3, DLK and MAPKKK5 [7–9,11,12].

There have been suggestions that MAPKKKs might be involved in signalling pathways other than the MAPK cascades. Overexpression of MEKK-1 and MEKK-3 has been shown to activate NF- κ B-dependent transcription in T cells and fibroblasts [13–15]. MEKK-1 might induce the degradation and phosphorylation of I κ B *in vivo* via an unidentified kinase in the I κ B multimolecular complex [16]. Another distantly related MAPKKK, NF- κ B-inducing kinase, also activates the NF- κ B pathway but does not activate any MAPK cascades [17].

The large number of MAPKKKs suggests that the MAPKKKs might represent a pivotal check-point in signalling pathways in which divergent stimuli transduce signals to specific MAPKKKs before amplification downstream to one or more MAPKK/MAPK cascades. Examples are the TGF β -specific activation of TAK-1 [18] and the tumour necrosis factor α (TNF- α), interleukin 1- and Fas-specific activation of NF- κ B-inducing kinase [17]. In addition, an uncharacterized MAPKKK, named I-MEKK, is induced by insulin in adipocytes and activates MKK-1 [19]. Furthermore tissue distribution and cellular compartmentalization might contribute to the specific functions of each MAPKKK in triggering distinct MAPK cascades.

We report here the identification of a human MAPKKK, named MAPKKK4, that preferentially activates the p38 cascade

Abbreviations used: ATF, activating transcription factor; CAT, chloramphenicol acetyltransferase; CRIB, Cdc42/Rac interactive binding; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; GST, glutathione S-transferase; HA, haemagglutinin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKK or MKK (MAP kinase kinase), mitogen-activated protein kinase kinase; MAPKKK or MEKK, mitogen-activated protein kinase kinase kinase; MTK, MAP three kinase 1; PH, pleckstrin homology; PHAS-I, phosphorylated heat- and acid-stable protein regulated by insulin or eIF-4E-binding protein; TNF, tumour necrosis factor.

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in fibroblasts. MAPKKK4 is also involved in the activation of the JNK and ERK pathways but not the NF- κ B pathway. It is activated by various physiological stress conditions. While the characterization of MAPKKK4 was in progress, a closely related murine MEKK-4 β was reported [20].

MATERIALS AND METHODS

Construction of MAPKKK4 expression plasmids

The catalytic domain of MAPKKK4 (Δ MAPKKK4) encompassing the 1278th codon to the C-terminus was isolated from a human T-cell lymphoma Jurkat cDNA library (Clontech, Palo Alto, CA, U.S.A.) by using PCR. The 856 bp fragment was subcloned into a modified pCDNA3 vector by *KpnI/XbaI* ligation downstream to a T7-peptide tag (MASMTGGQGMG). The kinase-inactive mutation (D $\rightarrow$ A) was introduced into Δ MAPKKK4 by overlap extension PCR together with an exogenous *NarI* site (silent), for mutant identification [21]. The second truncated version of MAPKKK4 (Δ MAPKKK4-L), 16 residues longer at the N-terminal end downstream to the T7-tag, was generated in the same way as Δ MAPKKK4 by PCR. All expression plasmids generated were sequenced to confirm the absence of undesirable PCR errors.

Other plasmids and recombinant proteins

Mammalian expression plasmids, obtained as gifts, were as follows: haemagglutinin (HA)-MKK-1 in pCEP vector from N. Ahn (University of Colorado, Boulder, CO, U.S.A.), HA-MKK-3 in pCR3.1 vector, HA-MKK-6 in pME vector and FLAG- Δ MAPKKK5 in pCDNA3 vector from Z. Yao (Amgen, Boulder, CO, U.S.A.), and HA-MKK-4 in pMT2 vector from J. R. Woodgett (Ontario Cancer Institute, Toronto, ON, Canada). The κ B-dependent chloramphenicol acetyltransferase (CAT) reporter plasmid contains two NF- κ B sites and a TATA box from the HIV-1 promoter, whereas the κ B-independent CAT reporter lacks the NF- κ B sites. They were kindly provided by M. Fenton (Boston University School of Medicine, Boston, MA, U.S.A.).

Kinase substrates used in the study were as follows: kinase-inactive His-MKK-1(K $\rightarrow$ M) was obtained as transformed *Escherichia coli* from N. Ahn (University of Colorado, Boulder, CO, U.S.A.); p38 α , activating transcription factor (ATF)-2(1-109), glutathione S-transferase (GST)-MKK-3 and MKK-6 purified from *E. coli* were generously provided by G. Kessler, C. Manthey and M. Kelley (Amgen, Boulder, CO, U.S.A., and Thousand Oaks, CA, U.S.A.); GST-c-Jun(1-169), GST-MKK-1, kinase-inactive GST-ERK1(K $\rightarrow$ A), ERK-induced phosphorylation-defective GST-MKK-4(T $\rightarrow$ A/S $\rightarrow$ A), kinase-inactive and ERK-induced phosphorylation-defective GST-MKK-4(K $\rightarrow$ R/T $\rightarrow$ A/S $\rightarrow$ A) and kinase-inactive GST-JNK2(K $\rightarrow$ R) were purchased from Upstate Biotech (New York, NY, U.S.A.); and the phosphorylated heat- and acid-stable protein regulated by insulin or eIF-4E-binding protein, PHAS-I, was purchased from Strategene (La Jolla, CA, U.S.A.). Some of the above GST-tagged substrates were eluted from the agarose beads with two cycles of 10 mM glutathione in kinase buffer at 4 $^{\circ}$ C for 10 min before use. Approx. 50 % of GST-tagged proteins were recovered in the eluates as detected by immunoblotting with anti-GST.

Anti-MAPKKK4 and other antibodies

Rabbit antisera, Ab1 and Ab2, were generated against two internal MAPKKK4 peptides, 83-96 (H₂N-TSPSTPRQMK-RMS-CO₂H) and 430-447 (H₂N-PSRPSKGNPEVEGDDT-

CO₂H) respectively. Specific antibodies were purified on corresponding peptide-conjugated Sulfolink agarose (Pierce, Rockford, IL, U.S.A.). For use in immunoprecipitation, specific antibodies were conjugated to CNBr-activated CL-4B agarose at 1 mg/ml (Pharmacia, Piscataway, NJ, U.S.A.).

Other antibodies used in the study were purchased as follows: monoclonal anti-T7 from Novagen (Madison, WI, U.S.A.); monoclonal anti-HA (clone 12CA5) from Boehringer Mannheim (Indianapolis, IN, U.S.A.); monoclonal anti-FLAG (M2) from Eastman Kodak (Rochester, NY, U.S.A.); rabbit anti-p38 α (C-terminus), anti-(JNK1/2) and anti-ERK2(C-terminus) from Santa Cruz Biotech (Santa Cruz, CA, U.S.A.); rabbit anti-I κ B and anti-(phospho-I κ B), rabbit anti-[(Thr/Tyr)-dual phosphorylated p38 α], anti-[(Thr/Tyr)-dual phosphorylated JNK1/2] and anti-(Tyr-phosphorylated ERK1/2) from New England Biolabs (Beverly, MA, U.S.A.).

