

## Molecular Cloning and Characterization of Human JNKK2, a Novel Jun NH<sub>2</sub>-Terminal Kinase-Specific Kinase

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At least three mitogen-activated protein kinase (MAPK) cascades were identified in mammals, each consisting of a well-defined three-kinase module composed of a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). These cascades play key roles in relaying various physiological, environmental, or pathological signals from the environment to the transcriptional machinery in the nucleus. One of these MAPKs, c-Jun N-terminal kinase (JNK), stimulates the transcriptional activity of c-Jun in response to growth factors, proinflammatory cytokines, and certain environmental stresses, such as short wavelength UV light or osmotic shock. The JNKs are directly activated by the MAPKK JNKK1/SEK1/MKK4. However, inactivation of the gene encoding this MAPKK by homologous recombination suggested the existence of at least one more JNK-activating kinase. Recently, the JNK cascade was found to be structurally and functionally conserved in *Drosophila*, where DJNK is activated by the MAPKK DJNKK (hep). By a database search, we identified an expressed sequence tag (EST) encoding a portion of human MAPKK that is highly related to DJNKK (hep). We used this EST to isolate a full-length cDNA clone encoding a human JNKK2. We show that JNKK2 is a highly specific JNK kinase. Unlike JNKK1, it does not activate the related MAPK, p38. Although the regulation of JNKK1 activities and that of JNKK2 activities could be very similar, the two kinases may play somewhat different regulatory roles in a cell-type-dependent manner.

Mitogen-activated protein kinase (MAPK) cascades consist of three protein kinases, a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). Kinase cascades such as these are widespread in animals and fungi (16, 30, 52). The general function of MAPK cascades is to relay environmental signals to the transcriptional machinery in the nucleus and thus to modulate gene expression (for reviews, see references 22, 23, and 52). At least three distinct MAPK cascades were identified in mammals. One leads to the activation of the extracellular signal-regulated kinases (ERKs), one leads to the activation of the Jun N-terminal kinases (JNKs), and another leads to the activation of p38 (for reviews, see references 3, 4, 6, 22, 42, and 52). The ERK cascade is mostly responsive to signals initiated by the binding of growth factors, such as epidermal growth factor (EGF) or platelet-derived growth factor, to their tyrosine kinase receptors (30). Although the JNK and p38 cascades are also activated by growth factors, their response to these signals is not as strong as that of the ERK cascade. More efficient activation of the JNK and p38 cascades is produced by proinflammatory cytokines, such as interleukin 1 (IL-1) or tumor necrosis factor (TNF), which have only a marginal effect on the ERK cascade. Most effective stimulation of the JNK and p38 cascades is observed following the exposure of cells to certain stresses, such as short wavelength UV irradiation or osmotic shock.

Multiple structurally similar protein kinases act in each of the steps of MAPK cascades. For instance, the ERK cascade contains ERK1 and -2 as MAPKs, MEK1 and -2 as MAPKKs, and Raf-1, A-Raf, and B-Raf as MAPKKKs (42). In the p38 cascade, the MAPKs are p38 $\alpha$  and  $\beta$  (13, 20, 27, 43), while the MAPKKs are MKK3 and -6 (5, 8, 14, 28, 40, 47). The identity

of the MAPKKKs that activate the p38 cascade is less clear. Some of the potential p38 MAPKKKs include apoptosis signal-regulating kinase1 (ASK1), TGF- $\beta$ -activated kinase 1, mixed lineage kinase 3, and MEKK1 (19, 51, 52a, 53). As most of these MAPKKKs also activate the JNK cascade and these results are mainly derived from transient transfection studies, the identity of the physiological p38 MAPKKKs is still nebulous. The JNK cascade contains three distinct gene products, JNK1, -2, and -3, each produced in several alternatively spliced forms (7, 12, 17, 21, 25, 35, 45). Only one kinase so far has been identified as the MAPKK for this cascade, JNKK1/SEK1/MKK4 (8, 28, 44). In addition to the JNKs, JNKK1 can phosphorylate and activate p38s (8, 28). There is substantial biochemical (32, 36) and genetic (37, 55) evidence for the existence of at least one additional JNK-activating kinase. At the MAPKKK level, several protein kinases were reported to be capable of activating the JNK cascade. The first and perhaps the most efficient activator of the JNK cascade identified is MEKK1, which was shown to directly phosphorylate and activate JNKK1 (28, 33, 54). Additional MAPKKKs that activate the JNK cascade include MEKK2, -3, and -4, MLK-3, and ASK1 (for a review, see reference 9 and references therein; 1, 10). As most of these results were achieved by transient transfection studies, the physiological functions of these upstream kinases remain to be identified.

Recently, a family of serine/threonine kinases was identified which have sequence homology with the budding yeast serine/threonine kinase STE20 (for reviews, see references 16 and 26). The mammalian STE20 like kinases include germinal center kinase (GCK), p65<sup>PAK</sup> and its homologs, hematopoietic progenitor kinase 1, and Nck-interacting kinases (18, 24, 31, 39, 49, 51). In yeast, STE20 was shown to act upstream of STE11, a MAPKKK in the yeast pheromone pathway. However, there is no evidence that STE20 acts as a MAPKKK directly activating STE11 (26). Most of the mammalian

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STE20-like kinases were shown to specifically activate the JNK and p38 cascades but not the ERK cascade.

The JNK and p38 MAPK cascades are evolutionarily conserved both in structure and function. The kinase domain of the budding yeast *Saccharomyces cerevisiae* HOG1 MAPK is 51% similar to that of p38 $\alpha$  (13). HOG1 is activated in response to osmotic stress, as is p38 (2, 13). The MAPKK that activates HOG1, PBS2, exhibits 59% similarity to its mammalian counterpart MKK3 (8). The MAPKKs that act at the top of the HOG1 cascade, SSK2 and SSK22, exhibit 37% identity to MEKK1 and 42% identity to MEKK4 (10, 29).

Recently, a JNK homolog, DJNK (*basket*), was identified in *Drosophila*, where it was shown to act in a pathway required for the dorsal closure of the embryo (41, 46). A JNKK1 homolog, DJNKK/*hemipterous* (*hep*), which also acts within the dorsal closure pathway, was identified as well (11). Interestingly, the *Drosophila* c-Jun homolog, DJun, is also required for dorsal closure, and it serves as a substrate for DJNK (12a). In addition to its role in development, JNK activity in *Drosophila* can be stimulated by endotoxic lipopolysaccharide, suggesting a role of the DJNK cascade in the insect immune defense system against bacterial infection (46).

To identify the "missing" JNK kinase, we screened ESTs of human genes for sequences that are similar to JNKK1 and DJNKK. One such sequence was identified and used to isolate a corresponding full-length cDNA clone. That cDNA encoded a putative MAPKK with greater similarity to DJNKK than to JNKK1. We expressed this polypeptide in *Escherichia coli* and showed it to be capable of phosphorylation and activation of both JNK1 and JNK2. Based on this function, we named this MAPKK JNKK2. Unlike JNKK1, the specificity of JNKK2 is more restricted, as it can not activate p38. Using both transiently expressed epitope-tagged proteins and antibodies that are specific to endogenous JNKK1 and JNKK2, we found that the two MAPKKs have the ability to respond to similar upstream stimuli but that the actual signaling pathways in which they function may be somewhat different.

## MATERIALS AND METHODS

**Molecular cloning of human JNKK2.** The expressed sequence tag (EST) database at National Center for Biological Information was searched with the text string "JNKK." A 200-bp EST fragment (H85962) was found, which upon translation contained a characteristic MAPKK motif, "YMAPER." Sequencing of the EST clone by a model 373A automated sequencer (Applied Biosystems) revealed a stretch of 500-bp open reading frame encoding putative kinase subdomains VIII, IX, X, and XI and a carboxy-terminal extension with an in-frame stop codon and about 1.5 kb of 3' untranslated region. Primers were designed based on the above-described sequence to amplify this 500-bp fragment by PCR. The PCR fragment was labelled with a random priming kit (Stratagene) and used to screen a human skeletal muscle cDNA library (Stratagene). Two overlapping clones were identified. Both contained complete kinase domains and in-frame stop codons at the 3' ends, but no in-frame stop codons were found at the 5' ends of these two cDNA clones. The EST database was searched again, and a new EST fragment (AA194047 and AA194205) was identified which overlapped with the 5' ends of both clones. Sequencing of this clone revealed an in-frame stop codon 5' to the first ATG.

