Cloning of MASK, a Novel Member of the Mammalian Germinal Center Kinase III Subfamily, with Apoptosis-inducing Properties*

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Received for publication, November 13, 2001
Published, JBC Papers in Press, December 6, 2001, DOI 10.1074/jbc.M110882200

We have cloned a novel human GCK family kinase that has been designated as MASK (Mst3 and SOK1-related kinase). MASK is widely expressed and encodes a protein of 416 amino acid residues, with an N-terminal kinase domain and a unique C-terminal region. Like other GCK-III subfamily kinases, MASK does not activate any mitogen-activated protein kinase pathways. Wild type MASK, but not a form lacking the C terminus, exhibits homophilic binding in the yeast two-hybrid system and in coimmunoprecipitation experiments. Additionally, deletion of this C-terminal region of MASK leads to an increased kinase activity toward itself as well as toward an exogenous substrate, myelin basic protein. A potential caspase 3 cleavage site (DESDS) is present in the C-terminal region of MASK, and we show that MASK is cleaved in vitro by caspase 3. Finally, wild type and C-terminally truncated forms of MASK can both induce apoptosis upon overexpression in mammalian cells that is abrogated by CrmA, suggesting involvement of MASK in the apoptotic machinery in mammalian cells.

The GCK family, together with the PAK family, comprises

* The work was supported in part by grants from the Danish National Research Foundation (to the Center for Experimental Bioinformatics) and by the Japan Science and Technology Corporation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB040057.

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† The abbreviations used are: GCK, germinal center kinase; PAK, p21-activated kinase; Ste20, Sterile 20; SOK1, Ste20-like oxidant-stress responsive kinase 1; JNK, Jun N terminus kinase; MAPK, mitogen-activated protein kinase; SOK1, Ste20-like oxidant stress response kinase-1; YSK1, yeast Ste20-related kinase 1; Mst, mammalian sterile twenty-like; Krs, kinase responsive to stress; MASK, Mst3 and SOK1-related kinase; EST, expression sequence tag; UTR, untranslated region; RT, reverse transcription; HA, hemagglutinin; TAK1, transformation activating kinase 1; TAB1, TAK1-binding protein 1; MBP, myelin basic protein; GST, glutathione S-transferase; ATF, activating transcription factor; EGFR, epidermal growth factor receptor; mAb, monoclonal antibody; IL, interleukin; SLK, Ste20-like kinase; HPK1, hematopoietic progenitor kinase 1; BAC, bacterial artificial chromosome; OSIR1, oxidative stress responsive 1; ERK, extracellular signal-regulated kinase; MEKK1, MAPK/ERK kinase 1; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; TNF, tumor necrosis factor; MOPS, 4-morpholinosopropanesulfonic acid; ELAM, endothelial leukocyte adhesion molecule; GFP, green fluorescence protein; WT, wild type; TRAF2, TNF receptor-associated factor 2.
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genome (1). Shortly after our first mention of MASK gene, another group described an identical gene as MST4 and showed that it is a kinase but did not assign any function (14). MASK is a protein kinase ubiquitously expressed in most tissues. Analogous to other GCK-III subfamily members, MASK was found to activate none of the MAPK pathways. The C-terminal non-catalytic region of MASK is involved in self-association and is inhibitory to its kinase activity. Using a polyclonal antibody generated against the C terminus of MASK, we show that it is expressed as a 47-kDa protein in several cell lines. MASK is cleaved in vitro by caspase 3 to generate a C-terminally truncated form presumably cleaved at a putative caspase 3 cleavage site. Most notably, both full-length and C-terminally truncated forms of MASK, but not a kinase dead version, induce apoptosis when overexpressed in MCF-7 human breast carcinoma cells and human embryonic kidney 293 cells. This apoptotic effect is abrogated upon coexpression of CrmA, a virally encoded inhibitor of caspsases. Finally, a kinase dead version of MASK cannot inhibit apoptosis induced by the TNF receptor. Taken together, our results implicate MASK in the apoptotic pathways in cells.

EXPERIMENTAL PROCEDURES

Screening and Cloning of MASK—To identify additional members of the GCK-III subfamily, we first identified several EST clones (DDBJ/EMBL/GenBank™ accession numbers: H03061, N22232, N75199, R79091, T64469, W16504, AA191319, AA395854, AA13812, AA48166, AA809779, and AA953667) that were similar, but not identical, to MST3 or SOKI/YISKI. Later a new cDNA clone AL511422 was used to extend the 5'-UTR region. In reference to the sequences of the EST clones, several PCR primers to amplify MASK cDNA were designed. For the forward primers, the following were generated: MASK(1F), 5'-GGGATCCCTCGAGCCACCTGCTCC-3'; MASK(114F), 5'-CAGAGAGAGAGGC-3'; MASK(1112F), 5'-CTACCGGAGCATATAAGCTGCTG-3'; MASK(1112F), 5'-CAGAGAGAGAGGC-3'; MASK(1584F), 5'-CTAAGCTTTAGGGCTGATTTT-3'; MASK(2640F), 5'-CTGAACTGACGTGTTAGTTC-3'. For the reverse primers; the following were generated: MASK(888R), 5'-TGGATCCCTGGTACCTTAC-3'; MASK(1935R), 5'-CCGAGGATCCCTTCATCTCT-3'; MASK(1935R), 5'-CCGAGGATCCCTTCATCTCT-3'; MASK(2527R), 5'-AAACCAACTGCGAGATTCATAT-3'; MASK(3256R), 5'-GACTGATCCTCTCTCTCTTGTATC-3'. The forward primers were described above, 5' and 3' rapid amplification of cDNA ends was also performed to verify the sequence obtained as previously described (15). The gene fragments were then assembled to represent a full-length MASK cDNA. One full-length clone was sequenced completely on both strands. All the PCR artifacts were excluded by comparing at least three independent clones.