Determination of tissue distribution by RNA blotting

A master dot-blot containing normalized quantities of poly(A)⁺ RNA species from 50 human tissues (Clontech, Palo Alto, CA, U.S.A.) was hybridized with a radiolabelled PCR fragment (372 bp long, codons 908-1040) from MAPKKK4 in accordance with the manufacturer's instructions. The hybridization signals were quantified by phosphorimaging (Molecular Dynamics, Sunnevale, CA, U.S.A.). A Northern blot containing poly(A)⁺ RNA species from eight tumour cell lines was also purchased from Clontech and probed similarly.

Cell transfection and protein expression

Human embryonic kidney-derived HEK293 cells in 10 cm plates were transfected with single plasmids or co-transfected with multiple plasmids at indicated doses by incubating with 24 μ l of LipofectAMINE (Life Technologies, Grand Island, NY, U.S.A.) in 4 ml of serum-free Dulbecco's modified Eagle's medium for 5 h (day 0), followed by incubation overnight in the presence of 10 % (v/v) fetal calf serum. When various doses of the T7-tagged Δ MAPKKK4 plasmid were used in the transfection, the T7-tag control plasmid was added to equalize the total amount of plasmid used in each plate. After replenishment with fresh culture medium on day 1, transfectants were harvested at 44-48 h after transfection (day 2) with or without pretreatments [osmotic shock, UV-C irradiation, TNF- α or epidermal growth factor (EGF), as indicated] in ice-cold lysis buffer containing 1 % (v/v) NP40, 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 μ g/ml pepstatin, 10 μ g/ml leupeptin, 1 mM Pefabloc, 50 mM glycerol 2-phosphate, 50 mM NaF, 1 mM sodium orthovanadate and 10 % (v/v) glycerol. NP40-insoluble materials and nuclei were removed by centrifugation at 23000 *g* and 4 $^{\circ}$ C for 10 min. Protein concentrations in the post-nuclear lysates were determined by bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.). Lysates containing equal amounts of protein were used for immunoprecipitation and immunoblotting. Specific protein expression was determined by immunoblotting and enhanced chemiluminescence (Amersham, Arlington Heights, IL, U.S.A.), followed by densitometry (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

The transfection efficiency of HEK293 cells with lipofectamine was determined by FACS analysis of cells transfected with green fluorescent protein in pCDNA3 vector, the same vector as MAPKKK4. Essentially all (100 %) cells were found to express green fluorescent protein but the expression levels varied from very high to very low. With such a widely varying range of expression in the transfectants, the number of folds of activation or inhibition measured should be taken as relative changes but not as an absolute efficiency of activation or inhibition by the

	I	II
MAPKKK4/MTK1	TFKWQGNKIGEGQYGVVTCISVDTELMAMKEIRF--QPNDHKTI	
MEKK4	TFKWQGNKIGEGQYGVVTCISVDTELMAMKEIRF--QPNDHKTI	
MAPKKK5	YDENGDRVVLGKGTGIVYAGRDLSNOVRIAIEI---PERDSRYS	
MEKK1	DAEWLKGQIGLGAFCSCYQADVTGTVMAVQVTVYNTSSQE	
MEKK2	PTNWRLGKLLGQGAFAFRVYVLCYDVTGRELAVKQVQF--NPSPETS	
MEKK3	PINWRRLGKLLGQGAFAFRVYVLCYDVTGRELASKQVQF--DPDSPETS	
Conserved	W G * * G V * V T G * *	

	III	IV	V
MAPKKK4/MTK1	KETA---DELKIFEGIKHPNLVRYFVGLHREE--MYIFMEYCDEG		
MEKK4	KETA---DELKIFEGIKHPNLVRYFVGLHREE--MYIFMEYCDEG		
MAPKKK5	Q---PLHEEIALHKHLKHNIVQYLGSPSE--NGFIKIFMEQVPGG		
MEKK1	EVVEALREIIRMMGHNLHPNIIIRMLGATCEKSN--YNIPIEWMAAG		
MEKK2	KEVNALECEIQLLKNLHERIVQYVGLRDPQEKTLISIFMELSPGG		
MEKK3	KEVSALECEIQLLKNLHERIVQYVGLRDRAEKILTTFMEYMPGG		
Conserved	* * * * V Y * I M * *		

	Via	
MAPKKK4/MTK1	TLEEVSR-L-G-LQ--EHVIRLVSKQITIAINVLHEHGIVHRDIKG	
MEKK4	TLEEVSR-L-G-LQ--EHVIRLVSKQITIAINVLHEHGIVHRDIKG	
MAPKKK5	SLSALLRSKVGPLKDNQETIGFVYTKQILEGLKYLHONQIVHRDIKG	
MEKK1	SVAHLLS-KYGAFLK--ESVIVNYTEQLLRGLSYLHENQIHRDVKG	
MEKK2	SIKDQLK-AVGALT--ENVTRKVTQRILEGVHLSNMIVHRDIKG	
MEKK3	SVKDQLK-AVGALT--ESVTRKVTQRILEGVHLSNMIVHRDIKG	
Conserved	- * --* V * * I ** *V***I**	

	Vib	VII	VIII
MAPKKK4/MTK1	ANIFLTSSGL-IKLGDFGCSVKLKNNAQTMPEGVNSTLGTAAAYMAP		
MEKK4	ANIFLTSSGL-IKLGDFGCSVKLKNNAQTMPEGVNSTLGTAAAYMAP		
MAPKKK5	DNLVINTYSGVLKISDFGTSKRLAGINPCT---ETFTGLTYMAP		
MEKK1	ANLLIDSTGQRLRIADFGAAARLASKGTGAGEFGQGLGTATFMAP		
MEKK2	ANILRDSFG-NIKLGDFGASKRLQITICL-SGTGMKSVTGYPMWSP		
MEKK3	ANILRDSFG-NIKLGDFGASKRLQITICL-SGTGIRSVTGYPMWSP		
Conserved	A* S G K * * * S * * * *		

	IX	X
MAPKKK4/MTK1	EVITRAKGEHGRAADIWSLGCVVIEVMTGKRPHW-EYE-HNFQIM	
MEKK4	EVITRAKGEHGRAADIWSLGCVVIEVMTGKRPHW-EYE-HNFQIM	
MAPKKK5	EIDKGP-RGYGAADIWSLGCITIEMATGKPPFVELGEPO--AAM	
MEKK1	EVL---RQQQYGRSCDVWSVGCATIEMACAKPPWNAEHSNHLALI	
MEKK2	EVI---SEGEGYGRKADISVACTVVEMLTEKPPW-AEPEA--MAAI	
MEKK3	EVI---SEGEGYGRKADISVACTVVEMLTEKPPW-AEPEA--MAAI	
Conserved	*VI G G *R A * * * G * * * T * *W E E	

	XI	
MAPKKK4/MTK1	YKVG-MGHKPPPIERLSPGKDFLSHCLESDDPKMRTASQLLDHSPV	
MEKK4	YKVG-MGHKPPPIERLSPGKDFLSHCLESDDPKMRTASQLLDHSPV	
MAPKKK5	FKVGMFKVHPEIPESMSAEAKAFILKCFEPDPDKRACANDLLVDEFL	
MEKK1	FKIASATTPASIPSHLSPLGRDVALRCLQLQDPDRPPSRELLKHFPV	
MEKK2	FKIATQPTNPPLPSHISEHGRDF-LKRIFFVEAKLRPSAEELLTHMFV	
MEKK3	FKIATQPTNPPLPSHISEHGRDF-LKRIFFVEAKLRPSAEELLTHMFV	
Conserved	* * * * DF * A * * H F	

Figure 1 Protein sequence alignment of the catalytic domains of human MAPKKK4 with other MAPKKs

The catalytic domains of human MAPKKK4 (accession number AA131807), murine MEKK-4 (accession number U85608), murine MEKK-1 (accession number L13103), murine MEKK-2 (accession number U43186), murine MEKK-3 (accession number U43187) and human MAPKKK5 (accession number U67156) were aligned by using the GENEWORKS program. The sequence of MTK1 is identical with that of MAPKKK4. Amino acid residues in murine MEKK-4 different from MAPKKK4 are boxed. Gaps are denoted by dashes. Consensus amino acid residues are marked by asterisks and those conserved between MAPKKK4 and three other MAPKKs are as indicated.

kinase construct (see, for example, Figure 5). The reason is that the higher expressers are activating or inhibiting better than the lower expressers, whereas the readout kinase activity signals represent the sum of these events in the cell population.