**Northern blot analysis.** Two commercial nylon membranes (Clontech) prebound with 2  $\mu$ g of gel-separated poly(A)<sup>+</sup> RNA samples isolated from various human tissues were hybridized with a 500-bp PCR fragment spanning the entire 3' end coding region of the *JNKK2* gene. The blots were prehybridized with QuikHyb hybridization solution (Stratagene) for 2 h and then probed with random-primed (Prime-it II; Stratagene) *JNKK2* probe at 65°C for 3 h. The blots were washed twice with 2 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% sodium dodecyl sulfate (SDS) at 60°C and twice with 0.1% $\times$  SSC plus 0.1% SDS at 60°C, respectively, before autoradiography.

**Construction of expression plasmids.** Both *JNKK1* and *JNKK2* cDNAs were subcloned into pcDNA3.1HisC (Invitrogen) between *Bam*HI and *Xho*I sites so that both genes were fused in-frame with an N-terminal Xpress tag. The *JNKK2* cDNA we used here encodes a truncated version of JNKK2 protein which starts with amino acids "PRSM." Hemagglutinin (HA)-tagged JNK and p38 have been described previously (28, 33). Xpress-tagged GCK and ASK were constructed and generously provided by Z. G. Liu and V. Baud. The expression plasmid

coding for human full-length MEKK2 was constructed by B. Su (47a). The expression plasmid coding for the kinase domain of MEKK1 was described previously (33).

**Antibodies.** The monoclonal anti-human JNKK1 antibody (no. 13671A) was generated at PharMingen (La Jolla, Calif.). The polyclonal JNKK2 antibody was raised in rabbit against a carboxy-terminal peptide (KDVMAKTESPRTSG). Anti-Xpress antibody (no. sc-499) was purchased from Santa Cruz Biotechnology, Inc.

**Construction of bacterial expression plasmids and purification of GST fusion proteins.** To make bacterial expression plasmids, we subcloned wild-type *JNKK* cDNAs into pGEX-KT (Pharmacia) between *Bam*HI and *Xho*I sites. The plasmids encoding fusion proteins were transformed into BL21(DE3). Bacterial cells containing glutathione *S*-transferase (GST) fusion proteins were induced at an optical density at 600 nm of 0.6 with 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), and induction was carried out for 3 h at room temperature. GST fusion proteins were purified on glutathione-Sepharose beads. GST-JNK1, GST-p38, GST-cJun(1-79), and GST-ATF2(1-92) were described previously (28).

**Cell cultures and transient transfection.** Both HeLa and 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and grown at 37°C with 5% CO<sub>2</sub>. Transient transfection was performed with Lipofectamine according to the manufacturer's recommendations (Life Technologies, Inc.). After 48 h, the transfected cells were treated with TNF- $\alpha$  (20 ng/ml) for 20 min, IL-1 (10 ng/ml) for 20 min, sorbitol (0.35 M) for 30 min, anisomycin (10  $\mu$ g/ml) for 30 min, UV (40 J/m<sup>2</sup>) followed by incubation at 37°C for 20 min, and EGF (15 ng/ml) for 30 min. For Jurkat cells, the stimuli used were anti-CD3 (10  $\mu$ g/ml), anti-CD28 (2  $\mu$ g/ml), phorbol-12-myristate-13-acetate (PMA) (10 ng/ml), and A23187 (0.5  $\mu$ g/ml). Cells were treated for 30 min before lysis (48). Cells were lysed in the lysis buffer (50 mM HEPES [pH 7.6], 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1.5 mM MgCl<sub>2</sub>, 30 mM *p*-nitrophenylphosphate, 100 mM NaF, 20 mM  $\beta$ -glycerolphosphate, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.1 mM sodium vanadate, and 10- $\mu$ g/ml leupeptin). The concentration of total soluble proteins in the cell extracts was quantitated by Bio-Rad protein assays.

**Protein kinase assays.** For in vitro-coupled kinase assay, 0.5  $\mu$ g of recombinant JNKK was incubated with 0.5  $\mu$ g of recombinant JNK or p38 in 25  $\mu$ l of kinase buffer (20 mM HEPES [pH 7.6], 20 mM  $\beta$ -glycerolphosphate, 10 mM *p*-nitrophenylphosphate, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 50  $\mu$ M sodium vanadate, 10  $\mu$ M ATP, and 10  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP) at 30°C for 20 min. One microgram of GST-cJun(1-79) or GST-ATF2(1-92) was then added along with an additional 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP, and the reactions were continued for another 20 min at 30°C. Reactions were stopped by the addition of concentrated Laemmli loading buffer. For in vivo-coupled kinase assay by immunoprecipitation, antibodies were added to 200  $\mu$ g of soluble cell extracts along with 40  $\mu$ l of 50% protein A-Sepharose slurry, and reactions were rotated at 4°C for 2 h. Protein A beads were washed twice with lysis buffer and twice with kinase buffer. Coupled kinase reactions were then carried out essentially the same way as described above. For kinase reactions with HA-tagged JNK, 12CA5 was added to 50  $\mu$ g of total soluble cell extracts along with 30  $\mu$ l of 50% protein A-Sepharose slurry. Protein A beads were washed twice with lysis buffer and twice with kinase buffer. Kinase reactions were initiated by adding 1  $\mu$ g of GST-cJun(1-79) in 25  $\mu$ l of kinase buffer containing 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. Reactions were carried out at 30°C for 20 min and terminated by addition of concentrated Laemmli loading buffer.

**Western blotting.** Twenty or thirty micrograms of total soluble cell extract was resolved on an SDS-polyacrylamide gel electrophoresis (PAGE) gel (10% polyacrylamide) and transferred to a nylon membrane (Immobilon-P; Millipore) at 350 mA for 4 h or at 150 mA overnight. The subsequent procedures were performed with the enhanced chemiluminescence detection kit (Amersham).

**Nucleotide sequence accession number.** The GenBank accession no. for the complete JNKK2 cDNA sequence is AF014401.

## RESULTS

**Molecular cloning of human JNKK2.** To search for additional JNK kinases, the six known mammalian MAPKKs and DJNKK were aligned with the Clustal W program (50). Although the MAPKKs share sequences that are conserved in all protein kinases (15), they also share certain small motifs which are unique to the MAPKK group. These characteristic motifs are useful indicators for the identification of new MAPKKs. One such motif, YMAPER in kinase subdomain VIII, was previously used to design degenerate primers for cloning new MAPKKs (8, 56). Since GenBank contains an increasingly large number of ESTs, we searched for MAPKK-related ESTs. One EST (H85962) whose conceptual translation contains the sequence YMAPER was thus identified. Blast search revealed that DJNKK (*hep*) and human JNKK1 were the closest sequences related to this EST. The EST clone was ordered and