Northern Blot Analysis—A human multiple tissue Northern blot (CLONTECH, Palo Alto, CA) containing immobilized poly(A) mRNA was used. A 612-bp fragment from the 3'-UTR of MASK transcript (nucleotides 1369–1980 of accession number AB040057) was used as a probe. The probe DNA was labeled with [α-32P]dCTP (Amersham Biosciences, Inc.) using the random primed labeling kit (Roche Molecular Biochemicals, Mannheim, Germany). Hybridization and washing steps were performed according to the manufacturer's instructions. The membranes were subsequently re-hybridized with a β-actin probe.

cDNAs and Constructs—The MASK cDNA was first cloned into a Gateway entry vector (Invitrogen, Gaithersburg, MD). The cDNA was then transferred using a Clonase reaction to generate a FLAG epitope-tagged version. A C-terminal deletion of MASK (deleting the C-terminal 99 amino acids) and a Myc epitope-tagged version was similarly generated. The FLAG epitope-tagged K53E mutant of MASK was generated by a PCR-mediated method (15) and subcloned directly into pCMVF vector provided by Dr. K. Matsumoto at Nagoya University. The HA epitope-tagged MASK was also subcloned into pCMVH vector provided by Dr. Matsumoto. The constructs for yeast two-hybrid system were obtained by transferring the cDNAs using a clonase reaction into yeast bait (pGBK7) and prey (pGAD7) vector (CLONTECH, Palo Alto, CA).

All the constructs were confirmed by sequencing. HA-p38, FLAG-TAK1, and TAB1 plasmids are as previously described (16). V5-tagged JNK1 was purchased from Invitrogen (Carlsbad, CA). MBP and GST-ATF2 were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). WT type EGF-R has been previously described (17).

Cotransfection—Transient transfection was performed according to the manufacturer's instructions. 293 and 293T cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum plus antibiotics. MCF-7 cells were grown in modified Eagle's medium with 10% fetal bovine serum plus antibiotics, non-essential amino acids, and pyruvate. Transfection of cells was performed as previously described (18). For single transfection assays, 1 × 10^6 cells per 6-cm dish were transfected with a total of 5 μg of cDNA. 5 μg of pCMV-HA empty vector was used as a negative control.

Antibodies and Growth Factors—Anti-FLAG M2 mAb was from Sigma Chemical Co. (St. Louis, MO), anti-HA and anti-Myc mAb were from Babco (Berkeley, CA), anti-V5 mAb was from Invitrogen (Carlsbad, CA), and anti-p38 rabbit polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA). IL-1 was purchased from Pro-mega (Madison, WI) and EGF from Upstate Biotechnology Inc. (Lake Placid, NY).

Preparation of Polyclonal Antibody—A synthetic peptide, CKKLEIK-FQKCDADESP, corresponding to the last 17 amino acids of the Mask was synthesized and conjugated to keyhole limpet hemocyanin by Boston Biologics (Woburn, MA). The purified protein was used to immunize rabbits for the generation of polyclonal antibody (Covance, Denver, PA).

Metabolic Labeling, Immunoprecipitation, and Western Blotting—For metabolic labeling, cells were washed and incubated overnight in cysteine- and methionine-free Dulbecco's modified Eagle's medium plus 35S-labeled cysteine plus methionine (Trans35S-label, ICN, Costa Mesa, CA). For immunoprecipitation studies, cells were lysed in lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate in the presence of protease inhibitors. The samples were centrifuged at 15,000 rpm for 4 min, and the clarified supernatants were used as cell lysates. The cell lysates were incubated with the respective antibodies against epitope tags, at the concentration recommended by the manufacturer, and 15 μl of a 50% slurry of protein G-Sepharose (Amersham Biosciences, Inc.) for 2 h to overnight. The mixtures were centrifuged at 15,000 rpm for 2 min. The pellets were washed three times and subjected to SDS-PAGE.

For testing dimerization of MASK in vivo, 293T cells were cotransfected with two versions of MASK (WT or ΔC) cDNA containing different epitope tags (i.e. FLAG and Myc) and metabolically labeled with [35S]methionine plus cysteine. STAM2 cDNA was cotransfected along with WT-MASK as a negative control. Cell lysates were prepared as previously described, and the supernatants were first immunoprecipitated overnight with anti-Myc antibody. After two washes with lysis buffer, the beads were resuspended in 1% SDS buffer and boiled for 5 min. Anti-FLAG antibody was added to the supernatant from this step, and the samples were left to immunoprecipitate overnight. Sample loading buffer was added to the beads directly, boiled for 5 min, and run on a SDS-PAGE gel. Loading controls were subjected to single immunoprecipitation, 5' and 3' RACE. Western Blot Analysis—A human multiple tissue Northern blot (CLONTECH, Palo Alto, CA) containing immobilized poly(A) mRNA was used. A 612-bp fragment from the 3'-UTR of MASK transcript (nucleotides 1369–1980 of accession number AB040057) was used as a probe. The probe DNA was labeled with [α-32P]dCTP (Amersham Biosciences, Inc.) using the random primed labeling kit (Roche Molecular Biochemicals, Mannheim, Germany). Hybridization and washing steps were performed according to the manufacturer's instructions. The membranes were subsequently re-hybridized with a β-actin probe.