Determination of kinase activation *in vivo* by kinase assays *in vitro*

Post-nuclear lysates (1 mg) from HEK293 cells transfected with T7-tag control, T7-tagged Δ MAPKKK4 or T7-tagged Δ MAPKKK4(D $\rightarrow$ A) plasmid were immunoprecipitated by agarose conjugated with anti-p38 α , anti-(JNK1/2) or anti-ERK2. The kinase activity of endogenous p38 α , JNK1/2 or ERK2 was determined by co-incubation of the immunoprecipitates with 5 μ g of ATF-2(1–109), 2 μ g of GST-c-Jun(1–169) or 4 μ g of PHAS-I respectively in 25 μ l of kinase buffer A (25 mM Hepes/25 mM MgCl₂/25 mM glycerol 2-phosphate/2 mM dithiothreitol/0.1 mM sodium orthovanadate) and 10 μ M [γ -³²P]ATP (10 μ Ci; Amersham, Arlington Heights, IL, U.S.A.) at 30 °C for 20–30 min.

To detect the activation *in vivo* of HA-tagged MKK-1, HA-tagged MKK-3, HA-tagged MKK-6 or HA-tagged MKK-4 co-

expressed with T7-tag control or T7-tagged Δ MAPKKK4 at the indicated plasmid doses, 0.2 mg of post-nuclear HEK293 transfectant lysates was immunoprecipitated with anti-HA and protein G-agarose (Pierce, Rockford, IL, U.S.A.). Immunoprecipitates of MKK-1, MKK-3, MKK-6 or MKK-4 were co-incubated with 1.4 μ g of kinase-inactive GST-ERK1(K $\rightarrow$ A), 5 μ g of p38 α , 5 μ g of p38 α or 1.2 μ g of kinase-inactive GST-JNK(K $\rightarrow$ R) respectively in 25 μ l of kinase buffer B (30 mM Hepes/20 mM MgCl₂/30 mM NaCl/25 mM glycerol 2-phosphate/1 mM dithiothreitol/0.1 mM sodium orthovanadate) and 10 μ M [γ -³²P]ATP (10 μ Ci) at 30 °C for 30 min.

All kinase reactions were stopped by the addition of equal volumes of 2 $\times$ Laemmli buffer containing 5% 2-mercaptoethanol. The substrates were resolved by SDS/PAGE and detected by autoradiography. The relative levels of phosphorylation signals were quantified by phosphorimaging (Molecular Dynamics, Sunnyvale, CA, U.S.A.) where indicated.

Determination of phosphorylation and activation of kinase substrates *in vitro*

T7-tag control, T7-tagged Δ MAPKKK4, T7-tagged Δ MAPKKK4(D $\rightarrow$ A) or FLAG-tagged Δ MAPKKK5 was immunoprecipitated by anti-T7-conjugated agarose or anti-FLAG-conjugated agarose from 0.2 mg of post-nuclear lysates or a normalized amount for T7-tagged Δ MAPKKK4(D $\rightarrow$ A) based on its expression level relative to that of T7-tagged Δ MAPKKK4. To determine the phosphorylation of substrates, the immunoprecipitates were co-incubated with 2 μ g of kinase-inactive His-MKK-1(K $\rightarrow$ M), 2 μ g of GST-MKK-3, 2 μ g of MKK-6 or 1.2 μ g of kinase-inactive GST-MKK-4(K $\rightarrow$ R/T $\rightarrow$ A/S $\rightarrow$ A) in 25 μ l of kinase buffer B and 10 μ M [γ -³²P]ATP (10 μ Ci) at 30 °C for 30 min.

To determine the activation of kinase substrates by immunoprecipitates *in vitro*, coupled kinase reactions were performed. For the activation of MKK-1, immunoprecipitates were co-incubated with 0.1 μ g of GST-MKK-1 together with 5 μ g of kinase-inactive GST-ERK1(K $\rightarrow$ A) bound on agarose, 0.1 μ g of GST-MKK-1 alone or 5 μ g of GST-ERK1(K $\rightarrow$ A) alone in 25 μ l of kinase buffer B and 25 μ M [γ -³²P]ATP (10 μ Ci) at 30 °C for 30 min. For the activation of MKK-4, immunoprecipitates were co-incubated with 0.5 μ g of ERK-induced phosphorylation-defective GST-MKK-4(T $\rightarrow$ A/S $\rightarrow$ A) together with 1 μ g of kinase-inactive GST-JNK(K $\rightarrow$ R), 0.5 μ g of GST-MKK-4(T $\rightarrow$ A/S $\rightarrow$ A) alone or 1 μ g of GST-JNK(K $\rightarrow$ R) alone under the same conditions as described above for MKK-1. For the activation of MKK-3 or MKK-6, immunoprecipitates were co-incubated with either 1 μ g of GST-MKK-3 or MKK-6 together with 10 μ g of p38 α , 1 μ g of GST-MKK-3 or MKK-6 alone, or 10 μ g p38 α alone under the same conditions as described above. All kinase reactions were analysed as described for kinase activity determination *in vivo*.

Determination of I κ B phosphorylation *in vivo* and NF- κ B reporter assay

I κ B phosphorylation *in vivo* was determined by immunoblotting with anti-I κ B against anti-(phospho-I κ B) followed by detection with chemiluminescence. Post-nuclear lysates (20 μ g) of HEK293 cells transfected with either T7-tag control or indicated doses of T7-tagged Δ MAPKKK4 plasmid were used. As a positive control, HeLa cell lysates with or without pretreatment with TNF- α were analysed in the same gels.

κ B-dependent or control κ B-independent CAT reporter plasmid (2 μ g) was co-transfected into HEK293 cells with 8 μ g of either T7-tag control or T7-tagged Δ MAPKKK4 plasmid. At 2

Figure 2 Tissue distribution of human MAPKKK4 mRNA

Dot-blots containing normalized quantities of poly(A)⁺ RNA species from 50 human tissues were probed for expression of MAPKKK4. Hybridization signals were quantified by phosphorimaging and normalized against the lowest expression level in kidney at an arbitrary value of 1.0.

days after transfection, total cell extracts were prepared with reporter lysis buffer. The CAT activity was determined by co-incubation of cell extracts with n-butyryl-CoA and 24 μ M [¹⁴C]chloramphenicol (0.15 μ Ci; NEN) at 37 °C for 6 h, followed by scintillation counting of the xylene-extracted butyrylated chloramphenicol in accordance with the manufacturer's instructions (Promega, Madison, WI, U.S.A.). Under these conditions, a linear standard curve was obtained on titration of CAT enzyme. As positive controls, HEK293 cells co-transfected with T7-tag control plasmid and either κ B-dependent or κ B-independent CAT reporter plasmid were pretreated with TNF- α for 9 or 22 h before cell lysis.

