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Cloning of rat MEK kinase 1 cDNA reveals an endogenous membrane-associated 195-kDa protein with a large regulatory domain

(mitogen-activated protein kinase pathways/membrane-associated protein kinase/pH domain/cysteine-rich region/proline-rich region)

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ABSTRACT **The coding sequence of rat MEK kinase 1 (MEKK1) has been determined from multiple, independent cDNA clones. The cDNA is full-length based on the presence of stop codons in all three reading frames of the 5′ untranslated region. Probes from the 5′ and the 3′ coding sequences both hybridize to a 7-kb mRNA. The open reading frame is 4.5 kb and predicts a protein with molecular mass of 161,225 Da, which is twice the size of the previously published MEKK1 sequence and reveals 801 amino acids of novel coding sequence. The novel sequence contains two putative pH domains, two proline-rich regions, and a cysteine-rich region. Antisera to peptides derived from this new sequence recognize an endogenous protein in human and rodent cells of 195 kDa, consistent with the size of the expressed rat MEKK1 clone. Endogenous and recombinant rat MEKK1 are enriched in membranes; little of either is found in soluble fractions. Expression of recombinant rat MEKK1 leads to activation of three mitogen-activated protein kinase modules in the order c-Jun N-terminal kinase/stress-activated protein kinase > p38 mitogen-activated protein kinase = extracellular signal-regulated kinase 2.**

The mitogen-activated protein/extracellular signal-regulated kinase (MAP/ERK) kinase pathway, a ubiquitous growth factor-activated protein kinase cascade, is involved in diverse processes ranging from the serum-dependent transcription of c-*fos* to the activation of phospholipase A_2 by epidermal growth factor (for reviews, see refs. 1 and 2). Efforts to elucidate the regulation of this pathway intensified when it was discovered that activation of the cascade by growth factors was dependent on Ras (3–5). Ras binds to isoforms of the protein kinase Raf, causing Raf to associate with membranes where it is activated by phosphorylation (6–9). Targeting Raf to membranes artificially is sufficient to activate it partially in fibroblasts (10). Once activated, Raf phosphorylates and activates MAP/ERK kinase (MEK) 1 and 2, the activators of the MAP kinases ERK1 and ERK2 (11–13).

Budding yeast have multiple MAP kinase pathways but apparently lack Raf-like kinases. Instead, the MEK activators in the yeast pathways are enzymes of which STE11, an enzyme in the pheromone response pathway, is the prototype (14, 15). Using a PCR strategy, Lange-Carter *et al.* (16) cloned a mammalian relative of STE11 that they named MEK kinase 1 (MEKK1) because it increased the activity of MEK1 in cells that expressed it. The mouse MEKK1 cDNA contained an ORF encoding 686 amino acids with 441 bp of putative 5′ untranslated region (UTR). Cotransfection of MEKK1 with c-Jun-N-terminal kinase/stress-activated protein kinase (JNK/SAPK) dramatically increased the activity of JNK/SAPK to phosphorylate c-Jun (17, 18). MEKK1 had a much smaller effect on the activity of cotransfected ERK2, in spite of a potent activation of MEK1 and MEK2 (19). These observations have led to the idea that MEKK1 may regulate the JNK/SAPK pathway but not the MAP kinase pathway.

We report the isolation of rat MEKK1 cDNA clones encoding an ORF of 1493 aa. Recombinant rat MEKK1 is the same size as the largest immunoreactive form of endogenous MEKK1, ≈195 kDa, in human and rodent cells. Expression of rat MEKK1 most potently activates JNK/SAPK, but at higher concentrations rat MEKK1 leads to increased activity of both ERK2 and p38 MAP kinase.