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RESULTS

Cloning of MASK—The occurrence of a novel kinase, MASK, in the human genome was first pointed out in our systematic phylogenetic analysis of the Ste20 group of kinases (1). While analyzing GCK-III subfamily kinases, we noticed a group of ESTs that were not related to MST3/SOK1/YSK1 genes (5–7). We designed several oligonucleotides to amplify full-length MASK cDNA by RTPCR and obtained a clone harboring a 3263-bp insert that was sequenced completely. The nucleotide sequence of the insert contained an open reading frame of 416 amino acids with the sequence upstream of the initiator methionine in good agreement with the Kozak consensus sequence for translation initiation (21). The predicted protein was most homologous to Mst3 and SOK1 kinases and, therefore, we have designated it MASK (Mst3 and SOK1-related kinase) (5, 6). After our first mention of the MASK gene (1), another group described an identical gene, Mst4, a kinase without any obvious function (14).

Sequence Analysis of MASK—The N-terminal region of MASK encodes a Ste20-like kinase domain comprising of 11 kinase subdomains (Fig. 1A) (22). A conserved motif called Ste20 signature sequence that is highly conserved in all Ste20 group kinases is also present in MASK (1). The kinase domain of MASK is most closely related to that of GCK-III subfamily kinases Mst3 (89% identity), SOK1/YSK1 (87%), Drosophila putative kinase CG5169 (85%), and C. elegans putative kinase TIA95.2 (79%) (1, 5–7). It is also 54–57% identical to GCK-II subfamily kinases such as mammalian Mast1/Krs2, Mast2/Krs1, Drosophila putative kinase CG11228, and C. elegans putative kinase C24A4.8.4 (1, 9–11). MASK is moderately related to other GCK family kinases (less than 50%) in its kinase domain (1). A notable feature of the GCK family kinases is the extensive sequence diversity in their C-terminal non-catalytic domains. The GCK-II subfamily is characterized by a short C-terminal region that is about 140 amino acids long (1). Like other members of this subfamily, MASK has a 142-amino acid long C-terminal non-catalytic region, which is moderately conserved among mammalian GCK-III subfamily kinases (about 42–46% identity with SOK-1 and Mst3, respectively) (1, 5–7). Because Drosophila and C. elegans putative orthologs are only predictions from the genomic sequence, their C-terminal residues remain uncertain, and are thus excluded from our comparison. The C-terminal region of MASK shows no significant similarity to those of GCKs in other subfamilies (1). Thus, MASK together with its mammalian homologs SOK1/YSK1 and Mst3 should make up a distinct subfamily. An examination of the C-terminal region of MASK revealed the presence of some characteristic motifs. The most upstream region in the C terminus is highly acidic, a feature also shared by other GCK-III members (1, 5–7). However, a part of this acidic region in MASK amino acid sequence DESDS resembles a putative targeting motif for caspase 3, an effector caspase activated during apoptosis (23). Caspase 3 cleavage sites are also found in other GCK family kinases such as Mast1, Mast2, SLK, and HPK1 (12, 13, 24, 25). The Multicopy program predicted residues 359–391 in the C terminus of MASK to form coiled-coils, which are implicated in self-association (Fig. 1A) (26). This has not been predicted for any of the members of GCK-III subfamily, although two members of the GCK-II subfamily are known to homodimerize through coiled-coil regions at their C termini. We find that the region that corresponds to the coiled-coil region is somewhat conserved between MASK, Mst3, and SOK-1 raising the possibility that they may all possess the ability to oligomerize.

A DNA data base search revealed that human the MASK gene is encoded by the BAC clone RP6–213H19 from human chromosome X (Fig. 1B). Comparison of MASK cDNA sequence with human genomic sequence revealed that the human MASK gene spans over 52.6 kb of genomic DNA (Fig. 1B). The gene location is Xq25 to 26.3. The coding region of the MASK transcript consists of 12 exons. All exon/intron boundaries matched the consensus sequences for splicing (Fig. 1C) (27).

Tissue Distribution of MASK mRNA—We examined the expression pattern of the MASK mRNA by probing a multiple tissue Northern blot. A major band ~3.3 kb was detected (Fig. 2), which is consistent with the size (3263 bp) of the clone that we amplified from brain cDNA. MASK transcript was detected in all tissues tested, with especially high expression detected in the placenta. A search of the human EST data base showed the presence of corresponding ESTs derived from almost every organ in the EST database confirming our conclusions based on the Northern blot analysis (data not shown). This ubiquitous expression pattern is reminiscent of that of SOK1/YSK1 and MST3 (with the exception of a brain-specific MST3 isoform reported recently) (5–7, 28). In other GCK subfamilies, functional division among subfamily members is often established by differential expression patterns (1). However, this is not the...
case with the GCK-III subfamily given that MASK shows a similar widespread expression pattern such as SOK1/YSK1 and MST3.

