

# MAPKKK6, a Novel Mitogen-Activated Protein Kinase Kinase Kinase, That Associates with MAPKKK5

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**MAPKKK5/ASK1 activates c-Jun N-terminal kinase (JNK) and p38 kinase signaling pathways and induces apoptosis when expressed in stably transfected cells. Using MAPKKK5 as bait in yeast two-hybrid screening, a novel protein that interacts with MAPKKK5 was identified and cloned. This novel protein is predicted to contain all 11 kinase subdomains and shares 45% amino acid identity with MAPKKK5 and thus is designated MAPKKK6. The interaction of MAPKKK6 with MAPKKK5 *in vivo* was confirmed by coexpression of MAPKKK5 and MAPKKK6 in 293 cells followed by immunoprecipitation. In contrast to MAPKKK5, which activated both JNK and p38 kinase pathways, MAPKKK6 only weakly activated JNK but not ERK or p38 kinase pathways.** © 1998 Academic Press

Mitogen-activated protein kinase (MAPK) cascades consist of a three-kinase module: MAPK, MAPK kinase (MKK), and MKK kinase (MAPKKK or MEKK) (reviewed in 1–3). These kinase cascades relay signals from cell surface to nucleus resulting in gene transcription. Growth factors and mitogens activate the extracellular regulated kinase (ERK) pathway (4), while stress stimuli and proinflammatory cytokines activate two closely related but distinct parallel pathways, c-Jun N-terminal kinase pathway (JNK) or stress-activated kinase pathway (SAPK) and p38 kinase pathways (5, 6).

Multiple upstream kinases (MAP kinase kinases or MAPKKKs), that activate the ERK, JNK and p38 kinase signaling pathways, have been identified. MEKK1 and MEKK4 preferably activated JNK when expressed at physiological levels (7–9). MEKK2, MEKK3, and Tpl-2 activated both JNK and ERKs but not p38 kinase pathway (10–12). MAPKKK5/ASK1,

TAK1, MLK3/SPRK, and MUK/DLK were shown to activate both p38 and JNK pathways (13–21). We reported previously the cloning of a new member of the MAPKKK family kinases, MAPKKK5 (13), also independently cloned as ASK1 (14); MAPKKK5/ASK1 activated JNK and p38 kinase pathways in 293 cells, but not the ERK pathway (13, 14). Recombinant MAPKKK5/ASK1 could directly phosphorylate MKK4, MKK3, and MKK6 *in vitro*, leading to their activation, indicating that MAPKKK5/ASK1 was an upstream activator in the JNK and p38 kinase pathways. Furthermore, MAPKKK5/ASK1 induced apoptosis when overexpressed in stably transfected cells and its catalytic inactive form inhibits TNF- $\alpha$ -induced apoptosis (14). The upstream activators of MAPKKK5/ASK1 remain unknown.

In an effort to elucidate the mechanisms by which MAPKKK5/ASK1 are regulated, we undertook studies to identify proteins that bind to MAPKKK5/ASK1 using the yeast two-hybrid system. We report here the isolation of a novel cDNA encoding a protein kinase that specifically interacts with MAPKKK5.

## MATERIALS AND METHODS

*Plasmid construction for the yeast two-hybrid system and yeast two-hybrid library screen.* Oligonucleotide primers were used in PCRs to amplify a 1371-bp DNA fragment encoding amino acids 648 to 1374 containing the kinase subdomains and the C-terminus of MAPKKK5 using MAPKKK5 cDNA as template. The primers used were 5'-AGCTGAGTCGACTGGTGAACACCATTACCGAAGAGA-3' and 5'-AGCTGAGTCGACGAAGATTAGATTGAGCAACAGTC-3'. PCR products were cut with *SaI* restriction enzyme and cloned into the *SaI* site of plasmid pGBT9 (ClonTech) to create an in-frame fusion with GAL4 DNA binding domain gene. The cloning junction was sequenced to confirm the fusion. The pGBT9-MAPKKK5 was transformed into yeast strain HF7c using the lithium acetate procedure and plated onto synthetic complete (SC) media lacking tryptophan. Plasmid DNA from HeLa cell cDNA library (ClonTech) was then transformed into the yeast strain containing the MAPKKK5 bait plasmid and plated on SC medium minus tryptophan, leucine, and histidine and grown at 30°C for 3–5 days. Transformants were assayed for  $\beta$ -galactosidase activity. Plasmid DNA was recovered by transformation into DH10B cells and sequenced on both strands.