MATERIALS AND METHODS

Cell Culture, Transfection, and Fractionation. Cells were grown and transfected as described (19). Whole cell lysates were prepared by lysis in 1× sodium dodecyl sulfate (SDS) electrophoresis sample buffer. Nondenatured, detergent lysates were prepared in 50 mM Hepes (pH 7.5), 0.15 M NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.1 M NaF, 1 mM Na_3VO_4, and 10 μg/ml each of leupeptin, pepstatin-A, and aprotinin. For subcellular fractionation, cells in 60-mm dishes were washed once with phosphate-buffered saline and lysed in buffer A (137 mM NaCl/8.1 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.2/2.7 mM KCl/2.5 mM EDTA/2 μg/ml aprotinin/2 μg/ml leupeptin) by Dounce homogenization. Intact nuclei were removed by two low-speed centrifugations at 370 × *g* for 10 min at 4°C. Cytosol was separated from membranes by centrifugation of the supernatant at 174,000 × *g* for 1 h. Membranes were washed once with buffer A and resuspended in 1 ml of lysis buffer (16). Material pelleted at low speed was combined, washed, and resuspended in buffer A, then Dounce homogenized, loaded on 50% sucrose, and centrifuged at 1600 × *g* for 10 min. The pellet containing nuclei was washed with buffer A and resuspended in 1 ml of lysis buffer (16). The fractions containing cytosol, membranes, and nuclei were mixed 1:1 with 2× electrophoresis sample buffer and 10 μl of each fraction was analyzed.

Antibodies and Immunoblotting. A monoclonal antibody to the hemagglutinin (HA) epitope (12CA5) was from Babco (Richmond, CA). Antibodies were prepared exactly as described (20) to four peptides shown in Fig. 1 that span the

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Abbreviations: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEKK1, MEK kinase 1; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; UTR, untranslated region; HA, hemagglutinin; GST, glutathione *S*-transferase.
Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (U48596).
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   1 MAAAAGDRAS SSGFPGAAAA SPEAGGGGGA LQGSGAPAAG AGLLRETGSA
  51 GREPADWRRQ QLRKVRSVEL DQLPEQPLFL TASPPCPSTS PSPEPADAAA
 101 GASGFQPAAG PPPPGAASRC GSHSAELAAA RDSGARSPAG AEPPSAAAPS
 151 GREMENKETL KGLHKMDDRP EERMIREKLK ATCMPAWKHE WLERRNRRGP
 201 VVVKPIPIKG DGSEMSNLAA ELQGEGQAGS AAPAPKGRRS PSPGSSPSGR
 251 SGKPESPGVR RKRVSPVPFQ SGRITPPRRA PSPDGFSPYS PEETSRRVNK
 301 VMRARLYLLQ QIGPNSFLIG GDSPDNKYRV FIGPQNCSCG RGTFCIHLLF
 351 VMLRVFQLEP SDPMLWRKTL KNFEVESLFQ KYHSRRSSRI KAPSRNTIQK
 401 FVSRMSNCHT LSSSSTSTSS SENSIKDEEE QMCPICLLGM LDEESLTVCE
 451 DGCRNKLHHH CMSIWAEECR RNREPLICPL CRSKWRSHDF YSHELSSPVD
 501 SPTSLRGVQQ PSSPQQPVAG SQRRNQESNF NLTHYGTQQI PPAYKDLAEP
 551 WIQAFGMELV GCLFSRNWNV REMALRRLSH DVSGALLLAN GESTGTSGGG
 601 SGGSLSAGAA SGSSQPSISG DVVEAFCSVL SIVCADPVYK VYVAALKTLR
 651 AMLVYTPCHS LAERIKLQRL LRPVVDTILV KCADANSRTS QLSISTLLEL
 701 CKGQAGELAV GREILKAGSI GVGGVDYVLS CILGNQAESN NWQELLGRLC
 751 LIDRLLLEIS AEFYPHIVST DVSQAEPVEI RYKKLLSLLA FALQSIDNSH
 801 SMVGKLSRRI YLSSARMVTT VPPLFSKLVT MLSASGSSHF ARMRRRLMAI
 851 ADEVEIAEVI QLGSEDTLDG QQDSSQALAP PRYPESSSLE HTAHVEKTGK
 901 GLKATRLSAS SEDISDRLAG VSVGLPSSAT TEQPKPTVQT KGRPHSQCLN
 951 SSPLSPPQLM FPAISAPCSS APSVPAGSVT DASKHRPRAF VPCKIPSASP
1001 QTQRKFSLQF QRTCSENRDS EKLSPVFTQS RPPPSSNIHR AKASRPVPGS
1051 TSKLGDASKN SMTLDLNSAS QCDDSFGSGS NSGSAVIPSE ETAFTPAEDK
1101 CRLDVNPELN SSIEDLLEAS MPSSDTTVTF KSEVAVLSPE KAESDDTYKD
1151 DVNHNQKCKE KMEAEEEEAL AIAMAMSASQ DALPIVPQLQ VENGEDIIII
1201 QQDTPETLPG HTKANEPYRE DTEWLKGQQI GLGAFSSCYQ AQDVGTGTLM
1251 AVKQVTYVRN TSSEQEEVVE ALREEIRMMS HLNHPNIIRM LGATCEKSNY
1301 NLFIEWMAGA SVAHLLSKYG AFKESVVINY TEQLLRGLSY LHENQIIHRD
1351 VKGANLLIDS TGQRLRIADF GAAARLASKG TGAGEFQGQL LGTIAFMAPE
1401 VLRGQQYGRS CDVWSVGCAI IEMACAKPPW NAEKHSNHLA LIFKIASATT
1451 APSIPSHLSP GLRDVALRCL ELQPQDRPPS RELLKHPVFR TTW*
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FIG. 1. The rat MEKK1 cDNA contains an ORF encoding a protein of 1493 amino acids. The amino acid sequence is shown. The rat MEKK1 ORF contains 801 residues of novel coding sequence N-terminal to the initiatior methionine (box) reported for the mouse MEKK1 cDNA clone (16). Four peptide sequences (underlined) were used to raise antibodies beginning with the most N terminal: N795, L613, L614, and M329 (near C terminus of kinase domain). Space restrictions prevented inclusion of the nucleotide sequence (Gen Bank accession no. U48596). The circled D is mutated to A in the D1369A MEKK1 mutant.