The Kinase Activity of MASK—The ability of MASK cDNA to code for the corresponding protein was first tested by transfecting a FLAG epitope-tagged version into 293T cells. The vector alone was transfected as a negative control (Fig. 3A). FLAG-tagged MASK migrated at ~55 kDa, which is greater than the expected molecular mass as deduced from its amino acid sequence. This difference is because of the addition of an epitope tag as well as the extra amino acids derived from the Gateway vector used in the cloning strategy. To check the expression of endogenous MASK, we probed cell lysates from four different cell lines in Western blots using a polyclonal antibody that was generated against a C-terminal peptide of MASK. As shown in Fig. 3B, MASK migrates on an SDS-PAGE gel at an apparent mass of 47 kDa in agreement with its predicted molecular weight. MASK was endogenously expressed in several cell lines tested that were derived from kidney, liver, breast, and T cells. This is consistent with the widespread expression of MASK observed in Northern blotting experiments.

The kinase activity of MASK was next tested by overexpression of vector or wild type (WT) MASK into 293 cells and immunoprecipitation with anti-FLAG antibody followed by an 

\textit{in vitro} kinase assay using MBP as an exogenous substrate. A mutant form of MASK with a mutation in a conserved lysine residue in the ATP binding pocket (MASK K53E) that should presumably be kinase dead was also included in this assay (22). A strongly phosphorylated band corresponding to MBP was detected in the WT MASK-transfected lane but not in vector.
control or kinase dead MASK (Fig. 3C). Also, a band corresponding to WT MASK was observed indicating that it is capable of undergoing autophosphorylation. Taken together, these results demonstrate that MASK cDNA encodes a functional kinase.

To examine the contribution of the C-terminal non-catalytic region to the kinase activity of MASK, we generated a version lacking 99 amino acids from the C-terminus of MASK (MASK ΔC) and compared its kinase activity to that of the wild type. Increased levels of autophosphorylation as well as MBP phosphorylation were observed for ΔC MASK as compared with WT, although their expression levels were comparable (Fig. 3D). This result demonstrates that the C-terminal region may negatively regulate the kinase activity of MASK. This finding is reminiscent of GCK-II subfamily kinases where the kinase activity is similarly inhibited by the C-terminal region (29). Because there is virtually no sequence homology between the C-terminal region of MASK and that of GCK-IIs, the inhibitory effect of MASK C-terminal region is likely to be carried out by a different mechanism (9–11).

Self-association of MASK—Presence of a short C-terminal region is a common structural feature of all GCK-III subfamily kinases (1, 5–7). However, no function has been assigned to this region. Because this region was found to contain a coiled-coil motif that is often utilized for oligomerization, we tested whether MASK was capable of forming homodimers by using the yeast two-hybrid system (30). We observed that WT MASK interacted with itself but not with two other control proteins, p53 or PAK5 (Table I). This association required the presence of the C-terminal region, because no interaction was observed when the interaction between WT MASK and ΔC MASK was tested.

To determine if homodimerization can also occur in vivo, we cotransfected FLAG or Myc epitope-tagged MASK constructs in 293T cells and metabolically labeled the cells (Fig. 4). An Myc-tagged version of an unrelated molecule, STAM2, was cotransfected with WT MASK as a negative control. FLAG-tagged WT MASK could only communoprecipitate with Myc-tagged WT-MASK. No association was observed when either one was truncated at the C terminus or when both were truncated. From these data, we conclude that MASK is capable of self-association and that the C-terminal region is required for this interaction. Given the enhanced kinase activity of the ΔC MASK shown above, this observation suggests that the kinase activity of MASK is regulated by the self-association of its C-terminal non-catalytic region.

Lack of Activation of MAPK and NF-κB Pathways by Overexpression of MASK—Most GCKs have been reported to activate either JNK or p38 MAPK pathways, but none of the GCK-III subfamily members have yet been reported to activate either MAPK pathway (1–7). This has stood as a common peculiar feature of the GCK-III subfamily. Previous reports in the literature present conflicting evidence about ERK activation by MST3. We therefore sought to study the effect of MASK overexpression on these MAPK pathways. 293 cells were co-transfected with wild type MASK and FLAG-tagged ERK2. V5 epitope-tagged JNK1, or HA-tagged p38. The respective MAPKs were immunoprecipitated and subjected to in vitro kinase assays. Transfection with wild type MASK did not result in the activation of ERK, JNK, or p38, although they were potently activated by cotransfection of EGFR with treatment of EGF or IL-1 or cotransfection of TAK1 and TAB1, respectively (Fig. 5, A–C). Because the C-terminally deleted form of MASK is more active as a kinase, it was possible that, although WT MASK did not activate these pathways, the ΔC mutant of MASK may be capable of activating them. We therefore also tested the ΔC mutant of MASK in these assays. As shown in Fig. 5 (A–C), we failed to observe any activation of these MAPK modules. Our results thus extend the data on GCK-IIIIs in their inability to activate these MAPKs as a common feature. One possible explanation for such behavior of GCK-III subfamily kinases is that they may act in a signaling pathway yet to be tested. Another possibility is that they can potentially activate a MAPK pathway but need to be activated in some manner. In this respect, our demonstration of self-association of MASK and inhibition of the kinase activity by its C-terminal region presents an interesting possibility that MASK is silenced by inter-molecular self-association. This may prevent MASK from interacting with its unknown effectors. Removal of the C-terminal region upon putative caspase 3 cleavage may act as a stimulus for MASK to participate in an as yet unknown signaling pathway.