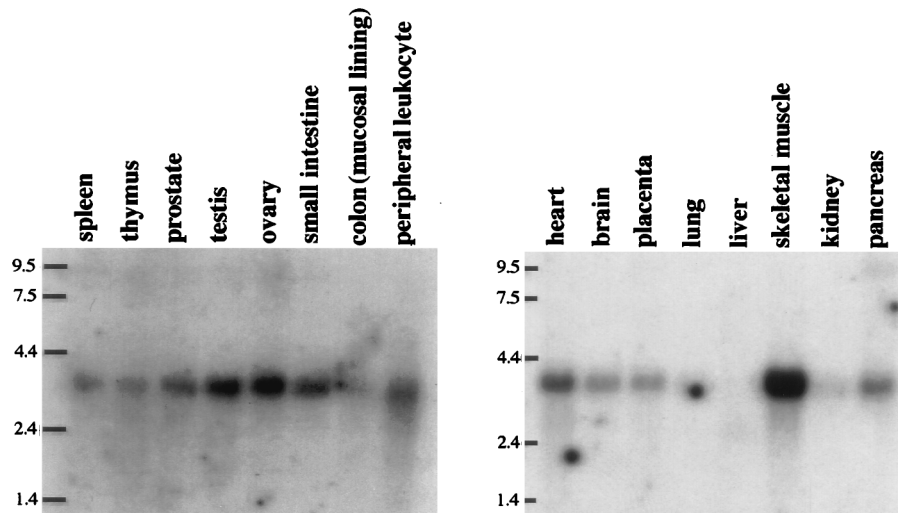


FIG. 1. Tissue distribution of JNKK2 mRNA. Nylon membranes containing 2  $\mu$ g of gel-separated, prebound poly(A)<sup>+</sup> RNA samples from various human tissues (Clontech) were hybridized with a 500-bp cDNA fragment of *JNKK2* labelled by random priming. Size markers (in kilobases) are indicated on the left.

sequenced. It had a 2.0-kb insert containing a 500-bp open reading frame, an in-frame stop codon, and a 1.5-kb 3' untranslated region. The 500-bp coding region contained kinase subdomains VIII, XI, X, and XI. Although it contained motifs that are characteristic of MAPKKs, it was distinct from all known mammalian MAPKKs. Since it was most closely related to DJNKK and human JNKK1, we named this putative MAPKK Jun N-terminal kinase 2, or JNKK2. To study its tissue distribution, the 500-bp coding region was amplified by PCR and used to probe Northern blots of human tissue RNA (Fig. 1). The *JNKK2* probe specifically recognized a 3.8-kb transcript that was ubiquitously expressed. As the JNKK2 was most abundant in skeletal muscle, we screened a human skeletal muscle cDNA library to isolate a full-length cDNA. Sequencing of this cDNA revealed the presence of an open reading frame for a polypeptide of 419 amino acids (Fig. 2A). Database search and multiple sequence alignment confirmed that this polypeptide was indeed a MAPKK that is most closely related to DJNKK (hep) (62% identity, 82% similarity), followed by human JNKK1 (52% identity, 68% similarity) (Fig. 2B).

**Activation of JNK by JNKK2 in vitro and in vivo.** To study the substrate specificity of JNKK2 in vitro, we subcloned the *JNKK2* cDNA into a pGEX vector (Pharmacia Biotech), and this *JNKK2* expression vector was transformed into *E. coli*. Recombinant GST-JNKK2 fusion protein was purified and tested for its ability to phosphorylate and activate JNK1. As shown by a coupled kinase assay, JNKK2, like JNKK1, phosphorylated and activated JNK1, which in turn phosphorylated its substrate GST-cJun(1-79) (Fig. 3A). JNKK2 was as efficient a JNK activator as JNKK1. As a control, JNKK2 did not phosphorylate JNK1(APF), a mutant of JNK1 in which the activating phosphoacceptor sites (Thr183 and Tyr185) were replaced by nonphosphorylatable residues (7). JNKK2 was also an efficient activator of GST-JNK2 in vitro (data not shown). Interestingly, JNKK2 did not phosphorylate and activate p38, while JNKK1 efficiently phosphorylated and activated p38, as measured by the ability of recombinant p38 to phosphorylate ATF2(1-92) (Fig. 3B). Like JNKK1, JNKK2 did not phosphorylate and activate ERK2, while MEK2 phosphorylated and activated ERK2 in vitro (data not shown).

To confirm the above results with JNKK1 and -2 isolated

from human cells, endogenous JNKK1 and -2 were immunoprecipitated from untreated or UV-irradiated HeLa cells with specific antibodies (see details in next section). The kinase activity of the JNKKs was assayed directly by their ability to phosphorylate recombinant MAPKs. As shown in Fig. 3C, activated endogenous JNKK1 from UV-irradiated cells phosphorylated both JNK1 and p38 in vitro, while JNKK2 isolated from UV-irradiated cells phosphorylated JNK1 but not p38. Neither JNKK1 nor JNKK2 phosphorylated ERK2 (data not shown). We also tested whether the kinase activity of transiently transfected JNK or p38 could be potentiated by cotransfected JNKKs. As expected, JNKK1 stimulated the kinase activity of both JNK1 and p38 in a cotransfection experiment, while JNKK2 stimulated only the activity of JNK1 under the same conditions (data not shown). These results indicate that JNKK2 is a specific activator of JNK1 and JNK2 and has negligible activity towards p38. JNKK1, on the other hand, activates both JNK and p38.

**JNKK1 and JNKK2 respond to the same set of extracellular stimuli.** To compare the regulation of JNKK2 to that of JNKK1, each cDNA was subcloned into an Xpress-tagged (Invitrogen) mammalian expression vector. The vectors were transiently transfected into 293 cells that, after 48 h, were either left untreated or exposed to several extracellular stimuli (TNF- $\alpha$ , IL-1 $\beta$ , hyperosmolarity, anisomycin, UV irradiation, and EGF [see Materials and Methods]). After a brief treatment period, the cells were lysed and the tagged JNKKs were isolated by immunoprecipitation with anti-Xpress (Santa Cruz Biotechnology Inc.). The activities of JNKK1 and JNKK2 were measured by a coupled kinase assay (28). While in 293 cells incubation with EGF resulted in a marginal increase in JNKK activity (data not shown), treatment with either TNF or IL-1 resulted in modest stimulation of kinase activity, and exposure to either increased osmolarity (0.35 M sorbitol), anisomycin, or UVC irradiation resulted in substantial JNKK activation (Fig. 4). No difference was observed in the response of either JNKK1 or JNKK2 to these stimuli. Similar results were obtained upon transient expression of the two JNKKs in HeLa cells (data not shown).

To rule out the possibility that these results were due to nonphysiological overexpression of the *JNKK* genes, we directly examined the regulation of endogenous JNKK1 and -2.



**A**  
1 MAASSLEQKL SRLEAKLKQE NRERRRIDL NLDISPQRPR PTLQLPLAND  
51 GGRSPSSSES SPQHPFPTTR PRHMLGLPST LFTPRSMESI EIDQKLQEIM  
101 KQTGYLTIGG QRYQAEINLD ENLGE MGSGT CGQVWKMRFR KTGHVIAVKQ  
151 MRRSGNKEEN KRILMDLDVV LKSHDCPYIV QCFGFTITNT DVFIAMELMG  
201 TCAEKLKERM QGPPIPERILG KMTVAIVKAL YYLKEKHGVI HRDVKPSNIL  
251 LDERGQIKLC DFGISGRGLVD SKAKTRSAGC AAYMAPERID PDPPTKPDYD  
301 IRADVWSLGI SLVELATGQF PYKNCKTDFE VLTKVLQEEP PLLPGHMGFS  
351 GDFQSFLKDC LTKDHRKRPK YNKLEHSFI KRYETLEVDV ASWFKDVMAK  
400 TESPRTSGVL SQPHLPFFR 419

**B**  
JNKK2 .....MAASSLEQKLSRLEAKLKQENR.....EARR.....  
JNKK1 .....MAAPSPSSGGGSGGSGSGTTP.....  
DJNKK .....MSTIEFETIGSRQLSLEAKLQAQNESHDQIVLSGARQPVVSGSVPSARVPPPLATSAS  
MKK3 .....  
MKK6 .....  
MEK1 .....  
MEK2 .....  
MEK5 MLWLAALGPPFAMENQVLVIRIKIPNSGAVDWTVHSGPQLLFRDVLVIGVLP EATTTAF

JNKK2 .....RIDLNLDISQRPRPTLQLPLANDGGSR.....  
JNKK1 .....PVGSPAELGHPALTSMQGKR.....  
DJNKK AATSATHAPSLGASVSVSGSISIAQRPAELVPVPHATLRSPTSSSSSSR SAFRSAAPATGL  
MKK3 .....MRK.....  
MKK6 .....MSQSKGKK.....  
MEK1 .....MPKKKEL.....TPILQLNPPAD.....  
MEK2 .....MLARRKELV.....LPALTINPTIAE.....  
MEK5 EYEDE.....DGDRITVRSDEEMKAMLSYYYSTVMEIQVNGQLIEP.....