MEKK1 sequence. The N795 antibodies were affinity purified as described (21); the N795 peptide antigen is less than 40% identical to any sequence in the GenBank data base. For detection of endogenous MEKK1, 100–200 μg of each whole cell or nondenatured detergent lysate was loaded onto 6% polyacrylamide gels in SDS. For overexpressed MEKK1, 20 μg of whole cell lysates were loaded. Gels were transferred onto nitrocellulose (Schleicher & Schuell) and developed using chemiluminescent detection (ECL; Amersham).

Protein Kinase Assays. The activities of cotransfected ERK2, JNK/SAPK, or p38 were determined in 12CA5 immunoprecipitates (19) using myelin basic protein, glutathione *S*-transferase (GST)-Jun, or GST-ATF2, respectively, as substrates in 50 μl of 10 mM Hepes (pH 8.0), 10 mM $MgCl_2$, 1 mM benzamidine, 1 mM dithiothreitol, 50 μM ATP (1 cpm per fmol) at 30°C for 60 min.

RESULTS

Isolation of cDNA Clones Encoding MEKK1. Multiple independent and overlapping clones encoding rat MEKK1 were isolated (Fig. 2). Four of the clones that were obtained from a size-selected, oligo(dT)-primed cDNA library contained inserts of over 4 kb, which included both N-terminal and catalytic domain sequence. Alignment of the cDNA clones yielded an ORF of 4.5 kb (Fig. 1), which included 2.5 kb of coding sequence not present in the mouse MEKK1 cDNA sequence. The C-terminal half of the rat sequence is 94% identical in predicted amino acid sequence and 90% identical in nucleotide sequence to the available mouse sequence, consistent with their being derived from the same gene from each species. The coding and 5′ and 3′ UTRs from the clones that contain the longest 5′ and 3′ regions together account for over 6.6 kb, close to the size of the MEKK1 mRNA (see below). Several cDNA clones contained the predicted initiator methionine. The longest 5′ cDNA contained upstream stop codons in all three frames, consistent with a full-length cDNA clone. The coding sequence predicts a protein of 1493 amino acids and 161,225 Da (Fig. 1). However, the recombinant and endogenous proteins both migrate at ≈195 kDa (see below).