We next tested the ability of MASK to activate NF-κB pathway. For this purpose, we performed luciferase assays using a reporter containing endothelial leukocyte adhesion molecule 1 (ELAM-1) promoter as readout. We found that overexpression of WT MASK or ΔC MASK in 293 cells did not activate the NF-κB pathway (Fig. 5D). TRAF2, a molecule known to be downstream of TNF receptor superfamily members, potently activated this promoter thus serving as a positive control (31).

Cleavage of MASK by Caspase 3 in Vitro—The sequence analysis presented above revealed a sequence that corresponds to a potential caspase 3 cleavage site (23). To test whether MASK serves as a substrate for caspase 3, we performed an in vitro caspase assay on MASK. 293 cells were transfected with WT MASK, and the transiently expressed MASK was immunoprecipitated and incubated with caspase 3-conjugated agarose beads. Treatment with caspase 3 resulted in the appearance of a smaller band, whose size corresponds to a cleavage form of MASK that is cleaved at the putative caspase recognition motif (Fig. 6A). Thus these results suggest that MASK can be cleaved by caspase 3 at the putative caspase target motif.
Interestingly, only a fraction of the immunoprecipitated MASK was cleaved by caspase 3. Longer incubations did not result in increase of a cleaved product (data not shown). We currently do not have an explanation for this finding, although it is possible that MASK protein is protected from cleavage by caspases by oligomerization or some other mechanism.

Overexpression of MASK Induces Apoptosis—To directly address the involvement of MASK in apoptosis, MCF-7 human breast carcinoma cells were cotransfected with vector control or wild type MASK (MASK WT) cDNA. The cells were metabolically labeled with [35S]methionine and cysteine and then immunoprecipitated with anti-FLAG-conjugated beads. The panel shows the gel after autoradiography to visualize MASK. Molecular mass markers are indicated on the right. B, MASK is endogenously expressed in various cell lines. Lysates from the indicated cell lines were resolved by SDS-PAGE. Subsequently, the proteins were transferred onto nitrocellulose and probed with anti-MASK antiserum. Molecular mass markers in kDa are shown on the left. C, lysates from 293 cells transfected with FLAG epitope-tagged MASK WT construct, a K53E mutant (MASK K53E), or a vector control were immunoprecipitated using anti-FLAG antibody beads and their kinase activities assayed using [γ-32P]ATP and MBP as substrates. Reaction products were separated by SDS-PAGE and were subjected to autoradiography. The top panel shows autophosphorylation of WT but not the kinase-dead mutant (K53E). The middle panel shows phosphorylation of MBP, and the bottom panel is a reprobing to confirm equal expression of the MASK constructs. D, lysates from 293 cells transfected with the indicated constructs were subjected to kinase assays as in C. The reaction products were separated by SDS-PAGE to examine the autophosphorylation (top panel) or phosphorylation of MBP (middle panel). The bottom panel shows the results from a parallel experiment to show equal expression of MASK WT and ΔC MASK proteins after metabolic labeling as in A.

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TABLE I
Deletion of the C-terminal region abolishes oligomerization of MASK in the yeast two-hybrid system

Interaction studies were performed by using the mating assay in the yeast two-hybrid method. Yeast strain PJ69–2A carrying the "bait" plasmid was mated with a Y187 yeast strain carrying the "prey" plasmid and plated onto selection plates as described under "Experimental Procedures." Interaction between pairs of proteins was revealed by growth on these deficient plates as well as by the development of blue color of the colonies.

| Bait plasmid | Prey plasmid |
|--------------|--------------|
| MASK WT      | Vector       |
| SV40 large T antigen | p53 |
|               | MASK WT      |
|               | MASK ΔC      |
|               | PAK5         |

- **MASK WT**
  - Vector: -
  - p53: -
  - MASK WT: ++
  - MASK ΔC: -
  - PAK5: -

- **SV40 large T antigen**
  - Vector: -
  - p53: + + +
  - MASK WT: -
  - MASK ΔC: -
  - PAK5: -

- **a**: no growth on the selective plates.
- **b**: ++, robust colonies with blue color on the selective plates.

**FIG. 4.** Homodimerization of MASK in vivo requires the C-terminal region. 293T cells were cotransfected with the indicated pairs of epitope-tagged constructs and metabolically labeled with [35S]methionine plus cysteine. Cell lysates were first immunoprecipitated with anti-Myc antibody. The immune complexes were washed, and bound proteins were eluted by boiling in 1% SDS. The samples were re-immunoprecipitated using anti-FLAG antibody to detect bound FLAG-tagged proteins as shown (top panel). The middle and lower panels show immunoprecipitation from parallel lysates to confirm expression of the indicated constructs.

were obtained when these studies of morphological changes were repeated in 293 cells (data not shown).

Because DNA fragmentation is another hallmark of apoptotic cells, we isolated the genomic DNA from vector, ΔC MASK, and TNF receptor-transfected cells and subjected them to agarose gel electrophoresis (32). As shown in Fig. 6D, the genomic DNA from the cells transfected with ΔC MASK showed a characteristic DNA fragmentation ladder that confirms apoptotic cell death. Thus we conclude that both WT and ΔC MASK can induce apoptosis upon overexpression in cells.