JNKK2 .....PSSSESSPQHETPPARPRHMLG.....LPSTLFTPRSMESIETIQKLQEIETKQ  
JNKK1 .....KALKLNFAFPFFKSTARFTLN.....PNPTGVQNPHELRLRTHSIES  
DJNKK RWTYTPPTTRVSRATEFLPLMLSSGGPGDVECTRPVILPLPTPPHPPVSEITDMKIKIIMEQ  
MKK3 .....F.....PAPNPTPPRNLDLSTRTFTITIGD  
MKK6 .....R.....NDELGLKIPKEAFEO.....PQTSSTPPRDLDLSSKACISITIGN  
MEK1 .....GSAVNGTSSAETN.....LEALQKKLLELELELDLQQRKRILEA  
MEK2 .....GRTSPTSEGASEAN.....LVDLQKLELELELDLQQRKRILEA  
MEK5 .....LQIFPRACKRFGERNIHGLKV.....TRAGPSQHSSPAVIDSLPSNQLK

JNKK2 TGYLTIGGQRYQAEINLDLENLQEMOSGTCQVWKMRFRKNGHVIAYKQNRRTSGNKEENK  
JNKK1 SGKLKISPEQHWDFTAEDLKLKDLGELGRGAYGSSVNMVHKKESQGYVAYKRTSTVDEKQK  
DJNKK TGKLNINGRQYPTDINDLKHLDLGLDGNQTSGNVVMHMLSNITIIAYKQNRRTSGNKEENK  
MKK3 .....RN.....FEVADLDLVIISLGGRLAIVGVVEKRVHVAQYVEMARRRATVNSQKQK  
MKK6 .....QN.....FEVADLDELFPMKGGALVGVVEKRVHVAQYVEMARRRATVNSQKQK  
MEK1 FLTQKQ.....KVGBELKDDDFEKLISELGAANGGVYFRVSHKPPESGLIMARKLHLEIKPAIRN  
MEK2 FLTQKA.....KVGBELKDDDFEKLISELGAANGGVYFRVSHKPPESGLIMARKLHLEIKPAIRN  
MEK5 SSAELKKILANGQMGNEQDITRYRDTLGHGNGGTIVYKAYHVFESGKELAYKVI LLDITLELQK

JNKK2 RLLMDDLVDVVKLSSHDCRYLYQCFETITNTIVFLAMELMG-TLAEELKLRMQG--GPTFE  
JNKK1 QLEMLDDEVWKSISDCRYLYQCFETITNTIVFLAMELMG-TLAEELKLRMQG--GPTFE  
DJNKK RLLMDDLVDVVKLSSHDCRYLYQCFETITNTIVFLAMELMG-TLAEELKLRMQG--GPTFE  
MKK3 RLLMDDLVDVVKLSSHDCRYLYQCFETITNTIVFLAMELMG-TLAEELKLRMQG--GPTFE  
MKK6 RLLMDDLVDVVKLSSHDCRYLYQCFETITNTIVFLAMELMG-TLAEELKLRMQG--GPTFE  
MEK1 QIIRLEQLVLEHCNCSFYIVYGFYGAFFSDEGEISICMELHMDGGGLDQVLEKAK--RITFE  
MEK2 QIIRLEQLVLEHCNCSFYIVYGFYGAFFSDEGEISICMELHMDGGGLDQVLEKAK--RITFE  
MEK5 QTIMSELETLVLEHCNCSFYIVYGFYGAFFSDEGEISICMELHMDGGGLDQVLEKAK--RITFE

JNKK2 RLLGKMTYAIYKALVYLKEKHGVIHRDVKPNSNILLDELRGQIKLCDFGISGRLYVDSKAKTR  
JNKK1 ELLGKITLITLAKALNHLKKNLKIHRDVKPNSNILLDRSGNIEKLCDFGISGRLYVDSKAKTR  
DJNKK QILGKVYVATYKALVYLKDKHGVVHRDVKPNSNILLDERGNIKKLCDFGISGRLYVDSKAKTR  
MKK3 DILGKEIAAVSTMRALVHLHSHKLSVHRDVKPNSNILLNKEGHHVMKCDFFGISGRLYVDSKAKTR  
MKK6 DILGKITLITLAKALVYLKDKHGVVHRDVKPNSNILLDRSGNIEKLCDFGISGRLYVDSKAKTR  
MEK1 QILGKVYVATYKALVYLKDKHGVVHRDVKPNSNILLDERGNIKKLCDFGISGRLYVDSKAKTR  
MEK2 ELLGKVYVATYKALVYLKDKHGVVHRDVKPNSNILLDRSGNIEKLCDFGISGRLYVDSKAKTR  
MEK5 HVLGRIAVAVYKALVYLKDKHGVVHRDVKPNSNILLDRSGNIEKLCDFGISGRLYVDSKAKTR

JNKK2 SAGCAAYMAFERIDPPDPTKPPDIDIRADVWSLGLSIVLELATAQGFYKKN.....  
JNKK1 DAGCRLPYMAFERIDP.SASRQCMELIRADVWSLGLSIVLELATAQGFYKKN.....  
DJNKK SAGCAAYMAFERID--PKRKKMIDIRADVWSLGLSIVLELATAQGFYKKN.....  
MKK3 DAGCKPYMAFERINP.ELNOKGYNSYKSDIWSLGLSIVLELATAQGFYKKN.....  
MKK6 DAGCKPYMAFERINP.ELNOKGYNSYKSDIWSLGLSIVLELATAQGFYKKN.....  
MEK1 FVGTRSYMAFERLQG...THYSVQSDIWSMGLSIVLELAVGRYPIPPDAKELELMFG  
MEK2 FVGTRSYMAFERLQG...THYSVQSDIWSMGLSIVLELAVGRYPIPPDAKELELIFGR  
MEK5 YVGTNAYMAFERISG...EQVGIHSYVWSLGLSIVLELAVGRYPIPPDAKELELMFG

JNKK2 .....CKTD FEVLAIKVLEPEFLLEFG--HMGF  
JNKK1 .....WNSVFDQLTQYVKGDDPFLGNSSEEREF  
DJNKK .....CNTDFEVLTKVLDSEFLCLFYGEQYVF  
MKK3 .....WGTPFQQLKQVVEEPPSFLQPAD--RF  
MKK6 .....WGTPFQQLKQVVEEPPSFLQPAD--KF  
MEK1 CQVEGDAAEETP--PRPRTFGRPLSSVGMDSRPMAIFELDTIYVNEPFKTFSG--VF  
MEK2 FVVDGEEGEPHS.SPKRPPGGRPLSSVGHGNDSRPMAIFELDTIYVNEPFKTFSG--VF  
MEK5 .....LQLLQCIYDEDSVLEFVG--EF

JNKK2 SGEFQSFYKDCLETRDHRKRFKYNKLEHSEFTKRYETLEVDVAVSWFKDVMAKTESPRTSG  
JNKK1 SPSFQINFLCLTRDESKRFKYNKLEHSEFTLMYETLEVDVAVSWFKDVMAKTESPRTSG  
DJNKK SPSFQINFLCLTRDHRKRFKYNKLEHSEFTLMYETLEVDVAVSWFKDVMAKTESPRTSG  
MKK3 SPSFQINFLCLTRDHRKRFKYNKLEHSEFTLMYETLEVDVAVSWFKDVMAKTESPRTSG  
MKK6 SAEEFVDFNSOCLKKNKSERPTQLMLQHEEFTLHESKOTDYAVSFLKLLIGD.....  
MEK1 SLEFFQDFNSOCLKKNKSERPTQLMLQHEEFTLHESKOTDYAVSFLKLLIGD.....  
MEK2 TPDFQDFNSOCLKKNKSERPTQLMLQHEEFTLHESKOTDYAVSFLKLLIGD.....  
MEK5 SEPFLVHFIITQCMRKKQKFERPAFELMGEHEFTIVQFN DGNAAVVSMMWVCRALEERRSQGPP

JNKK2 VLSQPHLPFFR  
JNKK1 MYVD.....  
DJNKK APEVT.....  
MKK3 .....  
MKK6 .....  
MEK1 AAGV.....  
MEK2 TAV.....  
MEK5 .....