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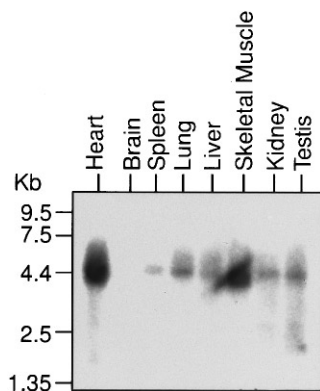
1	.....MAGPCPRSGAERAGSCWQDPLAVALSRGRQLAAPPGRCGA.....RSRPLSVVYVLTRE										54
1	MSTEADegITFSVPPFPAPSGFCTIPEGGICRRGGAAAVGEGEEHQLPPPPPGSFVNWVESAAAPGIGCPAATSSSSATRGSSVGGSSRRITVAVVINEA										100
55	PQGLEPREGTEAEPLRLCLREACAQVPRPRPPQLRSLPFGTLELGDTAALDAFYNADVVLVLEVSSSLVQPSLFYHLGVRESFSMTNNVLLCSQADLP										154
101	SQQQL.....VVAESEAQLSLREACTV.....GATLETLHFGLKDFGETTVLDRFYNADIAVEMSDAFRQPSLFYHLGVRESFSMANNIILYCDTNSD										190
155	DLQALREDVFKNSDCVGSYTLIPYVVTATGRVLCGDAGLLRGLADGL.VQAGVGTEALLTPLVGRLARLEATPTDSCGYFRETIRRDQARERFSGP										253
191	SLQSLKEITCQNTMCTGNFTVPMITPHNKVYCCDSSFMKGLTELMQPNFELLGLPICLPVDRFIQLLKVQAQASSQYFRESILINDIRKARNLYTGK										290
254	QLRQELARLQRRLDSEVLLSPDIIMNLLSYRDVQDYSAIIELVETLQALPTCDVAEQHNVCFFHYTFALNRRNRPGRDRAKALSVLLPLVQLEGSVAPDLY										353
291	ELAAELARIRQVDNIEVLTDIVINLLSYRDIQDYDSIVKLVELEKLPFTDLASHHHVKPHYAFALNRRNLPGRDRAKALDIMPMVQSEGVASDMY										390
354	CMCGRIYKDMFFSSGQDAGHREQAYHWYRKAFDVEPSLSHGINAAVLLIAAGQHFEDSKELRLIGMKLGCLLARKGCKVEKMQYVWDVGFYLGAIILAND										453
391	CLVGRYIKDMFLDSNFTDES RDHGASWFKKAFESPTLQSGINAYVLLLAAGHQFESSFELRKVGKLSLLGKGNLEKLSQVWEVGFPLGASVILAND										490
454	PTQVVLAAEQLYKLNAPIWYLVSMETFLLYQHF.RPTPEPPGPPRRRAHFWLHFLQSCQPFKTAQAQDQCLVLVLEMNKVLLPAKLEVRGTDVPVSTV										552
491	HMRVIAQASEKLFKLTAPWYLSIVETILYKHFVKLTTEQPVAKQELVDFWMDPLVEATKTDVTVV...RFPVLILEPTKIYQPSYLSINNEVEEKTI										586
553	TSLLEPETQDIPSSWTFPVASICVGSASKRDERCCFYALPPAQDVQLCFPSVGHGQWFCGLIQAQWVTPNDSTAPAEAEAGAGEMLEFDYEYETETGERL										652
587	SIWHVLPDDKKGIHEWNFSASSVRGVSSISKFEERCCFLYVLHNSDDFQIYFCTELHCKKFFEMVN..TITEEKGSTEBGDCESDLLEYDYEDENGDRV										684
653	VLGKGTGYGVYAGRDRHTRVRIAIKEIPERDSRFSQPLHHEEIALHRLRHKNIVRYLGSASQGGYKIFMEEVPGGSLSSLLRSVWGPLKDNESTISFYT										752
685	VLGKGTGYGVYAGRDLNSQVRIAIKEIPERDSRYSQPLHHEEIALHKLKHKNIVQYLSFSSENGFIKIFMEQVPGGSLSALLRSKWGPLKDNEQTIGFYT										784
753	RQILQGLGYLHNDHIVHRDIKGNVLIINTFSGLLKISDFGTSKRLAGITPCTETFTGTGLQYMAPEIIDQGRPYGKAADIWSLGTCTVIEMATGRPPFHEL										852
785	KQILEGLKYLHNDHIVHRDIKGNVLIINTYSGVLKISDFGTSKRLAGINPCTETFTGTGLQYMAPEIIDKGRPYGKAADIWSLGTCTVIEMATGKPPFYEL										884
853	GSPQAAMFQVMGYKVPMPSSLSAEAAQALLRTFEPDPRLRASAQTLGLDPPFLQPG...KRSRSPSSPRHAPRPSDAPSAS..PTPSANSTTQSQTFFCP										948
885	GEPQAAMFQVMGPKVHPEIPESMSAEAKAFILKCFEPDPKRAACANDLLVDEFLKVSSKKKKTKPKLSALSAGSNEYLRISISLPVPLVEDTSSSSEYGS										984
949	QAPSQH...PPSPPKRCLSYG.....GTSQLRVPEEPAEPEASP...EESGSLSLHQSERRAMLAALVEQELPALAENLHQEQK.EQGARLGR										1033
985	VSPDTELKVDPFVFKTRAKSCGERDVKGIRTLFLGIPDENFEDHSAPPSPPEEKDSGFFMLRKDSERRATLHRLITEDQDKIVRNLMESLAQGAEPKILKW										1084
1034	NHVEELLRLCLGAHIHTPNRRQLAQELRALQGRRLAQGLPALHRLFAFPDAVKQILRKQIRPHWMFVLDLSLRAVRAALGVLGPEVE.....KEA										1127
1085	EHITTLIASLREFVRSTDRKIIATTLTKLKLLEDLDFSHGISQVQVVLFGQDAVNKVLNRHNIKPHWMFALDSIIKRAVQTATITLVPBLRPHFSLASES										1184
1128	VSPRSEELSNEGDSQQSPGQQ.....SPLPVEPEQGPAPLMVQLSLLRAETDRLREILAGKEREYQALVQALQRLNEEARTY										1205
1185	DTADQEDLDVEDDHEEQPSNQTVRRPQAVIEDAVATSGVSTLSSTVSHDSQSAHRSNLVQLGRMKIETNRLLEELVRKEKELQALLHRAIEEKDQEIHL										1284
1206	VLAPPEP.....PTALSTDQGLVQWLQELNVDSGTIQMLLNHSFTLHTLLTYATRDLLIYTRIRGGMVCRIRWRAILAQAGSTPTVTSGP*										1289
1285	KLKSQPIEIPELPVFHLNSSGNTNDESELTDWLRVNGAEDTISRFLAEDYTLLDVLVYVTRDDLKCLRLRGMLCTLWKAIDFNKQT*.....										1375