Isolation of MEKK1 Genomic Clones. Two clones were isolated from a rat genomic DNA library with a probe from the 5′ end of the expected ORF. Both clones hybridized to the putative 5′ UTR of the cDNA (Fig. 3), confirming the authenticity of the most 5′ cDNA clone, which contained the stop codons.

Northern Blot Analysis of MEKK1. To obtain additional evidence that the 5′ sequences and those encoding the catalytic domain were from the same transcript, probes from the 3′ and 5′ ends of the rat ORF were hybridized to a Northern blot of poly(A)$^+$ RNA isolated from several rat tissues. Both probes hybridized to an mRNA of the same size, ≈7 kb (Fig. 4), that was most highly expressed in spleen, kidney, and lung. Both probes revealed a similar pattern of expression of the mRNA in the tissues examined, further suggesting that the probes hybridized to the same mRNA.

Expression and Immunoblotting Analysis of MEKK1. Recombinant rat MEKK1 expressed in 293 cells appeared as a group of proteins ranging from 195 to 200 kDa (Fig. 5 *A* and *C*). The same proteins were recognized by antibodies generated to several synthetic peptides including ones near its N and C termini (Fig. 5*A*). Unlike the wild-type protein, the catalytically defective mutant D1369A MEKK1 appeared as a single major species of slightly greater mobility than wild-type MEKK1 (Fig. 5*C*). The absence of the larger bands suggests that the wild-type protein undergoes a self-catalyzed modification that decreases its electrophoretic mobility. An affinity purified antibody to the N-terminal peptide also recognized a protein of ≈195 kDa in extracts of human 293, mouse RAW 264.7, Chinese hamster ovary, and rat PC12 cells (Fig. 5 *B* and *C*), the same size as the smallest band generated from MEKK1 and the predominant band from D1369A MEKK1. The recognition of this protein and additional smaller bands was blocked by preincubating the immune IgG with the antigenic peptide.

Localization of MEKK1 and Fragments. The localization of endogenous MEKK1, full-length recombinant rat MEKK1, and a catalytic domain fragment were examined by subcellular fractionation. The catalytic domain fragment was found predominantly in soluble and, to a lesser extent, in membrane fractions (data not shown). In contrast, both recombinant, full-length MEKK1 and the endogenous enzyme were absent from the soluble fraction, and were instead in the postnuclear, high-speed particulate fraction (Fig. 6). This suggested that the N-terminal domains of MEKK1 strengthen its association with membranes.