Because apoptosis induced by a number of stimuli is mediated by activation of caspases, we decided to test if caspase activation was required for the apoptotic effect observed in the case of MASK. CrmA is a gene product encoded by the cowpox virus that belongs to the serpin family of protease inhibitors (34). When CrmA was cotransfected into cells along with ΔC MASK, it was able to rescue the cells from the apoptotic effect caused by ΔC MASK (Fig. 7A).

Because MASK is a putative substrate of caspase 3, it is possible that it may be a downstream mediator of apoptosis induced by stimuli such as the TNF receptor. Because the kinase activity is required for the apoptotic effect of MASK, we used the kinase dead version as a potential dominant negative construct. We therefore cotransfected kinase dead MASK along with TNF receptor into 293 cells. As shown in Fig. 7B, overexpression of kinase dead MASK was unable to prevent TNF receptor-induced apoptosis, whereas cells transfected with CrmA were almost totally protected from cell death. In other experiments, we observed that the kinase dead mutant of MASK was also unable to rescue cells from staurosporine-induced apoptosis (data not shown).

**DISCUSSION**

The GCK family of protein kinases represents an emerging large family of protein kinases with eight subfamilies (1, 2). To study such a large family of protein kinases, a phylogenetic analysis is dispensable. Most of the GCK family of protein kinases characterized so far activate either JNK or p38 MAPK signaling pathways upon overexpression (1–4). However, there are several exceptions to this rule. LOK from the GCK-V subfamily does not activate either pathway, but its closely related homolog SLK activates JNK (25, 35). OSR1 from the GCK-VI subgroup is yet to be shown to activate either pathway, but its homolog PASK/SPAK activates p38 MAPK (36–38). Therefore, these kinases may be able to activate either pathway under different physiological conditions. Studies addressing the involvement of the most recent members of the GCK-VII subfamily, MYO2A and MYO2B, in MAPK signaling pathways are underway (39).2 The most notable exceptions are found in the GCK-III subfamily of kinases consisting of SOK1/YSK1 and Mst3 in addition to Mask, which is described in this report. All of them are active kinases when overexpressed but do not activate JNK or p38 MAPK pathways (5–7). However, it is possible that they participate in other less characterized MAPK pathways such as ERK3/4 or ERK5 (3).

Four GCK family kinases, Mst1/Krs2, Mst2/Krs1 (GCK-II), HPK1 (GCK-1), and SLK (GCK-V) have been reported to be directly or indirectly involved in apoptosis to date (1, 12, 13, 24, 25). They are suggested to be activated upon cleavage by caspase 3 to produce the free kinase domain with enhanced activity during apoptosis (1, 12, 13, 24, 25). This may also be the case with Mask for two reasons. First, both wild type and C terminus-truncated forms of Mask can induce apoptosis of cultured cells upon overexpression. Second, Mask can be cleaved by caspase 3 in vitro. Our findings indicate that the pro-apoptotic effect of Mask is enhanced by loss of the region C-terminal to the putative caspase cleavage site. In addition, this effect is abrogated upon treatment with a caspase inhibitor, CrmA. Because activation of the NF-κB pathway confers protection against apoptosis in several instances, the lack of activation of this pathway is consistent with the apoptosis-inducing ability of Mask (40). Given these observations, it is possible that Mask participates in the apoptotic cascade in

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2 A. C. Dose, personal communication.
cells. Whether this involvement in apoptosis is specific to MASK or common to other GCK-III subfamily members is yet to be tested. SOK1 is activated during the initial stages of chemical anoxia-induced necrotic cell death, but its involvement in apoptosis remains uncertain (8). Because the recognition motif for caspase 3 is present only in MASK, it may be that the induction of apoptosis is a property restricted to MASK. The demonstration that overexpression of MASK alone is sufficient to induce apoptosis is interesting for several reasons. Like other GCK-IIIs, MASK is ubiquitously expressed among tissues that do not undergo apoptosis under physiological conditions (5–7). Thus endogenous MASK is likely to be silenced so as not to induce apoptosis in the normal intracellular environment. Our demonstration that a fraction of the overexpressed MASK remains resistant to caspase 3 cleavage suggests that there might exist a protective mechanism that keeps MASK from participating in apoptotic events. One of such mechanisms is steric inhibition by oligomerization. Indeed, we have shown by yeast two-hybrid analysis and in vivo experiments that the direct self-association of MASK molecules requires its C terminus region. The C terminus region corresponding to 359–391 amino acids is predicted to form a coiled-coil motif,