	I	II	III	IV	V				
MAPKKK6	EtGerLvLGk	GtyGvVYagr	DrhTrvriAi	Kei.....p	erDSrfsQpL	hEEIaLhrrL	rHkNivYrLG	sa..sqggYL	KIFMEevPGG
MAPKKK5	EngdrvvLGk	GtyGiVYagr	DlnsqvriAi	Kei.....p	erDSrfsQpL	hEEIaLhKhL	KHkNivYrLG	sf..sengfi	KIFMEqvPGG
MEKK4	kwgrgnkiGe	GqyGkVYtCi	sVDTGelmAm	KeirF....q	PnDhrtiket	adelkifegi	KHpNlVrYfG	velhreE..m	yIFMEYcdeG
MEKK3	rrgKLLlLGq	GafGRVYlCy	DVDTGrelAs	KqvQF.gPdS	PBTsKEVsaL	ecEIqLlKnL	qHerIVqYyG	clRDraEKilL	tIFMEYmPGG
MEKK2	rlgKLLlLGq	GafGRVYlCy	DVDTGrelAv	KqvQF.nPeS	PBTsKEVsaL	ecEIqLlKnL	lHerIVqYyG	clRDpQyEktL	sIFMElsPGG
MEKK1	BwlKgqgiGL	GafsscyQaq	DVgTGtlmAv	KqytyvrrntS	sEgeevVeAL	rEEIrmghL	nHpNlrmLG	atceksn..y	nlFIeWmaGG
Con.	B-GK-L-LG-	GA-GRVY-C-	DVDTG---A-	K--QF--P-S	PEDSKEVQAL	-EEI-L-K-L	KH-NIV-YLG	-LRD--E--L	KIFMEY-PGG