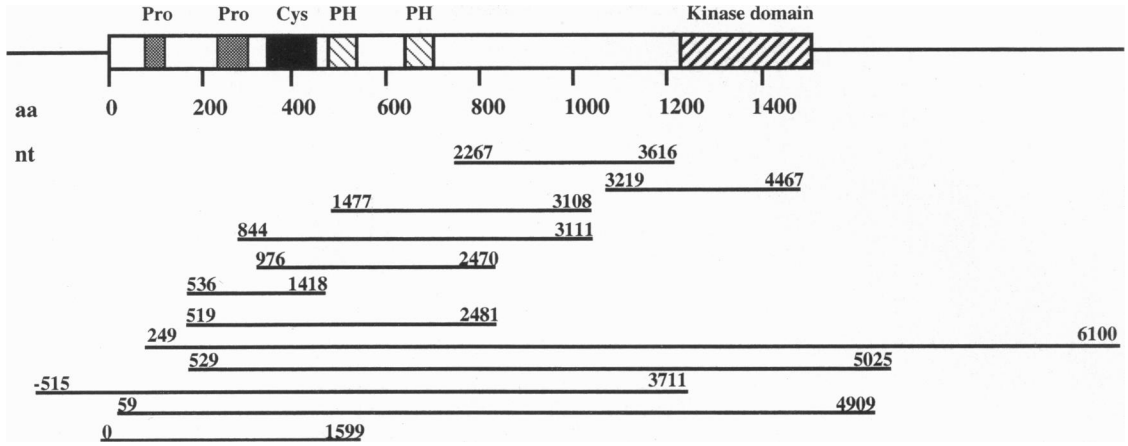

FIG. 2. Domain organization (*Upper*) and partial list of rat MEKK1 cDNA clones (*Lower*). The kinase domain is at the C terminus; N terminal to the kinase domain are two proline-rich regions (Pro) (residues 74–149 and 233–291) with multiple repeats of the form $PXPX_3P$, a cysteine-rich region (Cys), and two potential pleckstrin homology (PH) domains (residues 439–555 and 643–750). A nucleotide binding motif (not shown) is located at the beginning of the second proline-rich region. Four overlapping clones (one of which is shown; 3219–4467 nt) were isolated from an oligo(dT)-primed rat brain cDNA library (Stratagene) using 845 nt from mouse MEKK1 3′ coding sequence. A 5′ oligonucleotide probe from the above clone hybridized to a cDNA spanning 2267–3616 nt from the same library. This clone was used to screen a random-primed rat forebrain cDNA library (from J. Boulter) yielding nine independent clones containing an additional 565 amino acids of 5′ sequence. A 350-nt DNA fragment derived from 5′ rapid amplification of cDNA ends was isolated and used to screen a size-selected, oligo(dT)-primed rat brain cDNA library (also from J. Boulter). One of four independent clones included the initiator methionine and over 500 nt of 5′ UTR, with stop codons in all three reading frames. Using a 5′ probe from this sequence, multiple overlapping cDNA clones were isolated from two additional rat brain libraries. Several contained the initiator methionine as well as a small number of nt immediately upstream.

Activation of MAP Kinase Modules by MEKK1. Cotransfection of rat MEKK1 with JNK/SAPK, ERK2, and p38 caused activation of all three downstream protein kinases, assessed by phosphorylation of c-Jun, myelin basic protein, and ATF2, respectively (Fig. 7). If amounts of transfected MEKK1 cDNA were reduced, only JNK/SAPK was activated, indicating that MEKK1 most potently activates that pathway. Nevertheless, MEKK1 increased ERK2 and p38 activities by 2- to 5-fold.

DISCUSSION

Multiple independent overlapping cDNA clones and two genomic clones encoding rat MEKK1 revealed an ORF of 1493 amino acids. Hybridization of two genomic clones with probes corresponding to the 5′ coding region and 5′ UTR, Northern blot analyses with 5′ and 3′ cDNA probes, and immunoblots of the endogenous protein from multiple cell types support the conclusion that the complete coding sequence is represented in rat MEKK1.

Immunoblotting with an anti-peptide antibody shows that the largest form of endogenous MEKK1 in cultured cells from human and rodent cell types is 195 kDa. The C-terminal half of the rat MEKK1 sequence and the available mouse MEKK1 sequence are 94% identical and an antibody to an 18 residue peptide near the N terminus of MEKK1 detected proteins of similar size not only in rat, but also in mouse, hamster, and human cells, indicating that MEKK1 is highly conserved across species. The high degree of conservation suggests the newly identified sequence possesses important regulatory functions. In this regard, the cysteine-rich and proline-rich regions and the potential pleckstrin homology domains (24, 25) are candidates for roles in MEKK1 regulation.

Using antibodies raised against an N-terminal peptide or against C-terminal sequence (16), several immunoreactive species smaller than the full-length protein are detected in cell lysates. These smaller species also noted by others (16) may be proteolytic fragments (see below) or may be indicative of smaller forms of MEKK1 produced by translation initiation at downstream sites. A third possibility is that these smaller immunoreactive species represent crossreactivity of closely related family members. Our data cannot distinguish among these possibilities; however, we observe that recombinant full-length MEKK1 is susceptible to proteolysis in lysates. Species larger than the endogenous protein appear on overexpression of wild-type rat MEKK1. However, expression of a mutant protein that has no detectable kinase