Determination of activation *in vivo* of endogenous MAPKKK4 by kinase assay *in vitro*

The specificity of anti-MAPKKK4 antibodies was tested by peptide blocking before use in the immunoprecipitation of endogenous MAPKKK4. For both Ab1 and Ab2, purified antibody or antibody-conjugated agarose was preincubated with a 250-fold molar excess of the corresponding immunogenic peptide for 30 min at 22 or 4 °C respectively. Post-nuclear lysates (0.8 mg) of K562 cells were immunoprecipitated with preblocked or unblocked antibody-conjugated agarose and resolved by SDS/PAGE under reducing conditions. Immunoblotting with preblocked or unblocked antibodies was then detected by chemiluminescence.

K562 cells at exponential growth [(0.4–0.6) $\times 10^6$ cells/ml] were stimulated as follows, before cell lysis: no stimulus; osmotic

shock (0.6 M NaCl, 30 min), UV-C irradiation (254 nm, 85.7 mJ/cm², 30 min recovery), anisomycin (50 μ g/ml, 20 min), cyclophosphamide (10 μ M, 30 min), H₂O₂ (0.5 mM, 20 min), sodium arsenite (0.25 M, 20 min), phorbol ester (300 nM, 20 min) or NH₄Cl (30 mM, 20 min). Post-nuclear lysates (1 mg) was precleared with rabbit Ig-conjugated agarose before immunoprecipitation with Ab1-conjugated agarose. The MAPKKK4 activity in the immunoprecipitates was determined by phosphorylation of GST-MKK-3 *in vitro*, as described above, at 30 °C for 30 min.

RESULTS

Identification of MAPKKK4

A human expressed sequence tag (accession number AA131807) similar to the catalytic domain of MAPKKKs was identified from the dbEST database (NCBI, Bethesda, MD, U.S.A.). On further searching of the GENBANK database, this sequence was found in a 4990 bp sequence submitted under the description 'human myeloblast mRNA for KIAA0213 gene'. KIAA0213 encodes an open reading frame of 1491 residues with an in-frame stop codon corresponding to the C-terminus of the predicted protein. No in-frame methionine start codon is present at the N-terminus. A complete kinase catalytic domain with subdomains I to XI is located at the C-terminus. The catalytic domain is most similar at the protein level to MAPKKKs as follows: 42 % identical with murine MEKK-3 [22]; 41 % with human MAPKKK5 [10,11] and murine MEKK-2 [22]; and 33 % with murine MEKK-1 [23] (Figure 1). During the functional charac-

Figure 3 Overexpression of MAPKKK4 activates endogenous p38 α , JNK1/2 and ERK2 *in vivo* in HEK293 cells

Left panels: endogenous p38 α , JNK1/2 or ERK2 was immunoprecipitated from HEK293 cells (on a 10 cm plate) transfected with 8 μ g of plasmid of the T7-tag control (vector) or the T7-tagged catalytic domain of MAPKKK4 with [Δ MAPKKK4(D $\rightarrow$ A)] or without (Δ MAPKKK4) the kinase-inactive mutation. ATF-2(1–109), GST-c-Jun(1–69) or PHAS-I was incubated with the p38 α , JNK1/2 or ERK2 immunoprecipitates respectively in the presence of [γ - 32 P]ATP. The substrates were resolved by SDS/PAGE. The expression levels of Δ MAPKKK4 and Δ MAPKKK4(D $\rightarrow$ A) were compared by immunoblotting post-nuclear lysates with anti-T7. Right panels: similar immunoprecipitates were prepared to those described for the left panels for kinase assays *in vitro*, except that HEK293 cells transfected with the longer version of T7-tagged Δ MAPKKK4 (Δ MAPKKK4-L) were included. The expression levels of Δ MAPKKK4 and Δ MAPKKK4-L were compared as described above. Similar results were obtained in two independent experiments.

terization of KIAA0213, the sequences of the α and β isoforms of murine MEKK-4 were reported [20]. The α isoform is identical with the β isoform except for a 52-residue internal insertion in the non-catalytic domain. Alignment of the incomplete protein sequence of KIAA0213 with the complete sequence of murine MEKK-4 β isoform showed an overall 91% identity, with the catalytic domains most conserved at 98% identity. KIAA0213 might thus be the human homologue of murine MEKK-4 β and is referred to as MAPKKK4. On the basis of alignment with the murine MEKK-4 β sequence, the KIAA0213 sequence lacks approx. 60 codons at the 5' end.

The non-catalytic domain of MAPKKK4 has a partial Cdc42/Rac interactive binding (CRIB) domain with four conserved residues present instead of eight [24]. Many potential protein kinase C phosphorylation sites (S/TXX/R, K/RXXS/T, K/RXS/T, where X is any amino acid), cAMP-dependent protein kinase phosphorylation sites (RXS, RXXS) and tyrosine kinase phosphorylation sites (XD/EYX) are located in the non-catalytic domain of MAPKKK4 [25]. Segment-oriented and nearest-neighbour secondary structure analyses of MAPKKK4 (PSSP program at Baylor College of Medicine) identify a pleckstrin homology (PH)-resembling domain that can potentially form two antiparallel β -sheets containing seven β -strands with a 57-residue α -helix-promoting linker between β -strands 4 and 5 (residues 161–408). The long C-terminal α -helix (67 residues) following the β -strands contains a conserved Trp residue close to the end. However, it lacks the presence of at least one negatively charged residue at the -4 or -5 position to this Trp residue and the invariable large hydrophobic amino acid at the $+4$ position,

which are thought to stabilize the N-terminal β -sheets [26]. Similar invariable amino acids are absent from the region around the conserved Trp residue in murine MEKK-4. Secondary structure analyses of MEKK-4 predict the loss of β -strands 1 and 5–7 to be associated with sequence discrepancies from MAPKKK4. Whether these extended domains adopt a PH structural motif as proposed for MEKK-4 will be resolved when their tertiary structures are determined.

Tissue distribution of MAPKKK4

A radiolabelled PCR fragment of Δ MAPKKK4 was hybridized to a dot-blot containing poly(A)⁺ RNA species from 50 human tissues. The poly(A)⁺ RNA species were quantitatively normalized to eight different housekeeping genes so that the relative mRNA quantification was more accurate than most Northern blots. MAPKKK4 was found to be widely expressed, mostly in exocrine glands, haemopoietic tissues, heart, skeletal muscle, placenta, neural tissues, gastrointestinal tissues and reproductive organs (Figure 2). It is least expressed in lung, liver, kidney and fetal brain. Expression in these organs, however, is developmentally regulated and is high in fetal lung, fetal liver, fetal kidney and adult brain.

Northern blot analysis was also performed with several tumour cell lines (results not shown). MAPKKK4 was detected as a 6 kb poly(A)⁺ band at high levels in chronic myelogenous leukaemia K562 and colorectal adenocarcinoma SW480, and at moderate levels in HeLa cells, promyelocytic leukaemia HL-60 and melanoma G361.