FIG. 2. Amino acid sequence of JNKK2 and sequence alignment with other MAPKs. (A) The amino acid sequence of JNKK2 deduced from *JNKK2* cDNA sequence is presented in a single-letter code. Roman numerals on top of the amino acid sequence indicate protein kinase subdomains (15). The complete nucleotide sequence is available from GenBank (accession no. AF014401). (B) The amino acid sequence of JNKK2 aligned with those of six other human MAPKs and its closest homolog, DJNKK (hep), with the Clustal W program. Identical residues at similar positions in each MAPK are highlighted with a gray background. Conserved residues are boxed. Gaps are represented by “-” sign.

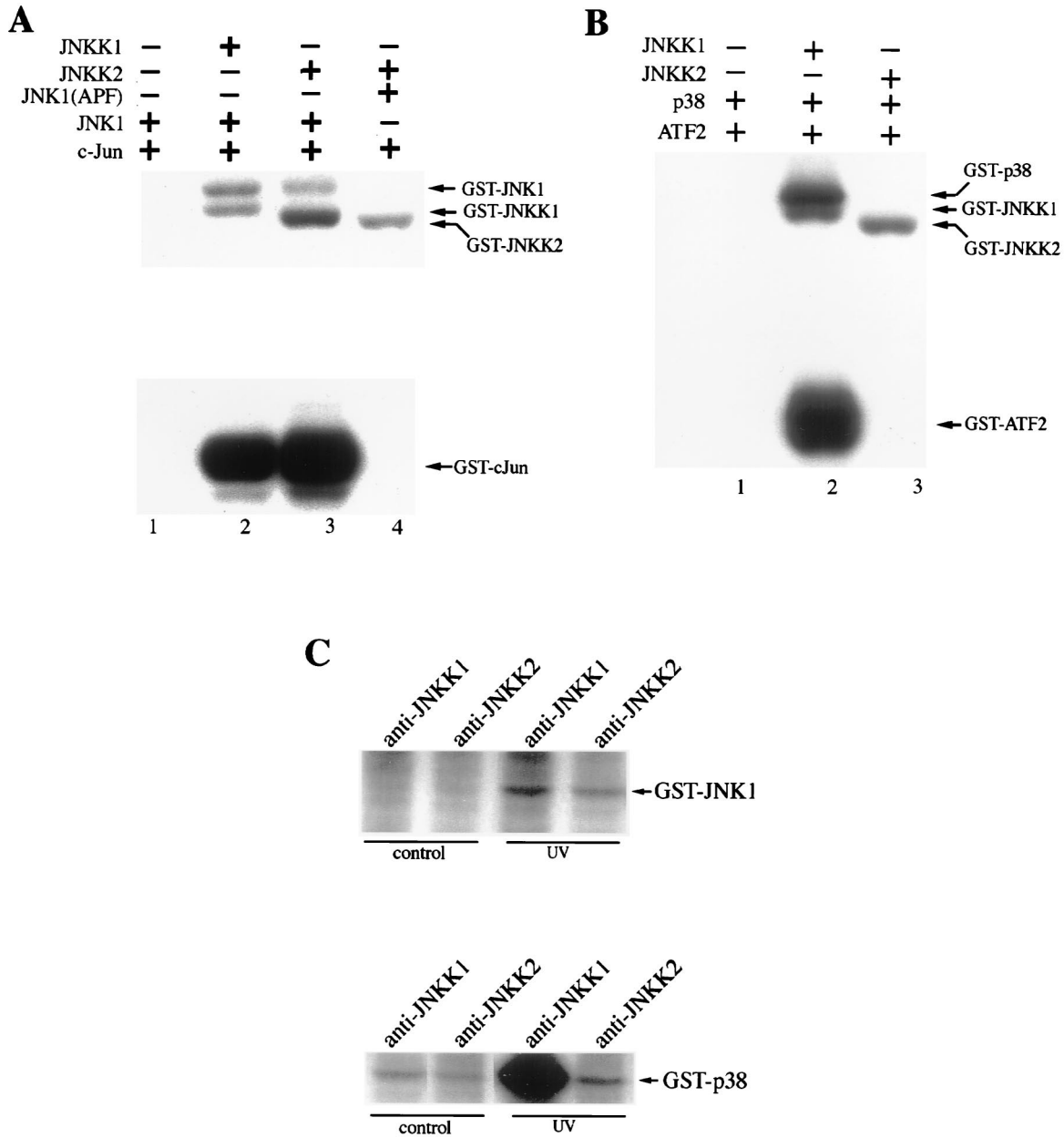


FIG. 3. Phosphorylation and activation of JNK by JNKK2. (A) Recombinant JNK1 (as GST-JNK1) or its mutant form GST-JNK1(APF) was preincubated with recombinant JNKK1 or JNKK2 (as GST fusion proteins) in kinase buffer plus  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , and then JNK activation was assayed by incubation with the JNK substrate GST-cJun(1-79). Direct incubation of GST-JNK1 with GST-cJun(1-79) was used as a control. (B) An experiment similar to that depicted in panel A was performed, except that GST-p38 and GST-ATF2(1-92) were used instead of GST-JNK1 and GST-cJun(1-79), respectively. (C) Endogenous JNKK1 and JNKK2 were immunoprecipitated from extracts of untreated (control) or UV-irradiated HeLa cells. After extensive washing, GST-JNK1 or GST-p38 in kinase buffer was added along with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . The antibodies used are indicated on top of the gel. In all cases, the reactions were separated by SDS-PAGE, and the phosphorylated proteins were visualized by autoradiography.

To do so, we used a previously generated monoclonal anti-JNKK1 antibody (PharMingen) and the polyclonal anti-JNKK2 antibodies raised against a carboxy-terminal peptide of JNKK2. The specificity of these two antibodies is demonstrated in Fig. 5. In panel A, purified recombinant GST-JNKK1 and GST-JNKK2 were resolved on SDS-PAGE and transferred to a nylon membrane, followed by immunoblotting with either JNKK1 or JNKK2 antibodies. It is clear that the JNKK1 antibody specifically recognizes only GST-JNKK1, while the JNKK2 antibody specifically recognizes GST-

JNKK2. In panel B, 293 cell lysates containing transiently transfected HA-tagged JNKK1 or JNKK2 were immunoprecipitated with either JNKK1 antibody, JNKK2 antibody, or preimmune sera in the presence of protein A-Sepharose beads. After extensive washing, anti-HA immunoblotting was carried out. HA-JNKK1 was detected only in the sample precipitated by anti-JNKK1 antisera (lane 3), whereas HA-JNKK2 was detected only in the sample precipitated by anti-JNKK2 antisera (lane 6). No cross-reaction was detected (compare lanes 3 and 4 and lanes 5 and 6). Neither HA-JNKK1 nor HA-JNKK2