**Fig. 5.** MASK does not activate ERK, JNK, p38 MAPK, or NF-κB pathways. A, lysates from 293 cells cotransfected with the indicated constructs and V5 epitope-tagged JNK1 were immunoprecipitated with the anti-V5 antibody and subjected to an in vitro kinase assay using [γ-32P]ATP and GST-ATF2 as a substrate. The cells in the lane labeled IL-1 were transfected with vector control but treated with IL-1 (10 ng/ml) for 20 min prior to lysis. Reaction products were separated by SDS-PAGE and subjected to autoradiography. Activity of JNK kinase is shown in the upper panel. The immune complexes were immunoprecipitated with anti-V5 antibody after metabolic labeling to ensure a similar input level (lower panel). B, lysates from 293 cells cotransfected with the indicated constructs and HA epitope-tagged p38 were immunoprecipitated with the anti-HA antibody and subjected to an in vitro kinase assay using [γ-32P]ATP and GST-ATF2 as a substrate. As a positive control, cells were cotransfected with TAK1 plus TAB1 plasmids. Reaction products were separated by SDS-PAGE and subjected to autoradiography. Activity of p38 kinase is shown in the upper panel, and the lower panel shows the results of Western blotting with anti-p38 antibody to confirm equal loading. C, lysates from 293 cells cotransfected with the indicated constructs and FLAG epitope-tagged ERK2 were immunoprecipitated with the anti-FLAG antibody and subjected to an in vitro kinase assay using [γ-32P]ATP and MBP as a substrate. As a positive control, cells were cotransfected with EGFR and stimulated with EGF for 15 min prior to cell lysis. Reaction products were separated by SDS-PAGE and subjected to autoradiography. Activity of ERK kinase is shown in the upper panel, and the lower panel shows the results of Western blotting with anti-FLAG antibody to confirm equal loading. D, 293 cells in 6-cm dishes were transfected with empty vector, WT-MASK, ΔC MASK, or TRAF2 plasmids along with NF-κB reporter plasmid, ELAM-luciferase, and β-galactosidase plasmid. Twenty-four hours later, the cells were lysed and luciferase and β-galactosidase activities measured. Relative luciferase activities normalized to β-galactosidase activities are shown.
which often mediates oligomerization (26, 30). Another possibility is involvement of inhibitory factors, which are yet to be found. To determine whether the oligomerization of MASK regulates apoptosis especially by preventing cleavage requires further studies.

The GCK-II subfamily kinases consisting of Mst1/Krs2 and Mst2/Krs1 are also known to induce apoptosis (12, 13). How-
ptosis, but its physiological phosphorylation substrate is still unidentified.

Points of similarity that we have noticed between MASK and Mst1 are their ability to oligomerize and the inhibitory effect on kinase activity of their respective C-terminal regions. Creasy et al. (29) have identified two distinct domains in the C-terminal region of Mst1, a dimerization domain and an inhibitory domain that reduces its kinase activity. Multicoil computer program predicts MASK to prefer a higher degree of oligomerization whereas Mst1 has been shown to dimerize by cross-linking experiments (26, 29). GCK-II, GCK-III, and GCK-VI subfamily kinases are often put together in the same category due to presence of a short C terminus region, but such an oversimplification should be avoided inasmuch as their C terminus regions are structurally distinct from each other (1).

While this report was in preparation, a report describing the cloning of a gene, designated MST4, that is identical to MASK was published (14). The cDNA reported for Mst4 is 1372 bp, whereas the length of MASK cDNA that we have cloned is 3263 bp. Given that our Northern blot shows a single band migrating at ~3.3 kb, it is likely that we have identified the full-length cDNA, whereas the Mst4 sequence represents a partial clone. Nevertheless, both of the cDNAs contain the entire open reading frame and code for the same protein of 416 amino acids. Additionally, Qian et al. (14) have described an alternatively spliced transcript that they designate as Mst4a, which encodes a protein of 354 amino acids. Although, the role of Mst4/MASK in apoptosis was not investigated, Qian et al. (14) also did not detect any activation of ERK, p38, or JNK MAPK pathways upon overexpression of Mst4/MASK.

In conclusion, we have identified and cloned a novel GCK family kinase, MASK, that belongs to the GCK-III subfamily. MASK shows widespread expression and does not activate ERK, p38, JNK, or NF-κB pathways. The C-terminal region of

Fig. 7. Apoptosis induced by MASK is abrogated by CrmA, and kinase-dead MASK does not prevent TNF receptor-induced apoptosis. A, 293 cells were transfected with ΔC MASK (2.5 μg) along with empty vector or CrmA (2.0 μg) constructs as indicated. 0.5 μg of a GFP plasmid was cotransfected to locate the transfected cells. For each condition, the upper panel shows fluorescence micrographs and the lower panel shows corresponding light micrographs. The arrows in the bottom panels indicate the transfected cells that are visualized in the fluorescent micrographs. B, 293 cells were cotransfected with TNFR (2.0 μg) and GFP (0.5 μg) plasmids along with 2.5 μg of empty vector, kinase-dead MASK, or CrmA as indicated. Transfected cells were visualized by fluorescence microscopy as shown.
MASK is essential for its self-association and has an inhibitory effect on its kinase activity. The C-terminal region can be cleaved by caspase 3 in vitro. Most importantly, MASK and its C-terminal truncated form can induce apoptosis upon overexpression, with the latter inducing a more potent apoptotic effect. These findings represent an important step toward elucidating the physiological role of MASK and other GCK-III subfamily kinases.

Acknowledgments—We thank Drs. Akihiro Kusumi, Kunihiro Matsumoto, and Takeshi Kobayashi for providing helpful suggestions.

