
DIGITAL ACCESS TO SCHOLARSHIP AT HARVARD
DASH.HARVARD.EDU


HARVARD LIBRARY
Office for Scholarly Communication

MST/MLK2, a Member of the Mixed Lineage Kinase Family, Directly Phosphorylates and Activates SEK1, an Activator of c-Jun N-terminal Kinase/Stress-activated Protein Kinase

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation	Hirai, Syu-ichi, Masaru Katoh, Masaaki Terada, John M. Kyriakis, Leonard I. Zon, Ajay Rana, Joseph Avruch, and Shigeo Ohno. 1997. "MST/MLK2, a Member of the Mixed Lineage Kinase Family, Directly Phosphorylates and Activates SEK1, an Activator of c-Jun N-Terminal Kinase/Stress-Activated Protein Kinase." Journal of Biological Chemistry 272 (24): 15167–73. https://doi.org/10.1074/jbc.272.24.15167.
Citable link	http://nrs.harvard.edu/urn-3:HUL.InstRepos:41483023
Terms of Use	This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 24, Issue of June 13, pp. 15167–15173, 1997
© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

MST/MLK2, a Member of the Mixed Lineage Kinase Family, Directly Phosphorylates and Activates SEK1, an Activator of c-Jun N-terminal Kinase/Stress-activated Protein Kinase*

(Received for publication, November 14, 1996, and in revised form, March 24, 1997)

Syu-ichi Hirai‡§, Masaru Katoh¶, Masaaki Terada¶, John M. Kyriakis‖, Leonard I. Zon, Ajay Rana‖, Joseph Avruch‖, and Shigeo Ohno‡§**

*From the ‡Department of Molecular Biology, Yokohama City University School of Medicine, 3-9, Fukuura, Kanazawa-ku, Yokohama 236, Japan, the ¶Genetics Division, National Cancer Center Research Institute, Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan, the ‖Diabetes Unit and Medical Services, Massachusetts General Hospital East, and the Department of Medicine, Harvard Medical School, Charlestown, Massachusetts 02129, and the **Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115*

c-Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs) are mitogen-activated protein kinase (MAPK)-related protein kinases that are involved in several cellular events, including growth, differentiation, and apoptosis. Mixed lineage kinases (MLKs) form a family of protein kinases sharing two leucine zipper-like motifs and a kinase domain whose primary structure is similar to both the tyrosine-specific and the serine/threonine-specific kinase classes. We have reported that a member of the MLK family, MUK/DLK/ZPK, can activate JNK/SAPK *in vivo*, and here we show that another member of the MLK family, MST/MLK2, activates JNK/SAPK. Both MUK/DLK/ZPK and MST/MLK2 cause a slight activation of p38/Mpk2 when overexpressed in COS-1 cells, whereas MST/MLK2, but not MUK/DLK/ZPK, activates extracellular response kinase (ERK) to a certain degree. The activity of SEK1/MKK4/JNKK, a MAPK kinase class protein kinase designated as a direct activator of JNK/SAPK, is also induced by MUK/DLK/ZPK or MST/MLK2 overexpression. Furthermore, recombinant MST/MLK2 produced in bacteria directly phosphorylates and activates SEK1/MKK4/JNKK *in vitro*, showing that MST/MLK2 acts like a MAPK kinase kinase. Taken together, these results suggest that MLK family members are MAPK kinase kinases preferentially acting on the JNK/SAPK pathway.

Protein kinases of the MAPK[1] family are involved in a variety of cellular responses to cytokines, hormones, and stress-inducing reagents. MAPKs are classified into at least three groups, including ERK, JNK/SAPK, and p38/Mpk2, and each group phosphorylates a different set of substrates, including other protein kinases and transcription factors (1–3). JNKs/SAPKs were first identified as c-Jun N-terminal kinases (JNKs) or stress-activated protein kinases (SAPKs), both of which are activated in response to UV irradiation, osmotic shock, heat shock, and treatment with tumor necrosis factor-α or antibiotics known to be inhibitors of eukaryotic protein synthesis (4–7). The activity of JNKs/SAPKs depends greatly on the phosphorylation of specific threonine and tyrosine residues located in kinase subdomain VIII catalyzed by upstream activators or MAPKK class protein kinases (5). One such activator, known as SEK1/MKK4/JNKK, has been cloned, and its activity has been shown to be greatly dependent on the phosphorylation of specific serine and threonine residues located between kinase subdomains VII and VIII, as reported for MEKs, activators of ERKs (8–10). The activation-phosphorylation of SEK1/MKK4/JNKK is catalyzed by upstream protein kinases or MAPKKK class protein kinases such as MEKK1 (9, 11). Since JNK/SAPK is induced by several kinds of cellular stimuli, diverse MAPKKKs may act upstream of the JNK/SAPK pathway. In fact, increasing numbers of protein kinases are now identified as MAPKKKs acting on SEK1/MKK4/JNKK, including MEKK2 and MEKK3 (12) and Tpl-2/Cot (13). However, the molecular mechanism that regulates these MAPKKKs remains mostly unknown. In addition to SEK1/MKK4/JNKK, at least two other stress-induced JNK/SAPK activators have been identified in column fractions of cell extracts, suggesting the presence of multiple pathways for JNK/SAPK activation (14, 15).

Recently, we reported that a protein kinase, MUK, activates the JNK/SAPK pathway when overexpressed in cultured cells (16). MUK is a rat homologue of mouse DLK (17) and human ZPK (18), whose kinase domain shows structural features of both the tyrosine-specific and serine/threonine-specific protein kinase classes. In addition to a unique kinase domain, MUK/DLK/ZPK contains two leucine zipper-like structures located proximal to the C-terminal end of the catalytic domain. These features are common to members of the mixed lineage kinase (MLK) family, including MLK1 (19), MST/MLK2 (20, 21), and SPRK/PTK1/MLK3 (22–24). Therefore, MUK/DLK/ZPK has been identified as the fourth member of the MLK family (16–18).

The finding that MUK/DLK/ZPK activates the JNK/SAPK pathway raises the intriguing possibility that other members of the MLK family also activate the JNK/SAPK pathway or other MAPK pathways. More recently, we have shown that SPRK/PTK1/MLK3 also activates the JNK/SAPK pathway preferentially through SEK1/MKK4/JNKK (25). However, the ability to activate SEK1/MKK4/JNKK directly has not been demon-

* This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan, the Cell Science Foundation, and the Naito Foundation. 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 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 81-45-787-2596/2597; Fax: 81-45-785-4140; E-mail: sh3312@med.yokohama-cu.ac.jp.