	VI	VII	VIII	IX	X	XI			
MAPKKK6	SLssLLRSvW	GpLKDNEStI	sfyTRQILqG	LgYLHdNhiv	HRDIKdGNvL	IntfsGLlKi	sDFGTsKRlA	gItPct....	BtfTGTlqYM
MAPKKK5	SLsaLLRSkV	GpLKDNEStI	gfYtKQILEG	LkYLHdNgIV	HRDIKdGNvL	IntysGvLKi	sDFGTsKRlA	gInPct....	BtfTGTlqYM
MEKK4	tLeevsR.l..	.gqL..EhVI	RlytKQItva	invLHehgIV	HRDIKGANif	ltSsGl.iKl	GDFGcSvKlK	maqtmpgev	nSTlGTaayM
MEKK3	SvkDqLk.aY	GALT..ESVt	RkYTRQILEG	mSYLHsNmIV	HRDIKGANIL	rDSaG.NvKl	GDFGASKRLq	tICm.sGTGi	rSVtGTpywM
MEKK2	SikDqLk.aY	GALT..EnVt	RkYTRQILEG	vHYLHsNmIV	HRDIKGANIL	rDSTG.NiKl	GDFGASKRLq	tICl.sGTGm	ksVtGTpywM
MEKK1	SvahLLs.kY	GAFK..ESVv	inYtQLlRG	LSYLHnEqIi	HRDvKGANIL	IDSTGqrLri	aDFGAaaraL	skgtgaGefq	gqllGTiafM
Con.	SL-DLLRS-Y	GALKDNESVI	R-YTRQILEG	LSYLH-N-IV	HRDIKGANIL	IDSTGGNLK-	GDFGASKRLA	-ICPC-GTG-	ES-TGT--YM

	VIII	IX	X	XI					
MAPKKK6	APEiIdGqP.	rGYGKAADIW	SLGCTVIEMA	TGrPPfHelg	spQ..AAMFq	vgmYkvhPpm	PSsLSaEaqa	FLLRtFEpDP	rLRASaqtLL
MAPKKK5	APEiIdGqP.	rGYGKAADIW	SLGCTVIEMA	TGKPPfyelg	EpQ..AAMFk	vgmYkvhPei	PesmsSaEaka	FiLkCFepDP	dkRacAndLL
MEKK4	APEVitrakG	EgHGRAADIW	SLGCVVIEMV	TGKRPPWH.Ey	E..hngfinkY	vg.mghkPPI	PerLSpEgka	FLshCLIESD	kiRwtAsqLL
MEKK3	sPEVI...sG	EGYGRKADVW	SLGCTVvEMl	TeKPPW.Aef	Ea..MAAIFK	iatQpTnPkL	PSHLSeghRd	F.Lkrifvea	rqRPsaEELL
MEKK2	sPEVI...sG	EGYGRKADIW	SvaCTVvEMl	TeKPPW.Aef	Ea..MAAIFK	iatQpTnPkL	PhHvSdytRd	F.Lkrifvea	klRPSAEELL
MEKK1	APEVl...rG	qqYGRScDvW	SvGCaiIEMA	caKPPWnaEk	hsnhlAlifK	iasatTaPsi	PSHLSpglRd	vavRCLElqP	qdrPpsrELL
Con.	APEVID-G-G	EGYGRAADIW	SLGCTVIEMA	TGKPPWHAEE	E-Q-MAA-FK	---Q-T-PPI	PSHLS-E-R-	FLLRC-E-DP	-LRPSAEELL

**FIG. 1.** Amino acid sequence comparisons. (Top) The amino acid sequence of MAPKKK6 was aligned with MAPKKK5 using the GAP program of the University of Wisconsin Genetics Computation Group. (Bottom) The putative catalytic domain of MAPKKK6 was aligned and compared with the catalytic domains of MAPKKK5, MEKK1, -2, -3, and -4 using the PILEUP and PRETTY program of the University of Wisconsin Genetics Computation Group. The kinase subdomains are indicated with Roman numerals. The conserved residues are in capital letters, while the nonconserved amino acid residues are shown in lowercase letters. The consensus sequence is also shown.