REFERENCES
1. Dan, I., Watanabe, N. M., and Kusumi, A. (2001) Trends Cell Biol. 11, 220–230
2. Kyriakis, J. M. (1999) J. Biol. Chem. 274, 5259–5262
3. Widmann, C., Gibson, S., Jarpe, M. B., and Johnson, G. L. (1999) Physiol. Rev. 79, 143–180
4. Kyriakis, J. M., and Avruch, J. (2001) Physiol. Rev. 81, 807–869
5. Schinkmann, K., and Illenis, J. (1997) J. Biol. Chem. 272, 28695–28703
6. Pombo, C. M., Bonventre, J. V., Molnar, A., Kyriakis, J. M., and Force, T. (1996) EMBO J. 15, 4537–4546
7. Osada, S., Iwasa, M., Saito, R., Mizuno, K., Suzuki, A., Hirai, S., and Ohno, S. (1997) Oncogene 14, 2047–2057
8. Pombo, C. M., Tsujita, T., Kyriakis, J. M., Bonventre, J. V., and Force, T. (1997) J. Biol. Chem. 272, 28972–28977
9. Creasy, C. L., and Chernoff, J. (1995) J. Biol. Chem. 270, 21695–21700
10. Creasy, C. L., and Chernoff, J. (1995) Gene 167, 303–306
11. Taylor, L. K., Wang, H., and Eriksen, R. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10099–10104
12. Lee, K. K., Murakawa, M., Nishida, E., Tsuhi, S., Kawasaki, S., Sakamaki, K., and Yonehara, S. (1998) Oncogene 16, 3029–3037
13. Graves, J. D., Gotob, Y., Draves, K. E., Ambrose, D., Han, D. K., Wright, M., Chernoff, J., Clark, E. A., and Krebs, E. G. (1998) EMBO J. 17, 2224–2234
14. Qian, Z., Lin, C., Kepiosta, R., LeBeau, M., and Rosner, M. R. (2001) J. Biol. Chem. 276, 22439–22445
15. Dieffenbach, C. W., and Dveksler, G. (1995) PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. Dan, I., Watanabe, N. M., Kobayashi, T., Yamashita-Suzuki, K., Fukagaya, Y., Kajikawa, E., Kimura, W. K., Nakashima, T. M., Matsumoto, K., Ninomiya-Tsuji, J., and Kusumi, A. (2000) FEBS Lett. 469, 19–23
17. Pandey, A., Fernandez, M. M., Steen, H., Blagoev, B., Nielsen, M. M., Roche, S., Mann, M., and Lodish, H. F. (2000) J. Biol. Chem. 275, 38633–38639
18. Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999) Nature 398, 252–256
19. Sun, X., Lee, J., Navas, T., Baldwin, D. T., Stewart, T. A., and Dixit, V. M. (1999) J. Biol. Chem. 274, 16871–16875
20. Liu, B., Fang, M., Lu, Y., Mendelsohn, J., and Fan, Z. (2001) Oncogene 20, 1913–1922
21. Kozak, M. (1987) Nucleic Acids Res. 15, 8125–8148
22. Hanks, S. K., and Hunter, T. (1995) FASEB J. 9, 576–596
23. Wolf, B. B., and Green, D. R. (1999) J. Biol. Chem. 274, 20049–20052
24. Chen, Y. R., Meyer, C. F., Ahmed, B., Yao, Z., and Tan, T. H. (1999) Oncogene 18, 7370–7377
25. Sabourin, L. A., and Rudnicki, M. A. (1999) Oncogene 18, 7566–7575
26. Wolf, E., Kim, P. S., and Berger, B. (1997) Protein Sci. 6, 1179–1189
27. Padgett, R. A., Grabowski, P. J., Konarska, M. M., Seiler, S., and Sharp, P. A. (1986) Annu. Rev. Biochem. 55, 1119–1150
28. Zhou, T. H., Ling, K., Guo, J., Zhou, H., Wu, Y. L., Jing, Q., Ma, L., and Pei, G. (2000) J. Biol. Chem. 275, 2513–2519
29. Creasy, C. L., Ambrose, D. M., and Chernoff, J. (1996) J. Biol. Chem. 271, 21049–21053
30. Burkhard, P., Stetefeld, J., and Strelkov, S. V. (2001) Trends Cell Biol. 11, 82–88
31. Rothe, M., Sarma, V., Dixit, V. M., and Goeddel, D. V. (1995) Science 269, 1424–1427
32. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) Br. J. Cancer 27, 253–262
33. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) Br. J. Cancer 27, 253–262
34. Ray, C. A., Black, R. A., Kronheim, S. R., Greenstreet, T. A., Sleath, P. R., Salvesen, G. S., and Pickup, D. J. (1992) Cell 69, 597–604
35. Kuramochi, S., Moriguchi, T., Kuida, K., Endo, J., Saka, K., Nishida, E., and Kusumi, A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13863–13868
36. Sabourin, L. A., and Rudnicki, M. A. (1999) Oncogene 18, 7566–7575
37. Wolf, E., Kim, P. S., and Berger, B. (1997) Protein Sci. 6, 1179–1189
38. Johnston, A. M., Naselli, G., Gonez, L. J., Martin, R. M., Harrison, L. C., and Burnside, B. (2000) FASEB J. 14, 2053–2062
39. Ushiro, H., Tsutsumi, T., Suzuki, K., Kayahara, T., and Nakano, K. (1998) J. Biol. Chem. 273, 22439–22445
40. Graves, J. D., Draves, K. E., Gotob, Y., Krebs, E. G., and Clark, E. A. (2001) J. Biol. Chem. 276, 14909–14915
Cloning of MASK, a Novel Member of the Mammalian Germinal Center Kinase III Subfamily, with Apoptosis-inducing Properties
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*J. Biol. Chem. 2002, 277:5929-5939.*
doi: 10.1074/jbc.M110882200 originally published online December 6, 2001

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