[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; ERK, extracellular response kinase; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase; MLK, mixed lineage kinase; GST, glutathione *S*-transferase; HA, hemagglutinin; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis.

Downloaded from http://www.jbc.org/ by guest on October 4, 2019

strated for either SPRK/PTK1/MLK3 or MUK/DLK/ZPK, leaving the possibility that another direct activator of SEK1/MKK4/JNKK may be involved. In this report, we show that MST/MLK2, like MUK/DLK/ZPK, activates JNK/SAPK and SEK1/MKK4/JNKK in COS-1 cells. Furthermore, we show that recombinant MST/MLK2 directly phosphorylates and activates a recombinant SEK1/MKK4/JNKK *in vitro*. These results show that MST/MLK2 and probably other members of the MLK family also are MAPKKKs that act preferentially on the JNK/SAPK pathway.

MATERIALS AND METHODS

Expression Vectors—The mammalian expression vectors for MUK (16), GST-SEK1 (8), and the epitope-tagged enzymes T7-tagged MUK, T7-tagged MUK/KN, T7-tagged JNK1, and T7-tagged ERK2 (16) and HA-tagged p38 (25) have been described elsewhere. The expression vector for MST was constructed with the mammalian expression vector SRα and human MST cDNA (20). T7-tagged MST was constructed with SRα, a DNA fragment encoding a His tag/T7 epitope derived from pBlueBac (Invitrogen), and MST cDNA. The first nine amino acid residues of the MST protein produced from this expression vector were replaced by a His tag/T7 epitope consisting of 40 amino acid residues. A kinase-defective mutant of MST, MST/KN, was constructed with the T7-tagged MST expression vector by in-frame deletion of residues 140–185, corresponding to kinase subdomains III–V.

Transfection and Stimulation of Cells—COS-1 cells were routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were transfected by an electroporation method using 16 μg of DNA for 5×10^6 cells seeded in four 10-cm dishes after the transfection. Cells were further cultured for 48 h before harvest. When required, 100 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate or 0.7 M NaCl was added 10 or 40 min before harvest. For UV irradiation, cells were exposed to 100 J of UV light/m^2 40 min before harvest.

Immunoprecipitation and Solid-phase Kinase Assay of Epitope-tagged JNK1, ERK2, and p38—Cells grown in 10-cm dishes were lysed in 250 μl of lysis buffer consisting of 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 50 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Deoxycholate and SDS were omitted when antibody against the hemagglutinin tag was used for immunoprecipitation. Immunoprecipitation of epitope-tagged enzymes was carried out at 4 °C for 1 h using 1 μg of mouse monoclonal antibody against either the T7 epitope (Invitrogen) or the HA tag (Boehringer Mannheim) pre-fixed on 5 μl of protein G-Sepharose 4 Fast Flow (Pharmacia Biotech Inc.). The Sepharose resin was washed five times with lysis buffer and finally washed with detergent-free lysis buffer, and aliquots were collected in two tubes: one was used for the solid-phase kinase assay, and the other was extracted with SDS sample buffer for Western blot analysis. For the solid-phase kinase assay, the epitope-tagged enzymes fixed on 2 μl of protein G-Sepharose were suspended in 20 μl of assay mixture containing 20 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 15 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 mM dithiothreitol, 25 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 1 μg of recombinant c-Jun, recombinant CREBP-1/ATF2, or bovine brain myelin basic protein (MBP) (Sigma M1891). The kinase reaction was carried out at 30 °C for 20 min and stopped by adding 25 μl of SDS sample buffer. The phosphorylation of each substrate was detected by SDS-PAGE, and the amount of ^{32}P incorporated was quantified with a Fuji BAS2000 image analyzer.

Production and Purification of Recombinant Proteins—Expression vectors for GST-SEK1, GST-JNK1, and GST-ERK1 were constructed with the bacteria expression vector pGEX-2T or pGEX-3X (Pharmacia) and cDNA encoding SEK1, the SEK1(K/R) mutant (9), MEK1, the MEK1(K/R) mutant (37), JNK1, or ERK1 (16). To construct the MalE-MST expression vector, a cDNA fragment encoding MST amino acids 10–757 was cloned into the pMAL-c2 vector (New England Biolabs Inc.). The expression vector for c-Jun was described elsewhere (16). The expression vector for GST-CREBP-1 was the kind gift of Drs. T. Maekawa and S. Ishii (38). The production of GST and MalE fusion proteins in bacteria (*Escherichia coli* DH1) was started by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside and continued for 18 h at 25 °C. The fusion proteins were purified according to the standard protocol using glutathione-Sepharose (Pharmacia) or amylose resin (New England Biolabs Inc.). Routinely, 1~5 mg of fusion proteins were recovered from 500 ml of bacteria culture.

In Vitro Assay of GST-SEK1 Expressed in COS-1 Cells—COS-1 cells grown in 10-cm dishes were lysed in 250 μl of G buffer containing 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. GST-SEK1 was recovered using 5 μl of glutathione-Sepharose. The resin was washed five times with G buffer and then washed one time with detergent-free G buffer. GST-SEK1 was eluted from the resin with 100 μl of 5 mM glutathione in detergent-free G buffer.

For the *in vitro* kinase assay, 5 μl of the GST-SEK1 solution were first incubated with 50 ng of GST-JNK1 in 20 μl of the assay mixture described above containing 50 μM ATP at 30 °C for 15 min. Then, 2 μl of substrate mixture containing 1 μg of c-Jun, 2.5 mM ATP, and 5 μCi of [γ-^{32}P]ATP were added, and the mixture was further incubated for 15 min. The reaction was stopped by adding 25 μl of SDS sample buffer, and the phosphorylation of c-Jun was quantified as described above.

In Vitro Kinase Assay of Recombinant MST—The *in vitro* phosphorylation of GST-SEK1(K/R) or GST-MEK1(K/R) by MalE-MST-(10–757) was carried out at 30 °C for 45 min in 20 μl of assay mixture containing 1 μg of GST-SEK1(K/R) or 2 μg of GST-MEK1(K/R) and 1 or 4 μg of MalE-MST-(10–757), adjusting the total amount of protein to 6 μg by adding MalE protein. The reaction was stopped by adding 25 μl of SDS sample buffer, and the proteins were analyzed by SDS-PAGE with Coomassie Brilliant Blue staining and autoradiography.