**FIG. 2.** Tissue distribution of MAPKKK6 mRNA. A tissue blot containing poly(A)<sup>+</sup> RNA from the indicated tissues was hybridized with radioactive MAPKKK6 probes as described under Materials and Methods. RNA size markers are shown on the left.

**cDNA library screening and Northern blot analysis of MAPKKK6.** A human skeletal muscle cDNA library in  $\lambda$ ZAPII phage vector was purchased from Stratagene. Replicate filters were prehybridized for 1 h at 68°C in ExpressHyb (Clontech) and hybridized overnight in the same solution to a 700-bp probe labeled with [ $\alpha$ -<sup>32</sup>P]dCTP. After hybridization, the filters were washed twice (2 × 30 min) in 0.1 × SSC/0.1% SDS at 55°C. Positive clones were isolated and sequenced on both strands. Filters containing poly(A)<sup>+</sup> RNA (2 mg per lane) from various tissues were purchased from Clontech. Filters were probed with a MAPKKK6 probe corresponding to the C-terminal coding region. Hybridization was performed at 68°C in Express Hybridization Buffer (Clontech) followed by three washings in 0.1 × SSC, 0.1% SDS at 55°C. Blots were exposed for 24 h at -70°C.

**Plasmid construction and expression.** Oligonucleotide primers were used in PCRs to amplify MAPKKK6. The primers added a FLAG epitope tag sequence at the 5' end. The PCR product was cloned into the mammalian expression vector pCR3.1 (Invitrogen). A catalytically inactive mutant of MAPKKK6 was created by substituting lysine 677 with a glutamic acid (K677E mutant) by site-directed mutagenesis using the overlapping PCR method as described (22). 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 50 U/ml penicillin, 50 mg/ml streptomycin. Cell transfection and harvesting were as previously described (23).

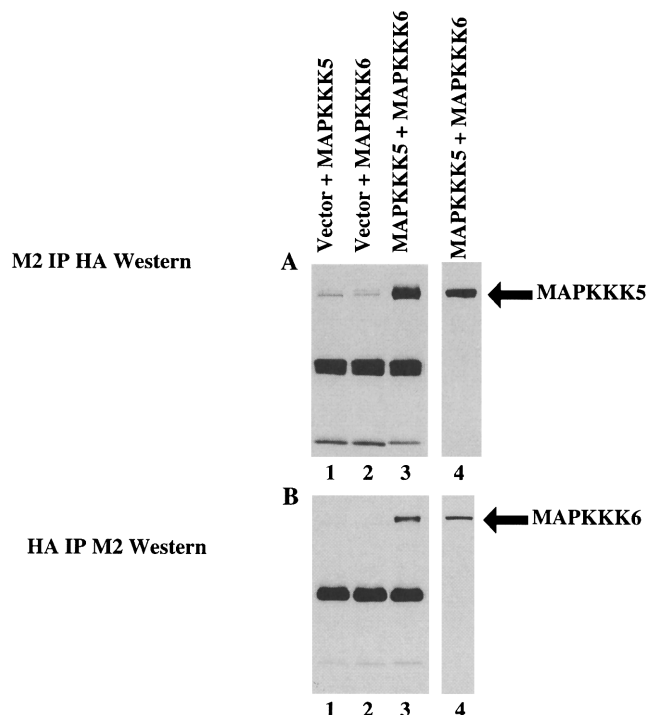
**Immunoprecipitation and Western blot analysis.** Cell Lysates containing 100  $\mu$ g of protein were immunoprecipitated with 5  $\mu$ g of anti-HA monoclonal antibody (mAb) 12CA5 (Berkeley Antibody Co.) or the anti-FLAG M2 mAb (Sigma) and protein A-Sepharose CL-4B beads (Pharmacia Biotech). For Western blot analysis, lysates containing equal amounts of total protein were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and electroblotted onto nitrocellulose membranes. The blots were then probed with mAb M2 or 12CA5, followed by biotinylated rabbit anti-mouse IgG and developed using the enhanced chemiluminescence detection system (Amersham Life Science Inc.).