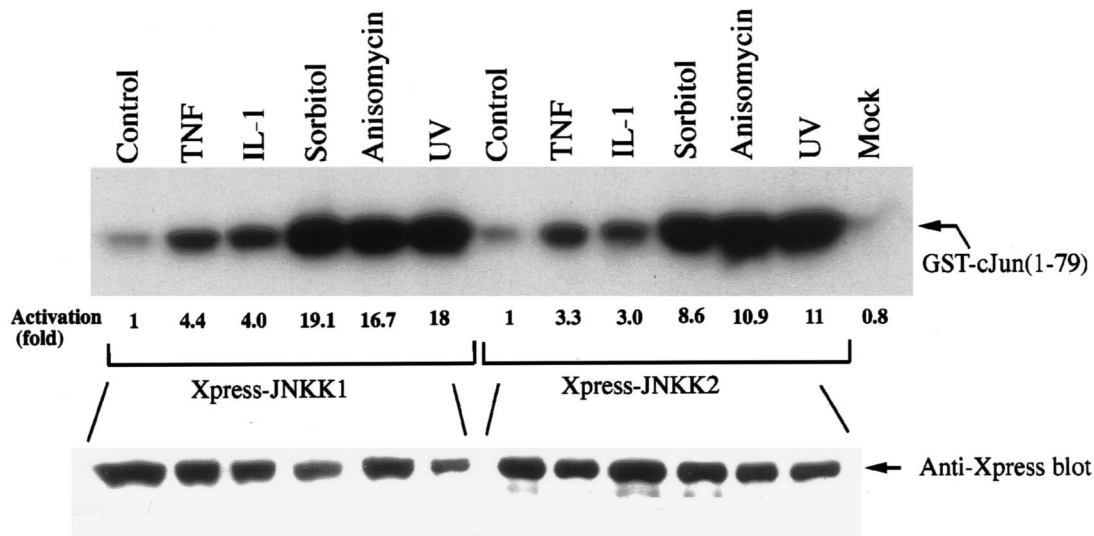


FIG. 4. Activation of transiently transfected JNKK1 and JNKK2 by extracellular stimuli. 293 cells were transfected with expression vectors encoding either Xpress-tagged JNKK1 or Xpress-tagged JNKK2. After 48 h, the cells were exposed to TNF- $\alpha$  (20 ng/ml) and IL-1 (10 ng/ml) for 20 min, sorbitol (0.35 M) and anisomycin (10  $\mu$ g/ml) for 30 min, or UVC (40 J/m<sup>2</sup>), followed by a 20-min incubation at 37°C, after which the cells were lysed. The tagged JNKKs were immunoprecipitated from 200  $\mu$ g of lysate, and their activities were tested with a coupled kinase assay with GST-JNK1 and GST-cJun(1-79) as substrates (see Materials and Methods). Phosphorylation of GST-cJun(1-79) was used as a final readout. One tenth of each lysate (20  $\mu$ g) was analyzed by immunoblotting to ensure that a similar amount of Xpress-tagged JNKKs was used in the kinase assay. Shown is a representative of two independent experiments. The fold activation relative to the basal activity in unstimulated cells transfected with either JNKK1 or JNKK2 was determined by phosphoimager analysis, and the value shown represents the average of two experiments. The standard deviations fall in the range of 10 to 25% of the mean values.

was detected when preimmune sera were used (lanes 7 and 8). The above-described immunoblotting analysis indicated that each antibody recognized only its cognate JNKK, without any detectable cross-reaction with the other JNKK.

HeLa, 293, or Jurkat cells were subjected to various extracellular stimuli, after which the cells were lysed, the endogenous JNKKs were immunoprecipitated, and their activity was measured by a coupled kinase assay. In HeLa cells, JNKK1

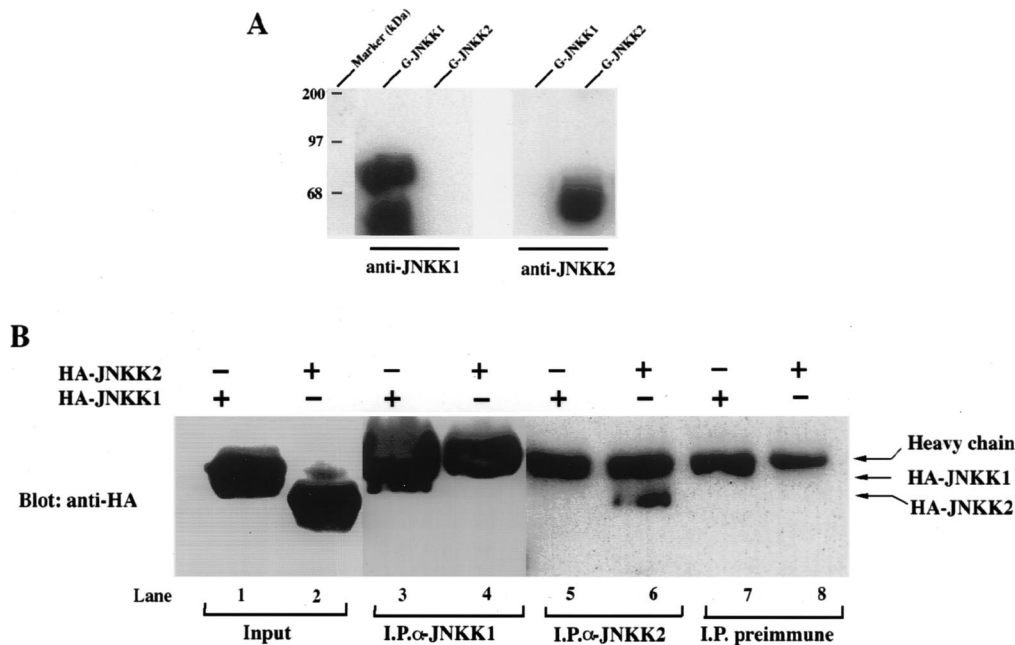


FIG. 5. Specificity of antibodies against JNKK1 and JNKK2. (A) Two identical sets of samples, each containing about 100 ng of recombinant bacterially expressed GST-JNKK1 and GST-JNKK2, were resolved on SDS-PAGE (10% polyacrylamide) and transferred to a nylon membrane. Each half of the membrane containing one set of samples was separately blotted with either JNKK1 or JNKK2 antibodies. Signals were detected with the enhanced chemiluminescence kit. (B) Fifty micrograms of 293 cell extract containing either HA-JNKK1 or HA-JNKK2 was immunoprecipitated with either JNKK1 antibody (lane 3 and 4), JNKK2 antibody (lane 5 and 6), or preimmune sera (lane 7 and 8), followed by extensive washing. Samples were resolved on 10% SDS-PAGE, transferred to a nylon membrane, and immunoblotted with anti-HA antibody (12CA5). Signals were visualized with the Amersham enhanced chemiluminescence kit. Lanes 1 and 2, HA-JNKK1 and HA-JNKK2 input (20  $\mu$ g), respectively.