The activation of GST-SEK1 or GST-MEK1 by MalE-MST-(10–757) was tested by a two-step kinase reaction. The first step was carried out at 30 °C for 45 min in 20 μl of assay mixture containing 25 μM unlabeled ATP, 50 ng of GST-JNK1 or GST-ERK1, 200 ng of GST-SEK1 or GST-MEK1, and 1 or 4 μg of MalE-MST-(10–757), adjusting the total amount of protein to 5.25 μg by adding MalE protein. The second reaction was started by adding 2 μl of substrate mixture containing 1 μg of c-Jun or myelin basic protein, 2.5 mM ATP, and 5 μCi of [γ-^{32}P]ATP and incubating the reaction mixture for a further 15 min. The phosphorylation of c-Jun or myelin basic protein was analyzed by SDS-PAGE as described above.

To test the ATP dependence of the activation of GST-SEK1 by MalE-MST-(10–757), 10 μg of MalE-MST-(10–757) and 2 μg of GST-SEK1 were incubated in 200 μl of assay mixture in the presence or absence of 50 μM ATP at 30 °C for 30 min. GST-SEK1 was then affinity-purified and assayed for JNK1 activation activity as described for the *in vitro* assay of GST-SEK1 expressed in COS-1 cells.

RESULTS

MST Activates the JNK/SAPK Pathway in COS-1 Cells—The ability of MST to activate three different MAPK-related protein kinase pathways (JNK/SAPK, p38/Mpk2, and ERK/MAPK) was tested in cotransfection experiments using COS-1 cells with immunoprecipitation of the epitope-tagged enzymes and solid-phase kinase assay using appropriate substrates as indicated in Fig. 1 (*A–C*). The overexpression of MUK and MST, like UV irradiation, caused a >10-fold induction of tagged JNK1 activity (Fig. 1*A*). The activity of tagged p38 was induced modestly (1.2~1.5-fold) by MUK or MST under conditions where osmotic shock with 0.7 M NaCl caused a >5-fold induction (Fig. 1*B*). The activity of tagged ERK2 was not induced by MUK at all (Ref. 16 and Fig. 1*C*), whereas it was induced modestly (2~3-fold) by MST, and 12-*O*-tetradecanoylphorbol-13-acetate stimulation caused a >10-fold induction (Fig. 1*C*). The immunoprecipitated enzymes were also analyzed by SDS-PAGE followed by Western blotting using anti-tag antibodies (Fig. 1*D*). Note that the amounts of enzymes used for the solid-phase kinase assay were roughly the same for each assay and that the activation of tagged JNK1 and tagged ERK2 could also be detected as a mobility delay by SDS-PAGE. Taken together, these data show that MST, like MUK, preferentially activates the JNK/SAPK pathway with a somewhat loose downstream specificity.

MST Activates SEK1 in COS-1 Cells—The activity of JNK/SAPK depends greatly on the phosphorylation of specific threonine and tyrosine residues catalyzed by upstream MAPKK class protein kinases such as SEK1 (7). Therefore, the ability of MUK and MST to activate SEK1 was tested in cotransfection experiments using COS-1 cells. GST-SEK1 was coexpressed with tagged MUK or tagged MST in COS-1 cells, and the