Figure 4 MAPKKK4 preferentially activates p38 α relative to JNK1/2 and ERK2 *in vivo* in HEK293 cells

Endogenous p38 α , JNK1/2 or ERK2 was immunoprecipitated from HEK293 cells (on 10 cm plates) stimulated by osmotic shock (0.6 M NaCl, 20 min), UV-C irradiation (254 nm, 85.7 mJ/cm², 20 min recovery) or EGF (100 ng/ml, 10 min), or transfected with either T7-tag control (vector) or increasing doses of T7-tagged Δ MAPKKK4 plasmid. Kinase assays *in vitro* were as described in the legend to Figure 3. The expression levels of Δ MAPKKK4 were detected by immunoblotting of post-nuclear lysates with anti-T7. The lower band was detected in vector-transfected cells and its identity is unknown. Similar results were obtained in two independent experiments.

Overexpressed MAPKKK4 activates p38 α , JNK1/2 and ERK2 *in vivo*

A transient transfection system was used to determine whether MAPKKK4 activates any of the MAPK cascades, p38, JNK and ERK *in vivo*. The partial MAPKKK4 cDNA lacking the N-terminal 60 residues was weakly expressed even under a strong cytomegalovirus promoter in human embryonic kidney-derived HEK293 cells and it exhibited very weak kinase activity (results not shown). It suggests that, like some other MAPKKKs, the non-catalytic portion of MAPKKK4 contains negative regulatory elements affecting its catalytic activity. A truncated MAPKKK4 that contains only the catalytic domain was then expressed as an N-terminally T7-tagged fusion kinase (Δ MAPKKK4) in HEK293 cells and found to be constitutively active. Endogenous p38 α , JNK1/2 and ERK2 immunoprecipitated from HEK293 cells overexpressing Δ MAPKKK4 were activated as detected by phosphorylation *in vitro* of ATF-2, c-Jun and PHAS-I respectively (Figure 3, left panels). These MAPKs were also phosphorylated *in vivo* as shown by immunoblotting with antibodies specific for Thr/Tyr-dual phosphorylated p38 α , Thr/Tyr-dual phosphorylated JNK1/2 and Tyr-phosphorylated ERK1/2 (results not shown). When the conserved Asp residue in the catalytic loop of subdomain IV was mutated to Ala, Δ MAPKKK4(D $\rightarrow$ A) overexpression did not activate p38 α , JNK1/2 or ERK2 (Figure 3, left panels). The expression level of Δ MAPKKK4(D $\rightarrow$ A) was 60 % of that of Δ MAPKKK4 as detected by immunoblotting and densitometry. The relatively lower expression level does not, however, account for the lack of downstream activation by Δ MAPKKK4 (compare with data in Figure 4). It suggests that the activation *in vivo* of the MAPKs is a result of the kinase activity of Δ MAPKKK4.

The activation of endogenous p38 α and ERK2 by Δ MAPKKK4 in HEK293 cells *in vivo* was in contrast with the lack of similar activity in HEK293 cells by the murine MEKK-

Figure 5 Inhibition of the activation of endogenous MAPKs in HEK293 cells by kinase-inactive MAPKKK4

HEK293 cells on 10 cm plates transfected with 8 μ g of either T7-tag control (vector or mock) or kinase-inactive T7-tagged Δ MAPKKK4(D $\rightarrow$ A) plasmid were treated with osmotic shock, UV-C irradiation, TNF- α or EGF. Immunoprecipitates of endogenous p38 α (A), JNK1/2 (B) or ERK2 (C) were assayed for kinase activity *in vitro* as described in the legend to Figure 3. Relative activation levels were quantified by phosphorimaging. The basal kinase activity levels in untreated vector or Δ MAPKKK4 transfectants were given the arbitrary value of unity. Similar results were obtained in two independent experiments.

4 [20]. We generated another truncated Δ MAPKKK4-L that had 16 additional residues between the T7-tag and the coding sequence and included the partial CRIB domain as in the murine truncated MEKK-4 construct. Consistently with the properties of Δ MAPKKK4, Δ MAPKKK4-L activated endogenous p38 α equally well (Figure 3, right panels). If the expression level of Δ MAPKKK4-L compared with Δ MAPKKK4 detected by immunoblotting is taken into consideration, Δ MAPKKK4-L was less potent in activating endogenous JNK1/2 and much less potent for ERK2.

Δ MAPKKK4 preferentially activates p38 α *in vivo*

Decreasing plasmid doses of Δ MAPKKK4 were transfected in HEK293 cells to determine the relative efficiencies of endogenous p38 α , JNK1/2 and ERK2 activation (Figure 4). None of the expression levels of p38 α , JNK1/2 and ERK2 were affected by transfection with Δ MAPKKK4 (results not shown). Endogenous p38 α was most highly activated by Δ MAPKKK4. It was significantly activated in the presence of 8 ng of plasmid; transfection with 80 ng or more of plasmid resulted in as high activation as by osmotic shock. Because the kinase assays *in vitro* were terminated within the linear range of the phosphotransfer reaction, it suggests that the endogenous p38 α was fully activated by transfection with 80 ng of plasmid. In comparison, endogenous JNK1/2 and ERK2 were activated to similar extents with 0.8 μ g of plasmid; their activation was insignificant at the 80 ng plasmid dose. In contrast, when Δ MAPKKK4 was co-transfected with p38 α , JNK1 or ERK2 in HEK293 cells, the relative activation efficiencies were in the order JNK1 p38 α ERK2 (results not shown). The difference in the activation efficiencies of endogenous and co-expressed MAPKs by Δ MAPKKK4 might be partly due to the different levels of basal activity of overexpressed MAPKs. Overexpressed p38 α showed high basal kinase activity, whereas overexpressed JNK1 and ERK2 showed no significant basal activity (results not shown).

Kinase-inactive MAPKKK4 inhibits activation of the p38 α , JNK1/2 and ERK2 cascades

HEK293 cells overexpressing the kinase-inactive Δ MAPKKK4(D $\rightarrow$ A) were treated by various stimuli and the activation of the endogenous MAPK cascades was compared with that in vector-transfected cells (Figure 5). Δ MAPKKK4(D $\rightarrow$ A) partly inhibited the activation of p38 α by UV-C irradiation, the activation of JNK1/2 by UV-C irradiation and TNF- α , and the activation of ERK2 by EGF. Inhibition of osmotic shock-induced p38 α and JNK1/2 activation was weaker, suggesting that this environmental stress probably induces MAPKs via more diverse kinase mediators than do other stimuli.