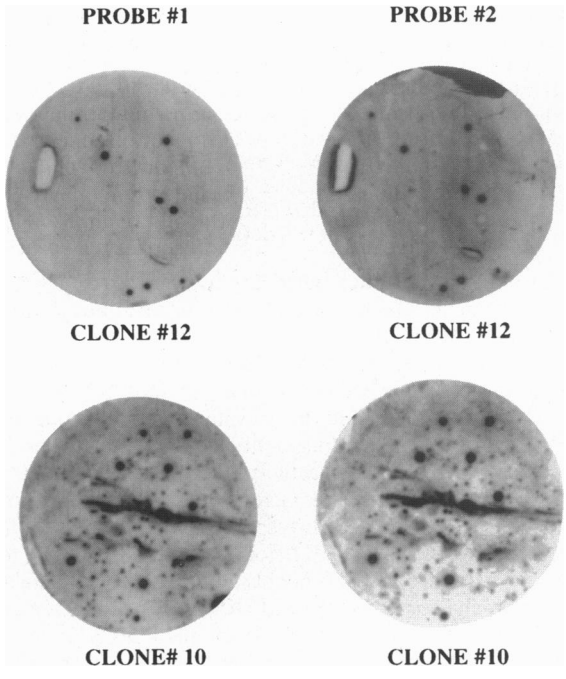

FIG. 3. Hybridization of probes from 5′ coding region (#1) and 5′ UTR (#2) to rat MEKK1 genomic clones (#12, #10). To confirm that the 5′UTR is derived from the MEKK1 gene, two MEKK1 genomic clones were isolated from a rat liver library (λGEM) with a 600-nt *Sac*I fragment downstream of the translation start site. Positive clones from the secondary screen were plated. Duplicate filters were prepared and hybridized with either the 600-nt fragment (probe #1) or a 500-nt *Eco*RI-*Pvu*I fragment that contains the 5′ UTR (probe #2), followed by washes at 65°C in 0.1 × SSC in 0.1% SDS. The two probes hybridized to the same colonies on duplicate filter lifts of both partially purified genomic clones, indicating that hybridized sequences were contained within the same genomic clone.

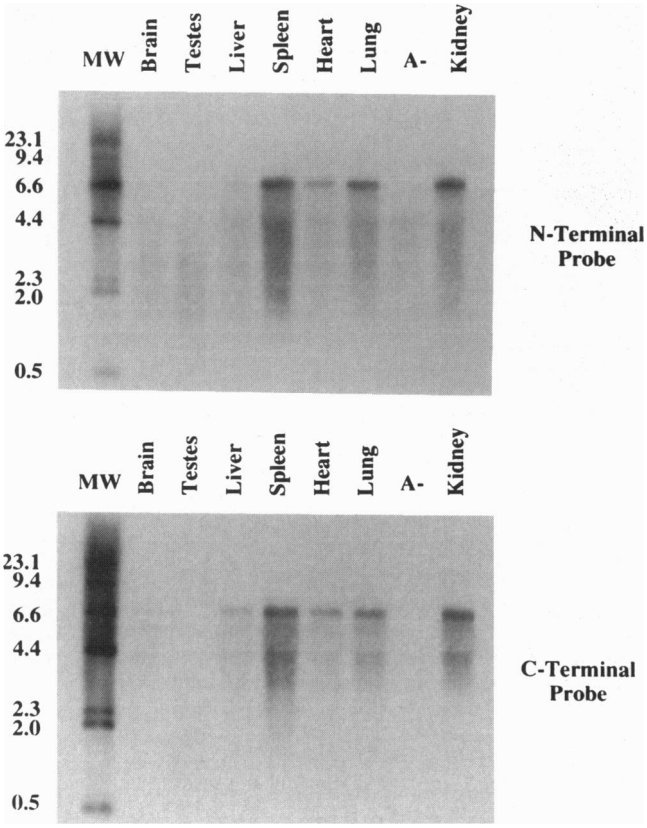

FIG. 4. Northern blot analysis of rat tissues with probes to 5′ (N-terminal) and 3′ (C-terminal) coding regions of MEKK1. Both probes hybridized to a band of ≈7 kb in 15 μg of poly(A)$^+$ RNA isolated from adult rat tissues. Poly(A)$^+$ RNAs were denatured in glyoxal and dimethylsulfoxide, size fractionated on 0.8% agarose gels, and transferred to Biotrans Plus membrane (ICN). The membrane was hybridized as described (22). Molecular weight markers (MW) are ^{32}P-labeled λ DNA digested with *Hin*dIII. Negative control (A)$^-$, 15 μg of poly(A)$^-$ RNA. A 2.0-kb *Nhe*I-*Bst*1107I fragment containing the initiator methionine and the downstream sequence was used as the N-terminal probe, and a 1-kb *Pst*I fragment containing most of the catalytic domain was used as the C-terminal probe.