**JNK/ERK/p38 kinase immunocomplex protein kinase assay.** Lysates from cells cotransfected with MAPKKK6 and HA tagged JNK1, Erk2, or p38 kinase plasmids were prepared and recombinant protein was immunoprecipitated with mAb 12CA5 and protein A beads. Beads were washed 3 times with lysis buffer and once with kinase buffer (25 mM Hepes, pH 7.4, 25 mM  $\beta$ -glycerophosphate, 25 mM MgCl<sub>2</sub>, 25 mM DTT, and 0.1 mM Na<sub>3</sub>VO<sub>4</sub>) and resuspended in 40  $\mu$ l of the same kinase buffer. The beads were then incubated with human GST c-Jun (1-169), PHAS-I or ATF-2 peptides and 1  $\mu$ l of [ $\gamma$ -<sup>32</sup>P]ATP at 30°C for 30 min. Reaction mixtures were then terminated and phosphorylated proteins were analyzed by SDS-PAGE.

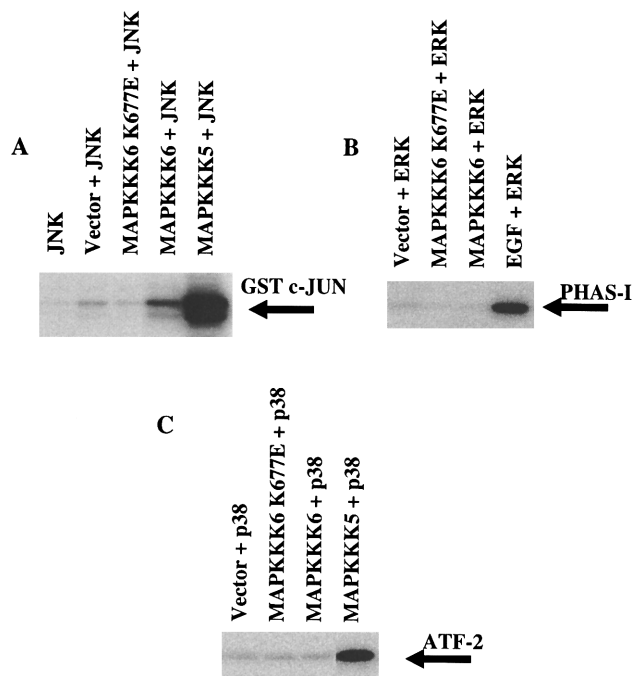
## RESULTS AND DISCUSSION

**Molecular cloning of MAPKKK6.** A yeast two-hybrid screen was used to identify proteins that interact with MAPKKK5. Several clones were identified and sequenced. One such clone termed Y11 appeared to contain novel cDNA sequence and was further characterized. Y11 was then transformed into yeast strain HF7c along with either MAPKKK5 bait plasmid or several other bait plasmids. Y11 was found specifically to interact with the kinase and c-terminal domain of MAPKKK5 but not with other kinases such as MKK6 or GLK (data not shown).

To isolate a full-length cDNA clone of Y11, we probed a human skeletal muscle cDNA library, a human heart cDNA library and a human brain library with the Y11 sequence. Several clones were isolated and sequenced and all found to contain overlapping sequences. The longest clone (5.0 kb) from a human skeletal muscle cDNA library contained a full-length cDNA sequence (Fig. 1). It was predicted to encode an open reading frame of 1280 amino acids. This novel cDNA was predicted to encode a novel serine/threonine kinase with high sequence homology to MAPKKK5 and thus we



**FIG. 3.** Interaction of MAPKKK6 with MAPKKK5 in 293 cells. A. 293 cells were cotransfected with vector and HA tagged MAPKKK5 (lane 1), vector and FLAG tagged MAPKKK6 (lane 2), HA tagged MAPKKK5 and FLAG tagged MAPKKK6 (lanes 3 and 4). Lysates were immunoprecipitated with anti-FLAG (A, lanes 1 to 3) or anti-HA (B, lane 1 to 3) mAb and protein A-Sepharose or not immunoprecipitated (lane 4). Proteins were separated on SDS-PAGE and transfected onto nitrocellulose membranes. Membranes were then blotted with either an HA mAb (A) or a FLAG mAb (B).