MAPKKK4 activates MKK-1, MKK-3, MKK-4 and MKK-6 *in vivo*

T7-tagged Δ MAPKKK4 was co-transfected with HA-tagged MKK-1, MKK-4, MKK-3 or MKK-6 in HEK293 cells to determine whether Δ MAPKKK4 activates these MKKs for p38 α , JNK1/2 and ERK2 *in vivo*. Δ MAPKKK4 activated co-expressed MKK-1 and MKK-4 as detected by the phosphorylation *in vitro* of kinase-inactive GST-ERK1(K $\rightarrow$ A) by MKK-1 immunoprecipitates and that of kinase-inactive GST-JNK2(K $\rightarrow$ R) by MKK-4 immunoprecipitates (Figure 6, upper panels). Δ MAPKKK4 also activated MKK-3 and MKK-6 as detected by the phosphorylation *in vitro* of p38 α by either immunoprecipitate (Figure 6, lower panels). On transfection of decreasing doses of the Δ MAPKKK4 plasmid to yield equivalent expression levels in both MKK-3 and MKK-6 co-

Figure 6 MAPKKK4 activates co-expressed MKK-1, MKK-4, MKK-3 and MKK-6 *in vivo* in HEK293 cells

Upper panels: HEK293 cells on 10 cm plates were co-transfected with 2 μ g of HA-tagged MKK-1 or HA-tagged MKK-4 plasmid together with 8 μ g of T7-tag control (vector) or T7-tagged Δ MAPKKK4 plasmid. Anti-HA immunoprecipitates of MKK-1 or MKK-4 were incubated with kinase-inactive GST-ERK1(K $\rightarrow$ A) or kinase-inactive GST-JNK2(K $\rightarrow$ R) respectively in the presence of [γ - 32 P]ATP. The substrates were resolved by SDS/PAGE. Lower panels: HEK293 cells on 10 cm plates were co-transfected with 2 μ g of HA-MKK-3 or 8 μ g of HA-MKK-6 plasmid together with 8 μ g of T7-tag control (vector) or various doses of T7-tagged Δ MAPKKK4 plasmid. Anti-HA immunoprecipitates of MKK-3 or MKK-6 were assayed for kinase activity *in vitro* with p38 α as a substrate, then analysed as above. The expression levels of T7-tagged Δ MAPKKK4 in all co-transfectants were determined by immunoblotting of post-nuclear lysates with anti-T7. For both the left and right panels, equal amounts of MKK-1, MKK-4, MKK-3 and MKK-6 were detected in post-nuclear lysates by immunoblotting with anti-HA (results not shown). Similar results were obtained in three independent experiments.

transfectants, MKK-3 was more activated (11.0-fold at the lowest plasmid dose) by Δ MAPKKK4 than was MKK-6 (3.3-fold at the lowest dose). In contrast with the activation of MKK-3, that of MKK-6 was only moderate by co-expressed Δ MAPKKK4 at the highest plasmid dose (4-fold compared with 60-fold). A similar profile of MKK-6 activation was detected with a smaller amount of MKK-6 immunoprecipitates, indicating that the moderate MKK-6 activation signal was not limited by the reaction conditions. The expression levels of co-expressed HA-tagged MKK-3 and MKK-6 were similar as detected by anti-HA immunoblotting (results not shown). Because MKK-3 is more widely expressed in different tissues than MKK-6 [27] and MKK-6 is the major p38 α activator in several cell types [28], MAPKKK4 probably has more ubiquitous effects on p38 α activation than other MAPKKs.

Figure 7 MAPKKK4 phosphorylates and activates MKK-1, MKK-4, MKK-3 and MKK-6 *in vitro*

HEK293 cells on 10 cm plates were transfected with 8 μ g of T7-tag control (vector), T7-tagged Δ MAPKKK4, kinase-inactive T7-tagged Δ MAPKKK4(D $\rightarrow$ A) or FLAG-tagged Δ MAPKKK5 plasmid. The expression levels of T7-tagged Δ MAPKKK4 and T7-tagged Δ MAPKKK4(D $\rightarrow$ A) were determined by immunoblotting of post-nuclear lysates with anti-T7, and normalized amounts were used in the subsequent kinase reactions *in vitro*. Left panels: anti-T7 or anti-FLAG immunoprecipitates were incubated with kinase-inactive His-MKK-1(K $\rightarrow$ M), kinase-inactive GST-MKK-4(K $\rightarrow$ R/T $\rightarrow$ A/S $\rightarrow$ A), GST-MKK-3 or MKK-6 in the presence of [γ - 32 P]ATP. The substrates were resolved by SDS/PAGE. Right panels: coupled kinase reactions were performed to detect the activation of MKKs. For MKK-1, anti-T7 or anti-FLAG immunoprecipitates were incubated with GST-MKK-1 plus kinase-inactive GST-ERK1(K $\rightarrow$ A), GST-MKK-1 alone, or GST-ERK1(K $\rightarrow$ A) alone. For MKK-4, immunoprecipitates were incubated with ERK-induced phosphorylation-defective GST-MKK-4(T $\rightarrow$ A/S $\rightarrow$ A) plus kinase-inactive GST-JNK2(K $\rightarrow$ R), GST-MKK-4(T $\rightarrow$ A/S $\rightarrow$ A) alone, or GST-JNK2(K $\rightarrow$ R) alone. For MKK-3 or MKK-6, immunoprecipitates were incubated with p38 α plus GST-MKK-3 or MKK-6, GST-MKK-3 or MKK-6 alone, or p38 α alone. The substrates were resolved by SDS/PAGE. Similar results were obtained in two independent experiments.

Phosphorylation and activation of MKK-1, MKK-4, MKK-3 and MKK-6 by MAPKKK4 *in vitro*

As shown above and reported previously by others, MAPKKKs typically activate MKKs and MAPKs *in vivo*. To determine that MAPKKK4 directly activates MKKs, Δ MAPKKK4 immunoprecipitates were tested for the phosphorylation and activation of *E. coli*-derived MKKs *in vitro* (Figure 7). Truncated MAPKKK5 (Δ MAPKKK5), which activates the p38 α and JNK pathways, was included as a control. Δ MAPKKK4 phosphorylated kinase-inactive His-tagged MKK-1(K $\rightarrow$ M), kinase-inactive GST-tagged MKK-4 (K $\rightarrow$ R/T $\rightarrow$ A/S $\rightarrow$ A), GST-tagged MKK-3 and MKK-6 (detected as a doublet), as did Δ MAPKKK5 (Figure 7, left panels). No phosphorylation of either of the MKKs was detected with the kinase-inactive Δ MAPKKK4(D $\rightarrow$ A), indicating that the MKKs were probably direct substrates for Δ MAPKKK4. However, the presence of other undetermined kinases in the immunoprecipitates cannot be ruled out. Δ MAPKKK4 also activated MKK-1, MKK-4, MKK-3 and MKK-6 *in vitro* in coupled kinase reactions (Figure 7, right panels). No activation signals were detected when either of the corresponding MKK or MAPK was omitted from the coupled kinase reactions. In comparison, Δ MAPKKK5 activated only MKK-4, MKK-3 and MKK-6, but MKK-1 was activated only weakly (Figure 7, right panels). This is consistent with a

previous report that Δ MAPKKK5 activates only the JNK and p38 α pathways [11]. The strong phosphorylation of MKK-1 by Δ MAPKKK5 *in vitro* probably occurred at sites irrelevant for kinase activation, analogously to the phosphorylation of MKK-1 by MEKK-1 *in vitro* [29].