Downloaded from http://www.jbc.org/ by guest on October 4, 2019

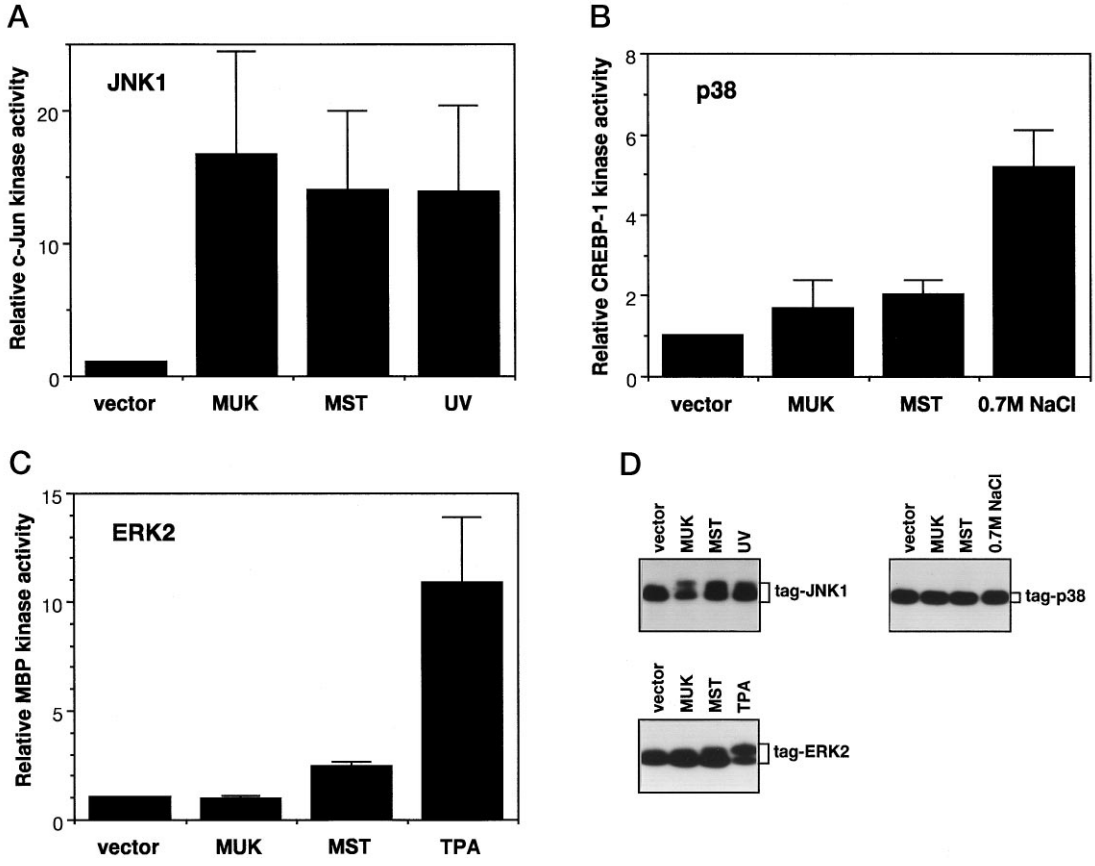

FIG. 1. **Activation of MAPK-related kinases by MUK and MST.** COS-1 cells were cotransfected with the expression vector for T7-tagged JNK1, HA-tagged p38, or T7-tagged ERK2 together with the expression vector for MUK or MST. Epitope-tagged enzymes were immunoprecipitated using appropriate antibodies, and activity was tested *in vitro*. *A*, the activity of T7-tagged JNK1 was tested using c-Jun produced in bacteria as a substrate. To obtain a potent activation of T7-tagged JNK1, vector-transfected cells were exposed to 100 J of UV light/m^2 40 min before the harvest. *B*, the activity of HA-tagged p38 was tested using GST-CREBP-1/ATF2 produced in bacteria as a substrate. To obtain a potent activation of HA-tagged p38, vector-transfected cells were treated with 0.7 M NaCl for 40 min before harvest. *C*, the activity of T7-tagged ERK2 was tested using bovine brain myelin basic protein as a substrate. As a control, vector-transfected cells were treated with 100 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (*TPA*) for 10 min before harvest. Each graph shows average values obtained from more than three independent transfection experiments. *Bars* indicate S.D. *D*, the immunoprecipitated enzymes were analyzed by Western blotting using anti-T7 tag or anti-HA tag antibodies. Typical patterns are shown.

activity of GST-SEK1 was measured *in vitro* after its recovery from the cells with a GST affinity resin. Two recombinant proteins, GST-JNK1 (a substrate for SEK1) and c-Jun (a substrate for JNK1), were produced in bacteria and used for the *in vitro* assay of GST-SEK1 activity; activity was finally detected as the incorporation of radiolabeled phosphate into c-Jun (Fig. 2*A*). The overexpression of tagged MUK or tagged MST in COS-1 cells caused a 10~30-fold activation of coexpressed GST-SEK1. On the other hand, no such activation of GST-SEK1 was observed when the tagged MST or tagged MUK mutant lacking a part of the kinase domain (*tag-MST/KN* and *tag-MUK/KN*) was overexpressed, although the expression of these mutants was confirmed by Western blot analysis using anti-tag antibody (Fig. 2*B*, *upper panel*). The incorporation of radiolabeled phosphate into c-Jun depended greatly on the presence of GST-JNK1, and very little activity was recovered from cells not transfected with the expression vector for GST-SEK1. Furthermore, roughly the same amount of GST-SEK1 was recovered from different sets of cells coexpressing different proteins (Fig. 2*B*, *lower panel*). Taken together, these results demonstrate the ability of MUK and MST to induce SEK1 activation in COS-1 cells.

Recombinant MST Directly Phosphorylates and Activates SEK1 in Vitro—The kinase domains of MLKs show considerable amino acid sequence homology to those of MAPKKK class protein kinases such as c-Raf and TAK. The MST kinase domain shares 35 and 40% amino acid identity with c-Raf and TAK, respectively. Furthermore, the activation of SEK1 *in vitro* by overexpressed SPRK immunoprecipitated from COS cells has been reported (25). These observations suggest that members of the MLK family are direct activators of SEK1, but do not rule out the possibility that they act farther upstream as activators of certain MAPKKKs. We then produced recombinant MST in bacteria as a fusion protein with maltose-binding protein (MalE) to test its ability to activate SEK1 *in vitro*. The MalE-MST-(10–757) fusion protein affinity-purified on amylose resin appeared as several bands ranging between 80 and ~140 kDa on SDS-PAGE (Fig. 3*A*). The uppermost band corresponds to the expected molecular size of MalE-MST-(10–757), whereas the other bands may represent partially degraded protein since these as well as the 140-kDa band react with anti-MalE antibody (data not shown). The ability of MalE-MST-(10–757) to phosphorylate recombinant GST-SEK1 or GST-MEK1 was first tested. To limit the autophosphorylation of GST-SEK1 or GST-MEK1, an invariant lysine residue located in kinase subdomain II of these kinases was mutated to arginine. MalE-MST-(10–757) could phosphorylate GST-SEK1(K/R) as well as MalE-MST-(10–757) itself (Fig. 3*B*). However, the phosphorylation of GST-MEK1(K/R) was hardly detected. In addition, neither MalE nor GST was phosphorylated. Therefore, SEK1 is a rather specific substrate for MST *in vitro*.