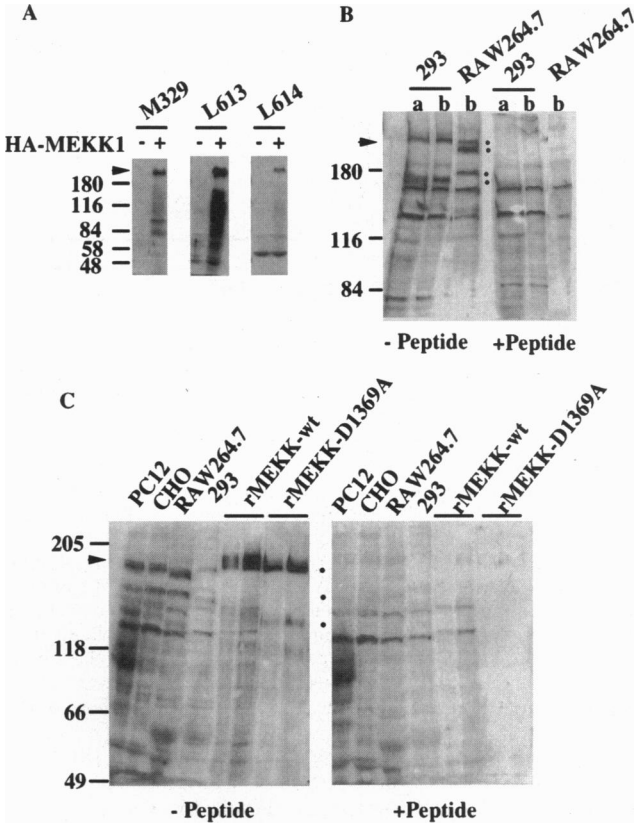

FIG. 5. Immunodetection of endogenous and transfected rat MEKK1. (*A*) HA-MEKK1 was detected in transfected (+) cells with antibodies M329, L613, and L614; −, the untransfected control. The major band at ≈195 kDa recognized by all three antibodies is indicated (arrowhead). Recombinant MEKK1 was expressed from a ligated cDNA to produce the full-length protein. (*B*) Endogenous MEKK1 proteins detected in 293 and RAW 264.7 cells with N795 antibodies. a, Whole cell lysates; b, nondenatured detergent lysates. (*C*) Endogenous MEKK1 proteins in PC12, Chinese hamster ovary, RAW 264.7, and 293 cells compared with transfected rat MEKK1 and D1369A MEKK1 detected with N795 antibodies. (*B* and *C*) The largest protein detected (arrowhead) is ≈195 kDa. Dots indicate endogenous proteins detected specifically in absence of competing peptide antigen. (*B*) The band at ≈80 kDa without peptide corresponds to that at ≈86 kDa with peptide. (*C*) The left panel uses no peptide antigen; the right panel uses 50 μg/ml antigenic peptide.

activity does not produce these larger forms, suggesting that the larger forms are generated by autophosphorylation of recombinant MEKK1.

When overexpressed, rat MEKK1 exceeds the capacity of anisomycin and other factors to increase JNK/SAPK activity (26). Thus, MEKK1 is able to activate kinase cascades in the absence of known regulators. Although several studies have shown an increase in MEKK activity in immunoprecipitates from cells exposed to tumor necrosis factor, hyperosmolarity, and other agents that activate the stress-responsive kinase pathways (27–29), the activity of the MEKK1 clone has not been shown to be increased with such agents (unpublished data). This is similar to the *in vitro* behavior of the yeast MEKK Ste11p (30). Based on a comparison of immunoblots of recombinant MEKK1 and the endogenous protein, MEKK1 appears to be of low abundance. We hypothesize that MEKK1 may be maintained in the low-activity state in cells by other proteins also of low abundance. Expression of very small amounts of the recombinant protein may saturate the regulatory component. Hence, the larger forms of MEKK1 that are generated when the protein is overexpressed in cells may reflect their deregulation. It is also possible that MEKK1 overexpression leads to the production of constitutively active proteolytic fragments that phosphorylate and activate the full-length protein.