MAPKKK4 does not activate the NF- κ B pathway

The NF- κ B cascade is another major signalling pathway induced by various stimuli that activate the MAPK cascades. MEKK-1 and MEKK-3 were previously reported to activate the NF- κ B pathway when overexpressed in transfectants [13–15]. To determine whether the NF- κ B pathway is activated by MAPKKK4, post-nuclear lysates from HEK293 cells overexpressing Δ MAPKKK4 at decreasing doses were immunoblotted with anti-I κ B and anti-(phospho-I κ B) to test for protein degradation and phosphorylation (Figure 8, upper panels). The expression level of I κ B was diminished at the high plasmid doses of Δ MAPKKK4 but no phosphorylation of I κ B was detected. As positive control, TNF- α -stimulated HeLa cells showed a distinct I κ B phosphorylation signal when the protein was degraded to a nearly undetectable level. This suggests that despite a lower steady-state I κ B protein expression level in the transfectants, no I κ B was activated. Co-transfection of HEK293 cells with

Figure 8 MAPKKK4 does not activate the NF- κ B pathway *in vivo*

Upper panels: HEK293 cells on 10 cm plates were transfected with either T7-tag control (vector) or various doses of T7-tagged Δ MAPKKK4 plasmid, as indicated. Post-nuclear lysates were immunoblotted with anti-I κ B or anti-(phospho-I κ B) (anti-P-I κ B). Cell lysates from unstimulated and TNF- α -stimulated HeLa cells were included as controls. Lower panel: HEK293 cells on 10 cm plates were transfected with 8 μ g of T7-tag control (vector) or T7-tagged Δ MAPKKK4 plasmid together with either 2 μ g of κ B-dependent (+ κ B) or κ B-independent (– κ B) CAT reporter plasmid. As positive controls, vector co-transfectants were treated with TNF- α for 9 or 22 h before analysis. Reporter transcription activities were measured in triplicate by scintillation counting and are presented as c.p.m. Results are representative of two independent experiments. Abbreviation: μ g.

Δ MAPKKK4 and a κ B-dependent reporter plasmid was further used to determine the activation of the NF- κ B pathway at the gene transcription level (Figure 8, lower panel). Consistent with a diminished steady-state expression of non-activated I κ B detected above, transfectants overexpressing Δ MAPKKK4 had lower reporter transcription activity compared with the vector-transfected cells. In contrast, 9 or 22 h pretreatment of vector-transfected HEK293 cells with TNF- α induced time-dependent reporter transcription activity. This activity was NF- κ B-responsive as no reporter activity was detected with the reporter plasmid lacking the NF- κ B-dependent site.

Activation of endogenous MAPKKK4 by various stress stimuli

K562 cells identified as having a high mRNA expression of MAPKKK4 were used to test the activation of endogenous MAPKKK4 by various stimuli. Rabbit antisera, Ab1 and Ab2, were raised against two internal peptides of MAPKKK4, and specific antibodies were purified. A specific 180 kDa protein with a molecular size predicted from the MAPKKK4 cDNA sequence was immunoprecipitated by Ab1 and detected by immunoblotting with Ab2 (Figure 9, upper panels). Both the

Figure 9 Activation of endogenous MAPKKK4 in K562 cells by various stimuli

Upper panels: K562 cells were subjected to immunoprecipitation with the anti-MAPKKK4 Ab1, with or without preblocking with excess Ab1 immunogenic peptide. Immunoprecipitates were then immunoblotted with another anti-MAPKKK4, Ab2, in the presence or absence of excess Ab2 immunogenic peptide. Post-nuclear K562 cell lysates were immunoblotted side by side for comparison. Lower panel: endogenous MAPKKK4 was immunoprecipitated with Ab1 from K562 cells pretreated with the indicated stress stimuli (see the Materials and methods section for doses and duration). Immunoprecipitates were then assayed for MAPKKK4 activity by phosphorylation of GST-MKK-3 *in vitro* as described for Figure 7 (left panels). Relative activation levels were determined by phosphorimaging and normalized against the basal MAPKKK4 activity from unstimulated cells with an arbitrary value of unity. Similar results were obtained in two independent experiments.

immunoprecipitation and immunoblotting were blockable by the corresponding immunogenic peptides, verifying that the 180 kDa protein was the endogenous MAPKKK4 in K562 cells. Ab2 immunoprecipitated the 180 kDa protein weakly. The major protein immunoprecipitated by Ab2 was approx. 110 kDa (results not shown) and was also detected as the major protein in the post-nuclear cell lysates by immunoblotting (Figure 9, upper panels). This 110 kDa protein was neither immunoprecipitated nor immunoblotted by Ab1, even though Ab1 immunoblotted the 180 kDa protein well. Several potential caspase-3 group-specific cleavage sites, DXDX, are present in MAPKKK4 [30], one of which predicts a cleavage product of 110 kDa recognizable by Ab2. Further studies will be required to determine whether MAPKKK4 is a substrate for caspases. Alternatively, the 110 kDa protein might represent a spliced isoform of MAPKKK4 or a cross-reactive unrelated protein. No alternative

splicing was suggested by Northern blotting analysis, although introns were detected close to the 5' end of the MAPKKK4 gene (R. Weaver

and P.-Y. Chan-Hui, unpublished work).

Endogenous MAPKKK4 was immunoprecipitated with Ab1 from K562 cells pretreated with various stress stimuli (see the Materials and methods section for details). The activity of MAPKKK4 immunoprecipitates was determined by phosphorylation *in vitro* of the physiological substrate GST-MKK-3 (Figure 9, lower panel). As with other MAPKKKs, MAPKKK4 immunoprecipitates showed a high basal activity *in vitro*. Activation of endogenous MAPKKK4 was detected at various levels dependent on the stimuli. It was most activated by treatment with NH_4Cl , sodium arsenite, anisomycin, cyclophosphamide and H_2O_2 . Osmotic shock and UV-C irradiation activated MAPKKK4 only slightly. MAPKKK4 was also activated by treatment of cells with phorbol ester, suggesting a possible involvement of protein kinase C in the regulation of MAPKKK4. No MAPKKK4 activation was detected in K562 cells pretreated with TNF- α , EGF or 10% (v/v) fetal calf serum for 0–30 min (results not shown).

DISCUSSION

We have identified MAPKKK4 from the dbEST and GenBank databases. When overexpressed in HEK293 cells, constitutively active truncated MAPKKK4 activated the endogenous p38 α , JNK1/2 and ERK2 cascades but not the NF- κ B pathway. It also activated endogenous p38 δ (P.-Y. Chan-Hui, unpublished work) [31,32]. MAPKKK4 activated co-expressed MKK-1, MKK-4, MKK-3 and MKK-6 *in vivo* and their *E. coli*-derived purified proteins *in vitro*. In HEK293 cells, the MKK-3/p38 α pathway was most activated by MAPKKK4 among the three MAPK cascades.