Downloaded from http://www.jbc.org/ by guest on October 4, 2019

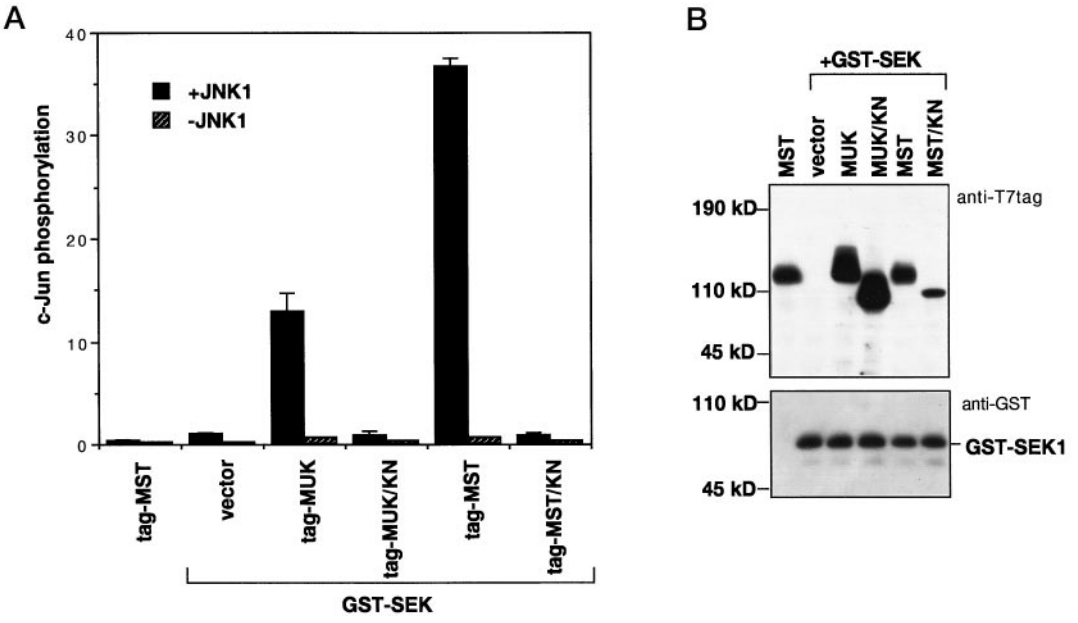

FIG. 2. **Activation of GST-SEK1 by MST and MUK in COS-1 cells.** COS-1 cells were cotransfected with the expression vector for GST-SEK1 together with the expression vector for T7-tagged MUK, MUK/KN, MST, or MST/KN. The activity of GST-SEK1 recovered from the COS-1 cells was tested *in vitro* using recombinant GST-JNK1 and c-Jun produced in bacteria. *A*, after the kinase reaction, proteins in the assay mixture were separated by SDS-PAGE, and c-Jun phosphorylation was quantified. Average values obtained with three different GST-SEK1 preparations are shown. *Bars* indicate S.D. *B*, the expression of T7-tagged MUK, MUK/KN, MST, or MST/KN in COS-1 cells was monitored by Western blot analysis using anti-T7 tag antibody (*upper panel*). An aliquot of GST-SEK1 recovered from COS-1 cells was analyzed by Western blotting using anti-GST antibody (*lower panel*). Typical patterns are shown.

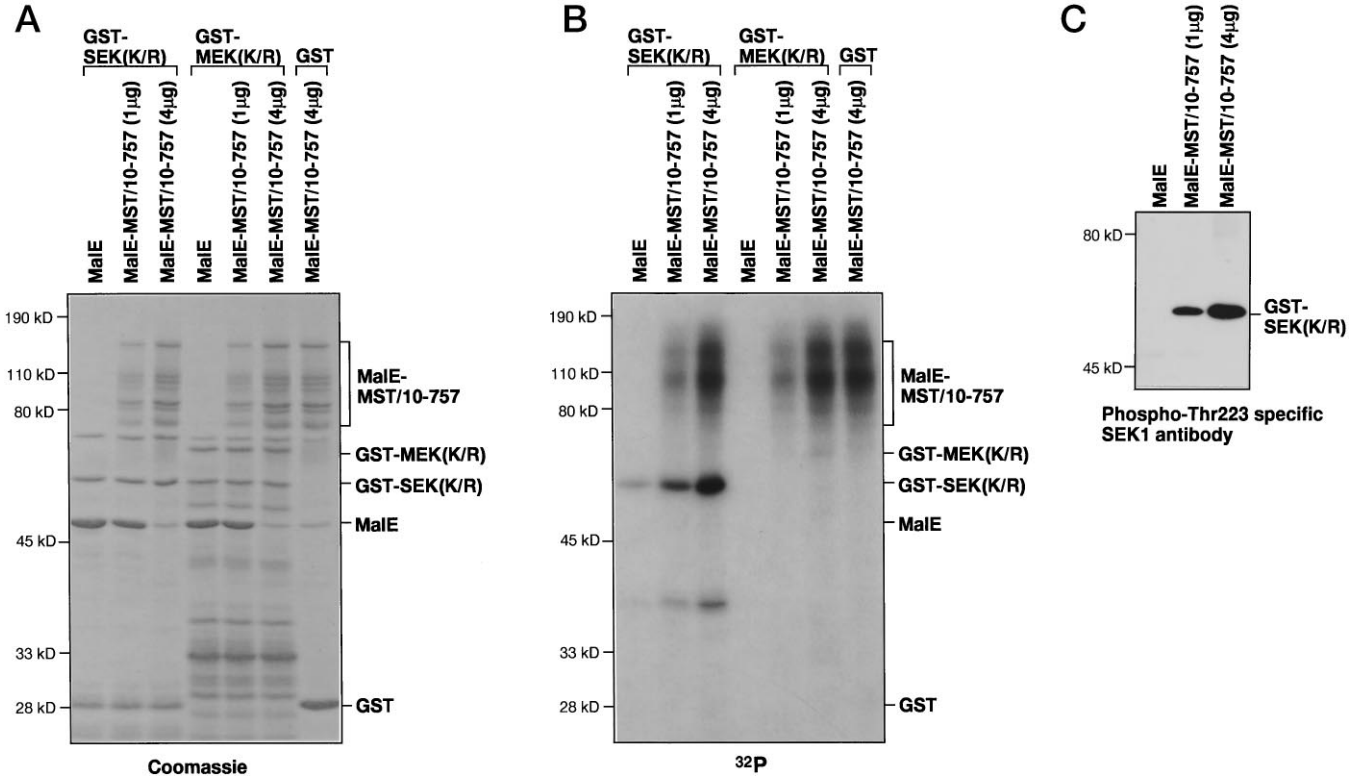

FIG. 3. **Phosphorylation of SEK1 by MST.** The indicated amounts of MalE-MST-(10–757) were incubated with 1 μg of GST-SEK1(K/R), 2 μg of GST-MEK1(K/R), or 3 μg of GST in an assay mixture containing [γ-^{32}P]ATP. The total amount of MalE fusion protein was adjusted to 6 μg by adding MalE protein to each lane. *A*, proteins in the assay mixture were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. In addition to the intact GST-SEK1(K/R) and GST-MEK1(K/R) proteins as indicated, partly degraded proteins can be seen. *B*, the gel was then exposed to x-ray film to detect phosphorylated proteins. *C*, the phosphorylation reaction was carried out using the indicated amounts of MalE-MST-(10–757), 1 μg of GST-SEK1(K/R), and unlabeled ATP, and proteins were analyzed by Western blotting using a Thr(P)-223-specific SEK1 antibody.

It has been reported that the activity of SEK1 is greatly dependent on the phosphorylation of serine and threonine residues at positions 219 and 223 of SEK1 (11). To test whether the threonine residue is phosphorylated by MalE-MST-(10–757), GST-SEK1(K/R) phosphorylated by MalE-MST-(10–757) was analyzed by Western blotting using a Thr(P)-223-specific