**FIG. 4.** JNK, ERK, and p38 kinase activities in cells transfected with MAPKKK6. 293 cells were transfected with HA epitope-tagged JNK1, or cotransfected with vector, MAPKKK6 K677E, MAPKKK6, or MAPKKK5 plus HA epitope-tagged JNK1. The cells were collected 48 h later, and immunocomplex kinase assays were performed with anti-HA mAb, using GST c-Jun (1–79) as substrate. (B). 293 cells were transfected with vector, MAPKKK6 K677E, MAPKKK6, plus HA epitope-tagged ERK. Cells in the last lane were stimulated with 5 ng/ml of EGF for 15 min. Immunocomplex kinase assays were performed with anti-HA mAb, using PHAS-I as substrate. (C) 293 cells were transfected with vector, MAPKKK6 K677E, MAPKKK6, or MAPKKK5 plus HA epitope-tagged p38 kinase. Immunocomplex kinase assays were performed with anti-HA mAb using ATF-2 (1–96) as substrate.

termed this novel cDNA MAPKKK6. Overall, MAPKKK6 is 45% homologous to MAPKKK5 as aligned by GAP program of GCG (Fig. 3A). The kinase domain of MAPKKK6 is 82% identical to that of MAPKKK5 at amino acid level. In addition to its homology to MAPKKK5, the putative catalytic domain of MAPKKK6 shares 37, 42, 43, and 42% amino acid identity to the kinase domains of MEKK1, MEKK2, MEKK3, and MEKK4 (Fig. 1). The expression of the MAPKKK6 was examined in a variety of human tissues by Northern blot analysis. The MAPKKK6 probe hybridized to a single species of mRNA of approximately 5.0 kb (Fig. 2). Among the tissues examined, strong hybridizing signals were observed in human heart and skeletal muscle. Weaker signals were detected in human lung, liver, kidney, testis, and spleen (Fig. 2).

**MAPKKK5 associates with MAPKKK6 in transfected 293 cells.** To determine whether MAPKKK5 could associate with MAPKKK6 in mammalian cells, 293 cells were cotransfected with an HA epitope tagged

MAPKKK5 and a FLAG epitope tagged MAPKKK6. Cells were lysed and MAPKKK6 was first immunoprecipitated with mAb to the FLAG epitope and then immunoblotted with mAb to HA epitope. Alternatively, MAPKKK5 was first immunoprecipitated with the HA mAb and then immunoblotted with FLAG mAb. In both cases, MAPKKK5 coprecipitated with MAPKKK6, indicating that MAPKKK5 associated with MAPKKK6 in cotransfected cells (Fig. 3).

**JNK, ERK, and p38 kinase activities in cells transfected with MAPKKK6.** To determine whether MAPKKK6 could activate JNK activity, 293 cells were cotransfected with mammalian expression vectors encoding portions of MAPKKK6 and an HA epitope tagged JNK. Recombinant JNK was then immunoprecipitated from cell lysates and used in a protein kinase assay with GST c-Jun protein as a substrate. As shown in Fig. 4A, transfection with MAPKKK6 resulted in three- to fourfold activation of JNK. Transfection with a kinase inactive form of MAPKKK6, in which lysine 677 in the ATP binding domain was mutated to a glutamic acid, resulted in no activation of JNK, indicating that the kinase activity of MAPKKK6 is required for the activation of JNK. Western blot analysis showed that JNK was expressed in comparable levels in all lanes (data not shown).

To determine whether MAPKKK6 could also function to activate the ERK and p38 kinase, 293 cells were transiently transfected with MAPKKK6 along with HA epitope-tagged ERK2 or p38 kinase. ERK2 or p38 kinase was then immunoprecipitated and its activity was assayed in immunoprecipitates using PHAS-I or ATF-2 peptide as substrate. Addition of EGF to 293 cells strongly activated ERK2 (Fig. 4B) and transfection of cells with MAPKKK5 activated p38 kinase. However, no increase in ERK2 or p38 kinase activity was observed when MAPKKK6 was overexpressed in 293 cells (Figs. 4B and 4C). Western blot analysis confirmed that both MAPKKK6, ERK2 and p38 kinase were expressed in the transfected 293 cells (data not shown). These data suggest that MAPKKK6 may not play a role in the MAPK/ERK or p38 kinase pathway.

## ACKNOWLEDGMENTS

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