Endogenous and expressed MEKK1 are associated with the particulate fraction of cells. However, MEKK1 from which the N-terminal half of the protein has been deleted is found primarily in soluble extracts. Expression of constructs of intermediate length comparable in size with mouse MEKK suggests that membrane association requires sequences in the middle of the enzyme. Thus, it is likely that internal sequences of MEKK1 target it to membranes (unpublished data).

Several studies implicate MEKK1 in the control of the JNK/SAPK pathway by cytokines and other agents. MEKK1 can also activate MEK1 and 2 from the related MAP kinase pathway *in vitro* or in transfected cells, and from p38 MAP kinase. This raises questions about which and how many kinase cascades it controls. Expression of rat MEKK1 most potently activates JNK/SAPK, but at higher concentrations leads to increased activity of both ERK2 and p38 MAP kinase. Our findings suggest that the extent of activation and concentration of MEKK1 in cells will have a major impact on its downstream consequences. The novel MEKK1 sequence identified here will provide for a more complete understanding of the regulation of MEKK1 function and the kinase cascades in which it operates.

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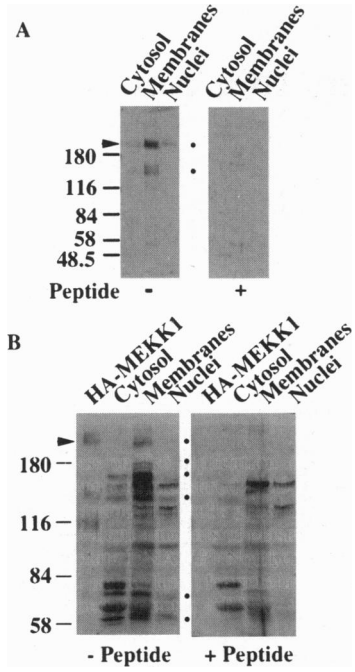

FIG. 6. Subcellular localization of MEKK1. (*A*) Transfected HA-MEKK1 detected in subcellular fractions of 293 cells by immunoblotting with N795 in the absence (*Left*) and presence (*Right*) of antigenic peptide. (*B*) Endogenous MEKK1 detected as in *A*. A membrane fraction containing HA-MEKK1 was loaded in the first lane of each panel. Arrowheads mark the largest protein species at ≈195 kDa.

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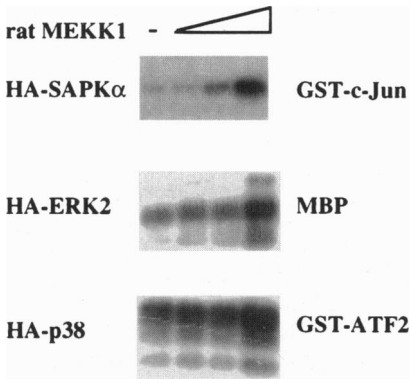

FIG. 7. Activation of three MAP kinase pathways by rat MEKK1. Increasing amounts (50 ng, 500 ng, and 5 μg), indicated by bar over autoradiograms, of rat MEKK1 cotransfected with 5 μg of HA-SAPK (*Top*), HA-ERK2 (*Middle*), and HA-p38 (*Bottom*). The vector was added to attain a total of 10 μg of DNA transfected per dish. Autoradiograms of kinase assays of the cotransfected kinases are shown. GST fusion proteins were purified on glutathione-agarose as described (23). cDNAs of rat ERK2, p38, and JNK/SAPK were subcloned into pCEP4HA as described (19). Immunoblotting confirmed that a similar amount of MEKK1 was expressed in each set of transfections.

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