Downloaded from http://www.jbc.org/ by guest on October 4, 2019

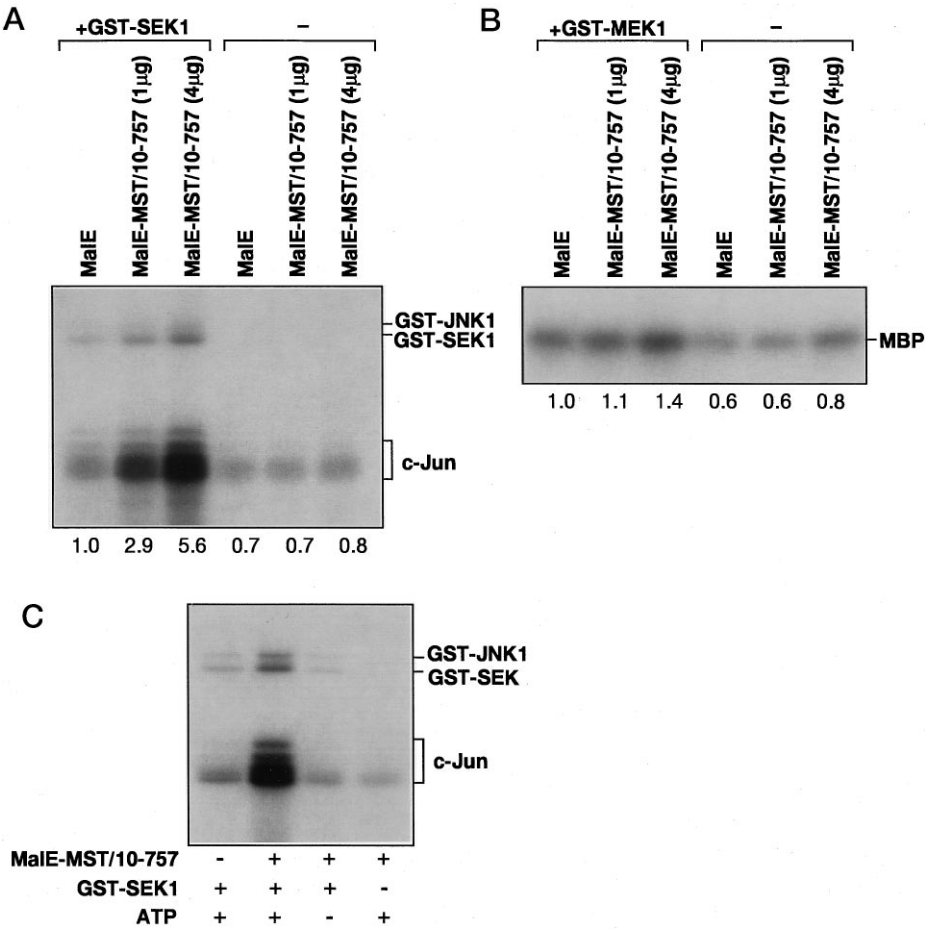

FIG. 4. **Activation of SEK1 by MST.** *A*, the indicated amounts of MalE-MST-(10–757) were first incubated at 30 °C for 45 min with unlabeled ATP and 50 ng of GST-JNK1 in the presence (*first three lanes*) or absence (*last three lanes*) of GST-SEK1 (200 ng). Then, 1 μg of c-Jun was added together with [γ-^{32}P]ATP, and the mixtures were incubated at 30 °C for 15 min. Phosphorylated c-Jun in the assay mixture was analyzed by SDS-PAGE followed by autoradiography. The values shown at the bottom are relative amounts of ^{32}P incorporated into c-Jun. *B*, the phosphorylation reaction was carried out as described for *A* using 200 ng of GST-MEK1, 50 ng of GST-ERK1, and 1 μg of MBP instead of GST-SEK1, GST-JNK1, and c-Jun, respectively. The values shown at the bottom are relative amounts of ^{32}P incorporated into MBP. *C*, MalE-MST-(10–757), GST-SEK1, and unlabeled ATP were mixed as indicated and incubated at 30 °C for 30 min. GST-SEK1 was then isolated with glutathione-Sepharose and incubated at 30 °C for 15 min with 50 ng of GST-JNK1 and unlabeled ATP. Finally, c-Jun was added together with [γ-^{32}P]ATP, and the mixtures were further incubated for 15 min. Phosphorylated c-Jun in the assay mixture was analyzed by SDS-PAGE followed by autoradiography.

SEK1 antibody (New England Biolabs Inc.). As shown in Fig. 3*C*, the reactivity of the antibody toward GST-SEK1(K/R) increased significantly after phosphorylation by MalE-MST-(10–757). Therefore, MalE-MST-(10–757) induces the phosphorylation of the threonine residue responsible for the activation of GST-SEK1.

We next tested whether MalE-MST-(10–757) activates GST-SEK1 *in vitro*. The activity of GST-SEK1 was estimated by its ability to activate GST-JNK1, whose activity in turn was estimated by its ability to phosphorylate c-Jun. When GST-JNK1 was preincubated with GST-SEK1 and MalE-MST-(10–757) in the presence of ATP, it phosphorylated c-Jun quite efficiently, whereas only limited phosphorylation was observed when MalE was used instead of MalE-MST-(10–757) (Fig. 4*A*, *first three lanes*). Notably, the enhancement of c-Jun phosphorylation induced by MalE-MST-(10–757) was totally dependent on the presence of GST-SEK1, and neither the direct activation of GST-JNK1 nor the direct phosphorylation of c-Jun by MalE-MST-(10–757) was observed (Fig. 4*A*, *last three lanes*). The basal incorporation of phosphate into c-Jun observed in the absence of GST-SEK1 depends on the intrinsic activity of GST-JNK1 since very little phosphorylation of c-Jun was observed in the absence of GST-JNK1 (data not shown). We also tested whether MalE-MST-(10–757) activates GST-MEK1 *in vitro*. The activity of GST-MEK1 was estimated by its ability to activate GST-ERK1, whose activity in turn was estimated by its ability to phosphorylate MBP. When GST-ERK1 was preincubated with GST-MEK1 and MalE-MST-(10–757) in the presence of ATP, the phosphorylation of MBP was slightly enhanced (Fig. 4*B*, *first three lanes*). However, a slight enhancement of MBP phosphorylation was also observed in the absence of GST-MEK1 (Fig. 4*B*, *last three lanes*), and MalE-MST-(10–757) itself can weakly phosphorylate MBP (data not shown). Therefore, the activation of GST-MEK1 by MalE-MST-(10–757) must be little if anything, as suggested by the very weak activity of MalE-MST-(10–757) in phosphorylating GST-MEK1(K/R) (Fig. 3*B*).

To test whether the activation of GST-SEK1 depends on phosphorylation by MST, GST-SEK1 was preincubated with MalE-MST-(10–757) in the presence or absence of ATP and assayed for JNK1 activation activity. As shown in Fig. 4*C*, incubation with MalE-MST-(10–757) in the absence of ATP did not cause the activation of GST-SEK1, whereas a significant activation of GST-SEK1 was caused by incubation in the presence of ATP. Therefore, MalE-MST-(10–757) acts like a MAPKKK and directly phosphorylates and activates GST-SEK1 *in vitro*.