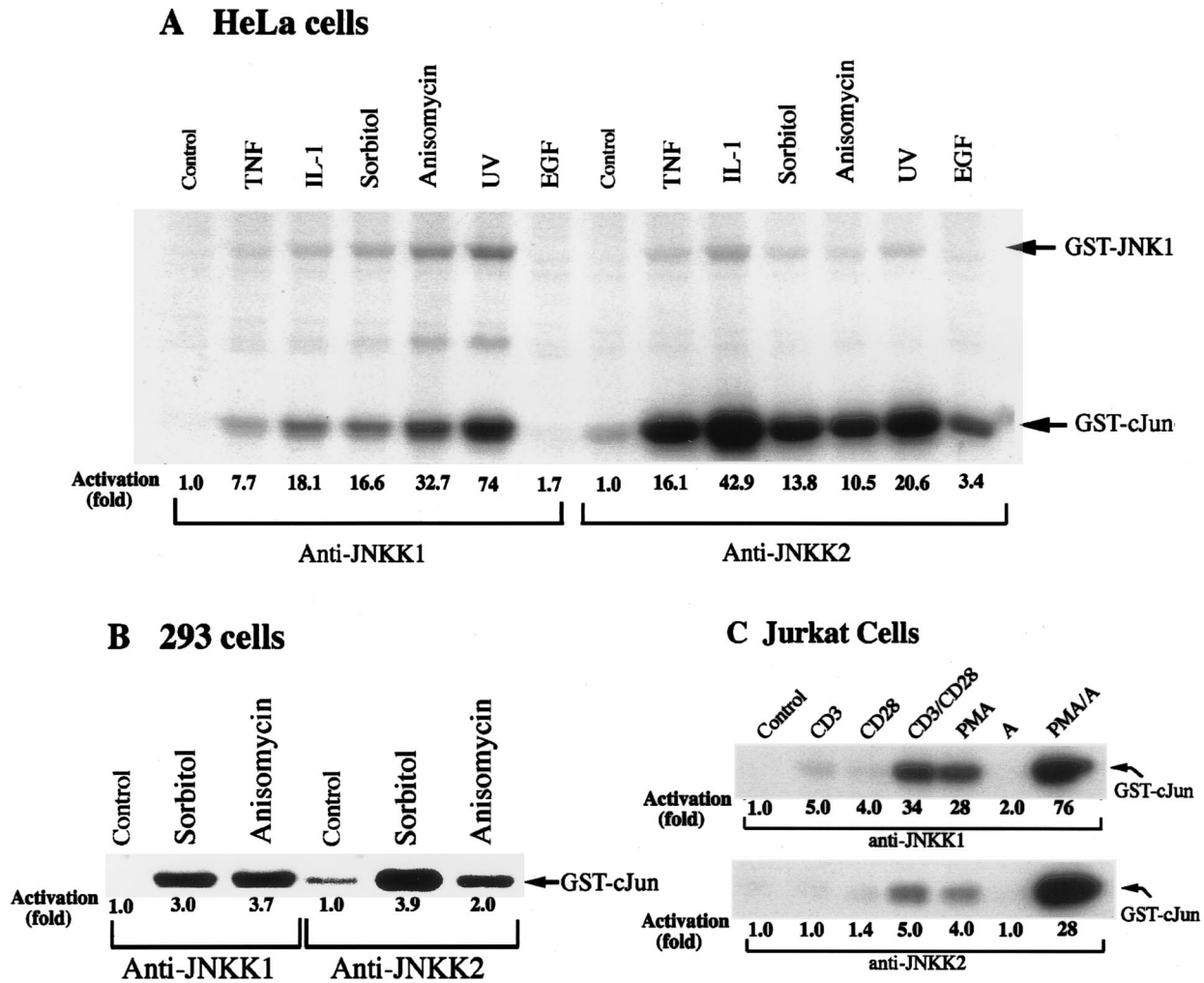


FIG. 6. Activation of endogenous JNKK1 and JNKK2 by extracellular stimuli. (A) HeLa cells were exposed to various extracellular stimuli as indicated (see Materials and Methods). Endogenous JNKK1 and JNKK2 were immunoprecipitated with specific antibodies from 200  $\mu$ g of total cell lysates, and their kinase activity was determined in a coupled kinase assay, with GST-JNK1 and GST-cJun(1-79) as substrates. (B) 293 cells were treated with either 0.35 M sorbitol or 10- $\mu$ g/ml anisomycin for 30 min, after which the cells were lysed and the activities of endogenous JNKK1 and 2 were assayed as described above. (C) Jurkat cells were either nonstimulated (control) or treated with anti-CD3 (10  $\mu$ g/ml), anti-CD28 (2  $\mu$ g/ml), anti-CD3 and anti-CD28, PMA (10 ng/ml), A23187 (0.5  $\mu$ g/ml), PMA and A23187, as previously described (48). After 30 min, the cells were collected and lysed, and JNKK1 and JNKK2 were isolated by immunoprecipitation with specific antibodies. The kinase activity of both JNKKs were determined in a coupled kinase assay as described above. A representative of two experiments is shown. The fold activation relative to that of unstimulated cells is the average of two experiments. The standard deviations range from 8 to 20% of the mean values.

activity was most effectively stimulated by either UVC irradiation or anisomycin treatment, and only modest stimulation was produced by TNF, IL-1, or exposure to high osmolarity (sorbitol), whereas no stimulation of JNKK1 activity was produced by treatment with EGF (Fig. 6A). JNKK2 activity, on the other hand, was most effectively stimulated by TNF, IL-1, and UV irradiation, while a somewhat weaker but still substantial response was produced by osmotic stress, anisomycin, or EGF. In 293 cells, however, due to high basal levels of JNKK activities, the stimuli that consistently resulted in a substantial increase in the activity of either JNKK1 or JNKK2 above the basal level were osmotic stress or anisomycin (Fig. 6B). No difference was observed in the response of the two JNKKs to either anisomycin or osmotic stress. In Jurkat cells, we compared the response of the two JNKKs to stimuli involved in T-cell activation and induction of IL-2 gene expression (48). Apart from the basal activity, which was high for JNKK2, no substantial difference was

observed in the response of the two JNKKs to various T-cell activators (Fig. 6C). As previously shown for JNK activity (48), either no response or a weak response was detected by measuring JNKK activity following stimulation of either the T-cell receptor (with anti-CD3) or the CD28 auxiliary receptor (with anti-CD28). However, costimulation of both receptors resulted in considerable JNKK1 and somewhat more modest JNKK2 activation. The more modest response of JNKK2, however, is most likely due to its high basal activity.

As a negative control, when GST-JNK1 was omitted in the above-described coupled kinase assays, GST-cJun(1-79) was not phosphorylated by immunoprecipitated JNKKs from activated cell extracts (data not shown). This control indicated that endogenous JNKKs were not coimmunoprecipitated with the JNKKs under the assay condition.

**Activation of JNKK2 by upstream kinases.** Next, we sought to determine which of several MAPKKs reported to be ca-



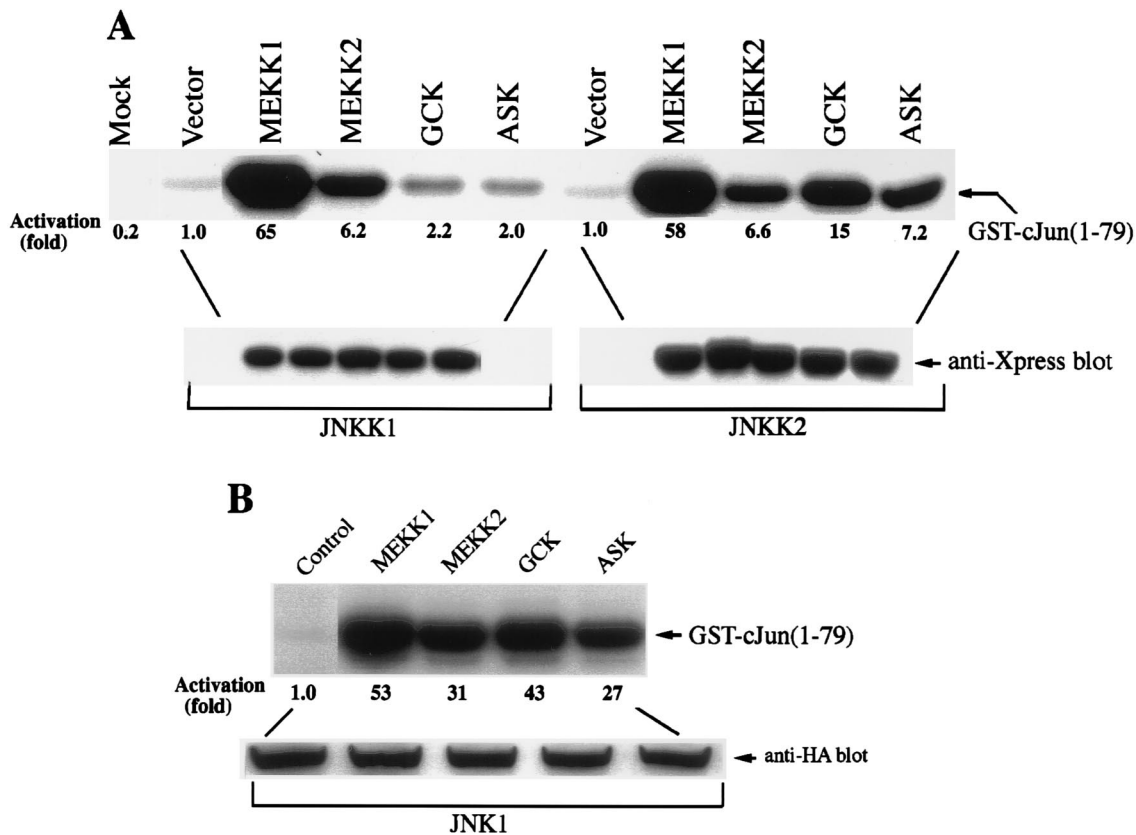


FIG. 7. Selective activation of JNKK1 and JNKK2 by upstream kinases. (A) 293 cells were cotransfected with 0.5  $\mu$ g of expression vectors encoding either an empty vector or the indicated upstream kinases and 1.5  $\mu$ g of an expression vector encoding Xpress-tagged JNKK1 or JNKK2. After 48 h, the transfected cells were lysed and JNKK1 and JNKK2 were immunoprecipitated with anti-Xpress. JNKK activity was determined in a coupled kinase assay, with GST-JNKK1 and GST-cJun(1-79) as substrates. (B) The experiment was carried out essentially the same as that described in panel A, except HA-JNK1 was used to substitute for Xpress-tagged JNKKs. HA-JNK1 was immunoprecipitated with antibody 12CA5, and its kinase activity was determined by direct phosphorylation of GST-cJun(1-79). One-tenth of each lysate was analyzed by immunoblotting to examine the expression levels of various epitope-tagged kinases. Shown is a representative of three experiments. The fold activation relative to that of cells cotransfected with an empty vector and an expression vector encoding either JNKK1, JNKK2, or JNK1 is the average of three experiments. The standard deviations vary between 10 to 20% of the mean values.