While the characterization of MAPKKK4 was in progress, a closely related murine MEKK-4 β was reported [20]. Like MAPKKK4, the truncated version of MEKK-4 containing the catalytic domain was shown to activate the endogenous JNK1/2 pathway when overexpressed in HEK293 cells. However, unlike MAPKKK4, Δ MEKK-4 did not activate the endogenous p38 α and ERK2 cascades. Because the catalytic domains of human MAPKKK4 and murine MEKK-4 share 98% identity and all putative activation and substrate recognition loops are conserved, the difference in substrate specificity is unlikely to be, though not impossible, a specific property of the murine protein. Δ MAPKKK4 and Δ MEKK-4 used for functional characterization differ in two ways. First, Δ MAPKKK4 was N-terminally T7-tagged and Δ MEKK-4 was C-terminally HA-tagged. It is possible that a free C-terminus of MEKK-4 is required for proper folding and functioning in MAPK pathways other than JNK. Secondly, a partial CRIB motif N-terminal proximal to the catalytic domain was included in the Δ MEKK-4 but not the Δ MAPKKK4 construct. Δ MEKK-4 was shown to interact with Rac1 and Cdc42, albeit in a GTP- and CRIB-independent manner [20,33]. Both Rac1 and Cdc42 are cytosolic proteins, partly associated with the cytoskeleton, which translocate to distinct intracellular membrane compartments: pinocytic vesicles for Rac1 and Golgi and secretory vesicles for Cdc42. Association with these proteins might result in the distinct compartmentation of Δ MEKK-4. This is supported by the recent localization of endogenous MEKK-4 to the Golgi apparatus in unactivated COS cells [33]. We generated another MAPKKK construct, Δ MAPKKK4-L, that contained 16 additional residues and included the partial CRIB motif. In contrast with Δ MAPKKK4, Δ MAPKKK4-L was less potent

in JNK activation than p38 α and only weakly activated ERK2. One plausible explanation is that compartmentation of Δ MAPKKK4-L via association with Rac1/Cdc42, a property possibly attributed to the addition of the 16 residues, led to a decreased accessibility to downstream substrates in the JNK1/2 and ERK2 pathways. Further analysis to determine whether Δ MAPKKK4 or Δ MAPKKK4-L binds Rac1/Cdc42 and whether these truncated proteins and the full-length protein display cellular compartmentation might help to explain the detected functional discrepancies between Δ MAPKKK4 and Δ MEKK-4. In contrast, Δ MAPKKK4-L and Δ MAPKKK4 each activated endogenous p38 α equally well. The discrepancy between Δ MAPKKK4s and Δ MEKK-4 in the activation of p38 α might reflect subtle differences in experimental conditions such as lysis buffer, transfection efficiency and HEK293 sublines.

The complete 5' cDNA sequence of MAPKKK4 was described in the full-length cloning of MAP kinase 1 (MTK1), the human homologue of yeast (*Saccharomyces cerevisiae*) ssk2/ssk22, during the preparation of this paper [34]. MTK1 is identical with MAPKKK4 in sequence except for a 49-residue internal insertion in the non-catalytic domain that is 67% identical with that in the α isoform of murine MEKK-4. Full-length untagged MTK1 activated co-expressed p38 α and JNK1 but not ERK2 when overexpressed in COS or HeLa cells. The discrepancy between Δ MAPKKK4 and MTK1 in ERK2 activation might be explained by the possible compartmentation via Rac1/Cdc42 association as discussed above and is supported by our results with MAPKKK4-L. Alternatively, but not exclusively, other elements in the non-catalytic domain might negatively regulate the activation of ERK2 pathway and the N-terminal truncation of MAPKKK4 has relieved it from such constraints. Intriguingly, unlike Δ MAPKKK4, N-terminal GST-tagged truncated MTK1 purified from yeast activated the reconstituted p38 α and JNK1 pathways but not the ERK2 pathway *in vitro*. It is possible that, apart from negative regulation, some unidentified mammalian cell-specific molecules associated with the catalytic domain might positively regulate the activation of the ERK2 pathway by MTK1/MAPKKK4.

The efficiency of Δ MAPKKK4 in activating the JNK pathway is also different from that of full-length MTK. On titration of plasmid doses in transfection experiments, full-length MTK1 activated co-expressed p38 α and JNK equally well in HeLa cells. In our titration experiments, however, we found that Δ MAPKKK4 activated the endogenous p38 α more efficiently. The difference might be attributable to the different methods of transfection used. We found that Δ MAPKKK4 activated JNK2 more efficiently than p38 α when co-transfected, and overexpressed p38 α had a much higher basal kinase activity in HEK293 cells than overexpressed JNK, which showed undetectable activity (P.-Y. Chan-Hui, unpublished work). Under these conditions, MAPKKK4-induced activation of co-expressed p38 α might be quantitatively limited by the available MKK-3 and MKK-6. The same conditions might exist in HeLa cells co-transfected with MTK1 and might explain the equal activation efficiencies of p38 α and JNK that we observed. Taking the above into consideration, we believe that the activation of endogenous MAPKs represents more a quantitatively reliable observation than that of overexpressed MAPKs and that Δ MAPKKK4 activates the p38 pathway more efficiently than those of JNK and ERK. Meanwhile, we cannot exclude the possibility that the N-terminal truncation of MAPKKK4 has removed some recognition sites for downstream signalling molecules in the JNK and ERK pathways. Further experiments with full-length MAPKKK4 to activate the endogenous MAPK pathways will extend our observations.

We found that kinase-inactive Δ MAPKKK4, containing the catalytic domain, partly inhibited the activation of endogenous p38 α , JNK1/2 and ERK2 stimulated by stress, TNF or EGF in HEK293 cells. It probably inhibited by sequestering the downstream MKK effectors from endogenous MAPKKK4 and other MAPKKKs or by sequestering upstream activators of MAPKKK4. These results suggest that, in the absence of regulatory mechanisms contributed by the N-terminal non-catalytic domain, MAPKKK4 is capable of activating all three MAPK cascades. Studies with kinase-inactive full-length MTK1, an α isoform of MAPKKK4, however, showed inhibition of stress-induced activation of co-expressed p38 α in COS cells but not stress-induced activation of JNK1 or phorbol ester-induced activation of ERK2 [34]. Further experiments with full-length constructs will be required to determine whether MAPKKK4 is functionally linked to substrates in all three MAPK pathways *in vivo* (such as via cell-activation-induced membrane translocation of Rac1/Cdc42) and whether MAPKKK4 is their physiological upstream activator.

Endogenous MAPKKK4 was activated to various degrees on treatment of K562 cells with stress stimuli such as osmotic shock, UV-C irradiation, anisomycin, H₂O₂, sodium arsenite, cyclophosphamide and NH₄Cl. Most of these stress stimuli are pleiotropic and many induce overlapping effects in the cells. These effects can be categorized as endoplasmic reticulum stress, oxidative stress, DNA damage and non-specific cell surface receptor activation. They have previously been shown to activate the JNK and p38 α pathways, whereas some were reported to activate ERK [35–37]. As a potential upstream activator in these MAPK pathways, MAPKKK4 might function as an important signalling transducer for physiological stress.

So far, MAPKKK4 is the only MAPKKK capable of activating all three MAPK cascades. Recent evidence has shown that the p38 α and JNK pathways are involved in apoptotic responses triggered by growth factor withdrawal [38,39], Fas ligand binding [40,41], B-cell antigen receptor cross-linking [41], heat shock [42], DNA-damaging agents [42], and UV-C and γ radiation [42,43]. In contrast, ERK activation has generally been associated with mitogenic and differentiation responses [44]. Under certain conditions in which ERK is activated by stress stimuli, it is thought to be involved in cellular recovery after damage [37]. The balance between the activation of the stress-induced p38 α /JNK pathways and that of the mitogenic ERK pathway might indeed be a determinant factor in cell survival and apoptosis [38]. As a broadly distributed kinase, MAPKKK4 might serve as a stress responder to harmful conditions and prepare the cells for cell cycle arrest and cellular repair, followed by either recovery or apoptosis depending on the prevalence of other anti-apoptotic or pro-apoptotic signals.

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