DISCUSSION

We show here that a member of the MLK family, MST/MLK2, activates MAPK pathways. We have already reported that other members of the MLK family, MUK/DLK/ZPK and SPRK/PTK1/MLK3, also activate MAPK pathways (16, 25). All these kinases activate the JNK/SAPK and p38/Mpk2 pathways when overexpressed in COS cells, while the activation of p38/Mpk2 is rather modest. However, only MST/MLK2 slightly activates the ERK pathway. We also show that recombinant MST/MLK2 produced in bacteria directly phosphorylates and activates SEK1 *in vitro*. Therefore, MST/MLK2 is a novel MAPKKK, and together with MST/MLK2, all members of the MLK family may be classified into the MAPKKK class of protein kinases that act preferentially on the JNK/SAPK pathway.

All members of the MLK family share a well conserved kinase domain, especially MLK1, SPRK/PTK1/MLK3, and

Downloaded from http://www.jbc.org/ by guest on October 4, 2019

MST/MLK2, showing 75–77% amino acid identity. An additional member of the MLK family, MUK/DLK/ZPK, has a somewhat divergent kinase domain, sharing 46~48% amino acid identity with the others. Among protein kinases outside the MLK family, all show the highest amino acid identity (34~40%) to the kinase domains of TAK and c-Raf, which are known to act like MAPKKKs (26, 27). This agrees with our conclusion that MLKs act like MAPKKKs. However, the kinase domains of MLK family members share rather limited numbers of amino acids (29~34%) with MEKKs, MEKK1, MEKK2, and MEKK3, which also act like MAPKKKs (12, 28). In addition, the amino acid sequence identity with MEKKs is comparable to that with protein kinases situated farther upstream such as PAK1 (29) and GCK (30) (28–33%). The amino acid sequence identity is also low between the kinase domains of MEKKs and c-Raf or TAK, ranging from 26 to ~31%. Therefore, MEKKs are rather distantly related to other MAPKKKs, including Raf, TAK, and MLKs. Instead, a relatively high number of amino acid residues (31~34%) are shared between the kinase domains of MEKKs and Tpl-2/Cot, a recently identified MAPKKK that activates the ERK and JNK/SAPK pathways (13), suggesting the presence of at least two groups of MAPKKKs carrying structurally distinguishable kinase domains. These two groups are also characterized by their sequence similarity to tyrosine kinases such as c-Abl and c-Met; the kinase domains of Raf, TAK, and MLKs share 29~37% amino acid sequence identity with these tyrosine kinases, whereas the kinase domains of MEKKs and Tpl-2/Cot share rather limited amino acid sequence identity, ranging from 24 to ~27%. In contrast to the considerably diverged features of MAPKKK class protein kinases, MAPKK class protein kinases carry highly conserved kinase domains, showing at least 40% amino acid sequence identity among them (9). Therefore, MAPKKKs may have evolved from two or more different ancestral protein kinases, whereas all MAPKKs may have originated from a single ancestor.

The selective activation of target MAPK pathways by MAPKKK depends on the substrate specificity of each MAPKKK. The substrate specificity might be determined not only by the direct interaction of a MAPKKK and its substrate(s), MAPKK(s), but also by an additional factor, such as STE5 protein of budding yeast, that acts as an adapter molecule for a certain set of protein kinases (31). The rather weak activity of MST/MLK2 in phosphorylating SEK1/MKK4/JNKK *in vitro* could be explained by the lack of such an additional factor as well as by the structural abnormality of MST/MLK2 produced in bacteria. In any case, the ability of MST/MLK2 to activate SEK1/MKK4/JNKK indicates that the activation of JNK/SAPK by MST/MLK2 *in vivo* is mediated by SEK1/MKK4/JNKK. In addition, some other MAPKKs that have not been cloned yet (14, 15) could also be involved in the activation of JNK/SAPK by MST/MLK2 *in vivo*.

The specific response of each MAPK pathway to several different extracellular stimuli may depend on which MAPKKK(s) is activated by the stimulation. Furthermore, it may also depend on which MAPKKKs and MAPKKs are expressed in the cell. Both MST/MLK2 and SEK1/MKK4/JNKK are highly expressed in skeletal muscle and brain among the human tissues tested, implying that they play an important role in the activation of JNK/SAPK in these tissues (20, 21). In addition, several tumor cell lines show high levels of MST expression, implying some function of MST/MLK2 in cellular transformation (20). Notably, MST/MLK2 can partly activate the ERK pathway, which plays an important role in cellular transformation (32–34). However, the question of whether the constitutive activation of MST causes cellular transformation remains to be answered.

The mechanisms by which MST/MLK2 or other members of the MLK family are activated remain unclear. Overexpressed MST is active by itself, and stimulation of cells by serum, UV light, or osmotic shock does not produce any synergistic effect with overexpressed MST.[2] Furthermore, the overexpression of other members of the MLK family, MUK/DLK/ZPK and SPRK/PTK1/MLK3, as well as other MAPKKKs such as MEKKs and Tpl-2/Cot also causes a potent activation of JNK/SAPK without any extracellular stimuli (12, 13, 16, 25). Therefore, the activity of these protein kinases may be regulated by a mechanism that does not function well with overexpressed enzymes. The control of mRNA or protein synthesis is a candidate for such a mechanism. In addition, the activity could be regulated by a limited amount of enzymatic or nonenzymatic inhibitor.

MST/MLK2, as well as other members of the MLK family, contains two leucine zipper-like motifs proximal to the C-terminal end of the kinase domain and a Rac/Cdc42-binding motif (35) proximal to the C-terminal end of the leucine zipper-like motifs. In addition, MST/MLK2 and SPRK/PTK1/MLK3 have SH3 domains proximal to the N-terminal end of the kinase domain. These motifs may contribute to the interaction, if any, with other proteins. Recently, it was reported that an upstream protein kinase, HPK1, interacts with SPRK/PTK1/MLK3 through the SH3 domain (36). However, the functional significance of these motifs remains to be clarified.

Acknowledgments—We thank Drs. Toshio Maekawa and Shunsuke Ishii for the gift of the GST-CREBP-1 expression vector. We also thank Kumi Noda for excellent technical support.