pable of activation of the JNK cascade can activate JNKK2. The Xpress-tagged *JNKK1* or *JNKK2* expression vectors were transiently cotransfected into 293 cells along with expression vectors for either *MEKK1*, *MEKK2*, *GCK*, or *ASK*. The tagged JNKKs were immunoprecipitated with anti-Xpress, and their activity was measured by a coupled kinase assay. As shown in Fig. 7A, MEKK1 was the most potent JNKK1 activator, followed by MEKK2. GCK and ASK, on the other hand, led to only a small increase in JNKK1 activity. MEKK1 was also the most potent JNKK2 activator, but in this case, GCK and ASK stimulated JNKK2 as efficiently as MEKK2 did. Each of these MAPKKs efficiently stimulated JNK1 kinase activity under the same cotransfection condition, in agreement with previous reports (Fig. 7B) (19, 28, 33, 39).

## DISCUSSION

Previous studies led to the identification of the JNK-activating MAPKK, JNKK1/SEK1/MKK4 (8, 28, 44). JNKK1 also phosphorylates and activates p38 at least as efficiently as it activates the JNKs (8, 28). Following the molecular characterization of JNKK1/SEK1/MKK4, biochemical (32, 36) and genetic (37, 55) analyses of JNK activation have strongly suggested the existence of at least one more JNK-activating kinase. Most importantly, inactivation of the *JNKK1/SEK1/*

*MKK4* locus by homologous recombination and derivation of T cells that are homozygous for these mutations indicated that JNK activity was no longer responsive to anisomycin or heat shock treatment, while the response to osmotic shock or UV irradiation was almost fully retained (37, 55). We therefore searched for a MAPKK that could be the missing JNK-activating kinase. The experiments described above indicate that the MAPKK we identified, JNKK2, is indeed a JNK-activating kinase. Furthermore, JNKK2 is more specific than JNKK1; while JNKK1 activates both JNK and p38, JNKK2 activates only the JNKs. This specificity is demonstrated by the results of two experiments: first, recombinant JNKK2 activates only JNK1 and JNK2 but not p38; second, JNKK2 immunoprecipitated from UV-irradiated HeLa cells activates JNK1 and JNK2, whereas JNKK1 isolated from the same cells can activate both JNKs and p38 when they are tested *in vitro*. Before we submitted this work, Tournier et al. (51a) published a study about the molecular cloning of MKK7, a murine homolog of JNKK2. Like human JNKK2 described here, MKK7 specifically phosphorylates and activates JNK but not p38.

It is interesting that JNKK2 is more closely related to DJNKK, the *Drosophila* JNK-activating kinase (11), than JNKK1 is (82% versus 68% similarity, respectively). Genetic analysis indicates that like DJNK (41, 46), DJNKK functions in dorsal closure (11) and the phenotype of the DJNKK loss-of-



function mutation is strikingly similar to that of DJNK loss of function (41, 45). While one of the major roles of the JNK cascade in *Drosophila* appears to be in dorsal closure of the embryo, the exact cellular and biological function of the JNK cascade in mammals remains to be identified. Unfortunately, loss of the JNKK1/SEK1/MKK4 function results in early embryonic lethality in mice, restricting the analysis of its functions to mosaic animals (37, 55). So far, the consequences of loss of JNKK1 function were described only for ES cells and T cells. In *SEK1*<sup>-/-</sup> ES cells, JNK activation was abolished only in response to anisomycin, heat shock, or MEKK1 cotransfection, while JNK activation by UV irradiation or hyperosmotic shock was not compromised (37, 55). The latter result is not difficult to comprehend considering the existence of JNKK2 which may substitute for JNKK1 to activate JNKs in response to upstream stimuli. However, if this is the case, it is puzzling that the loss of JNKK1 resulted in almost complete loss of the response to anisomycin and MEKK1, as both JNKK1 and JNKK2 appear to be equally responsive to these stimuli, at least in human cell lines. One possible explanation is that the regulation of JNKK2 activity and its ability to activate JNK in response to various upstream stimuli is cell-type specific, due to the specific organization of individual components within this signaling cascade. Although JNKK2 is activated substantially in response to anisomycin or MEKK1 in 293 and HeLa cells, it may not respond well to these stimuli in ES cells, suggesting that JNKK2 and JNKK1 may be differentially regulated by upstream activators in different cell types. Another possibility is that JNKK2 is not efficiently expressed in ES cells and that another unidentified JNKK, which does not respond to anisomycin or MEKK1, is expressed in these cells. Further work is needed to clarify this issue.

Based on transient transfection experiments and on analysis of the endogenous enzymes in several cell lines, it appears that, while JNKK1 and JNKK2 can be responsive to the same set of extracellular stimuli, the exact signaling pathways in which they function may vary from one cell type to another. Thus, in HeLa cells, endogenous JNKK2 is considerably more responsive to EGF, IL-1, and TNF than JNKK1 is, whereas in the Jurkat T-cell line, the two JNKKs exhibit very similar responsiveness to T-cell activators, such as antibodies that activate the TCR and the auxiliary CD28 receptor or PMA plus Ca<sup>2+</sup> ionophore.

Loss of the JNKK1/SEK1 function was found to cause a reduction in the number of CD4<sup>+</sup>CD8<sup>+</sup> double-positive immature thymocytes, to lead to increased sensitivity to induction of thymocyte cell death by activation of either CD95 (Fas) or CD3 (TCR), and to cause decreased proliferation and IL-2 production in T cells in response to CD28 costimulation and PMA/Ca<sup>2+</sup> ionophore activation (38). The decreased but not completely abolished T-cell proliferation and IL-2 production could be very well explained by our result that both JNKK1 and JNKK2 are activated in response to CD3/CD28 costimulation or PMA/Ca<sup>2+</sup> ionophore activation (Fig. 6C). Loss of JNKK1 does not affect JNKK2-mediated JNK activation which, together with other signals, can still lead to increased AP-1 activity and increased IL-2 production (48). It was also noted that JNK activation by PMA plus Ca<sup>2+</sup> ionophore in immature thymocytes from *SEK1*<sup>-/-</sup> mice was completely abolished, whereas JNK activation in peripheral T cells from *SEK1*<sup>-/-</sup> mice was as good as that in wild-type mice (38). A possible explanation for this result is that JNKK1 is expressed throughout T-cell development, whereas JNKK2 expression in T cells is developmentally controlled; it may not be expressed in immature thymocytes but is expressed in mature peripheral T cells. Further investigation is under way to address this issue.

The exact molecular basis for these differences is not yet

clear, but it is likely to be caused at least in part by differential expression of upstream activators, including MAPKKKs. For example, we find that JNKK2 is more responsive to the upstream kinases ASK and GCK than JNKK1 is, whereas both JNKKs exhibit similar responsiveness to MEKK1 or MEKK2. The variation in the relative responsiveness of JNKK1 and JNKK2 from one cell type to another is of considerable interest and serves as yet another indication of the existence of a higher-order subcellular organization of MAPK cascades (34). Future efforts will be targeted toward understanding the molecular basis for this functional organization.

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