REFERENCES

1. Nishida, E., and Gotoh, Y. (1993) *Trends Biochem. Sci.* **18,** 128–131
2. Davis, R. J. (1994) *Trends Biochem. Sci.* **19,** 470–473
3. Cano, E., and Mahadevan, L. C. (1995) *Trends Biochem. Sci.* **20,** 117–122
4. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) *Genes Dev.* **7,** 2135–2148
5. Dérijard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. (1994) *Cell* **76,** 1025–1037
6. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, R. F., Avruch, J., and Woodgett, J. R. (1994) *Nature* **369,** 156–160
7. Galcheva-Gargova, Z., Dérijard, B., Wu, I.-H., and Davis, R. J. (1994) *Science* **265,** 806–808
8. Sánchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M., and Zon, L. I. (1994) *Nature* **372,** 794–798
9. Dérijard, B., Raingeaud, J., Barrett, T., Wu, I.-H., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) *Science* **267,** 682–685
10. Lin, A., Minden, A., Martinetto, H., Claret, F.-X., Lange-Carter, C., Mercurio, F., Johnson, G. L., and Karin, M. (1995) *Science* **268,** 286–290
11. Yan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R., and Templeton, D. J. (1994) *Nature* **372,** 798–800
12. Blank, J. L., Gerwins, P., Elliott, E. M., Sather, S., and Johnson, G. L. (1996) *J. Biol. Chem.* **271,** 5361–5368
13. Salmerón, A., Ahmad, T. B., Carlile, G. W., Pappin, D., Narsimhan, R. P., and Ley, S. C. (1996) *EMBO J.* **15,** 817–826
14. Moriguchi, T., Kawasaki, H., Matsuda, S., Gotoh, Y., and Nishida, E. (1995) *J. Biol. Chem.* **270,** 12969–12972
15. Meier, R., Rouse, J., Cuenda, A., Nebreda, A. R., and Cohen, P. (1996) *Eur. J. Biochem.* **236,** 796–805
16. Hirai, S.-i., Izawa, M., Osada, S.-i., Spyrou, G., and Ohno, S. (1996) *Oncogene* **12,** 641–650
17. Holzman, L. B., Merritt, S. E., and Fan, G. (1994) *J. Biol. Chem.* **269,** 30808–30817
18. Reddy, U. R., and Pleasure, D. E. (1994) *Biochem. Biophys. Res. Commun.* **202,** 613–620
19. Dorow, D. S., Devereux, L., Dietzsch, E., and De Kretser, T. (1993) *Eur. J. Biochem.* **213,** 701–710
20. Katoh, M., Hirai, M., Sugimura, T., and Terada, M. (1995) *Oncogene* **10,** 1447–1451
21. Dorow, D. S., Devereux, L., Tu, G. F., Price, G., Nicholl, J. K., Sutherland, G. R., and Simpson, R. J. (1995) *Eur. J. Biochem.* **234,** 492–500
22. Gallo, K. A., Mark, M. R., Scadden, D. T., Wang, Z., Gu, Q., and Godowski, P. J. (1994) *J. Biol. Chem.* **269,** 15092–15100
23. Ezoe, K., Lee, S.-T., Strunk, K. M., and Spritz, R. A. (1994) *Oncogene* **9,** 935–938
24. Ong, Y. L., Leung, I. W. L., Heng, H. H. Q., Tsui, L.-C., and Lassam, N. J. (1994) *Oncogene* **9,** 1745–1750
25. Rana, A., Gallo, K., Godowski, P., Hirai, S.-i., Ohno, S., Zon, L., Kyriakis, J. M.,

[2] S.-i. Hirai, unpublished data.

Downloaded from http://www.jbc.org/ by guest on October 4, 2019

and Avruch, J. (1996) *J. Biol. Chem.* **271,** 19025–19028
26. Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K. (1995) *Science* **270,** 2008–2011
27. Daum, G., Eisenmann-Tappe, I., Fries, H.-W., Troppmair, J., and Rapp, U. R. (1994) *Trends Biochem. Sci.* **19,** 474–480
28. Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J., and Johnson, G. L. (1993) *Science* **260,** 315–319
29. Manser, E., Leung, T., Salihuddin, H., Zhao, Z., and Lim, L. (1994) *Nature* **367,** 40–46
30. Pombo, C., Kehrl, J. H., Sánchez, I., Katz, P., Avruch, J., Zon, L. I., Woodgett, J. R., Force, T., and Kyriakis, J. M. (1995) *Nature* **377,** 750–754
31. Choi, K.-Y., Satterberg, B., Lyons, D. M., and Elion, E. A. (1994) *Cell* **78,** 499–512
32. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) *Cell* **77,** 841–852
33. Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woude, G. F., and Ahn, N. G. (1994) *Science* **265,** 966–970
34. Sun, H., Tonks, N. K., and Bar-Sagi, D. (1994) *Science* **266,** 285–288
35. Burbelo, P. D., Drechsel, D., and Hall, A. (1995) *J. Biol. Chem.* **270,** 29071–29074
36. Kiefer, F., Tibbles, L. A., Anafi, M., Janssen, A., Zanke, B. W., Lassam, N., Pawson, T., Woodgett, J. R., and Iscove, N. N. (1996) *EMBO J.* **15,** 7013–7025
37. Ueda, Y., Hirai, S.-i., Osada, S.-i., Suzuki, A., Mizuno, K., and Ohno, S. (1996) *J. Biol. Chem.* **271,** 23512–23519
38. Maekawa, T., Sakura, H., Kanei-Ishii, C., Sudo, T., Yoshimura, T., Fujisawa, J.-i., Yoshida, M., and Ishii, S. (1989) *EMBO J.* **8,** 2023–2028

Downloaded from http://www.jbc.org/ by guest on October 4, 2019

MST/MLK2, a Member of the Mixed Lineage Kinase Family, Directly Phosphorylates and Activates SEK1, an Activator of c-Jun N-terminal Kinase/Stress-activated Protein Kinase

Syu-ichi Hirai, Masaru Katoh, Masaaki Terada, John M. Kyriakis, Leonard I. Zon, Ajay Rana, Joseph Avruch and Shigeo Ohno

J. Biol. Chem. 1997, 272:15167-15173.
doi: 10.1074/jbc.272.24.15167

Access the most updated version of this article at http://www.jbc.org/content/272/24/15167

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 15 of which can be accessed free at
http://www.jbc.org/content/272/24/15167.full.html#ref-list-1

Downloaded from http://www.jbc.org/ by guest on October 4, 2019