A cDNA encoding a novel member of the mitogen-activated protein kinase kinase (MAPKK) family, MAPKK6, was isolated and found to encode a protein of 334 amino acids, with a calculated molecular mass of 37 kDa that is 79% identical to MKK3. MAPKK6 was shown to phosphorylate and specifically activate the p38/MKP2 subgroup of the mitogen-activated protein kinase superfamily and could be demonstrated to be phosphorylated and activated in vitro by TAK1, a recently identified MAPKK kinase. MKK3 was also shown to be a good substrate for TAK1 in vitro. Furthermore, when co-expressed with TAK1 in cells in culture, both MAPKK6 and MKK3 were strongly activated. In addition, co-expression of TAK1 and p38/MKP2 in cells resulted in activation of p38/MKP2. These results indicate the existence of a novel kinase cascade consisting of TAK1, MAPKK6/MKK3, and p38/MKP2.

Mitogen-activated protein kinase (MAPK)1 and its direct activator, MAPK kinase (MAPKK), have been shown to function in a wide variety of biological processes (1–4). Recent studies identified novel subgroups of the MAPK superfamily that include stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) (5, 6) and p38/MPK2 (7–9), in addition to classical MAPKs. SAPK/JNK, p38/MKP2, and MAPK are characterized by having the Thr-Pro-Tyr (TPY), the Thr-Gly-Tyr (TGY), and the Thr-Glu-Tyr (TEY) sequences, respectively, as the dual phosphorylation motif (1–10). Further, several subgroups of the MAPKK superfamily have been identified, such as MKK3 (11), MKK4/SEK1/JNKK (11–14), and MEK5 (15, 16). MKK3 is a specific activator for p38/MPK2 (11), MKK4/SEK1/JNKK (11–14), and MEK5 (15, 16). MKK3 is a specific activator for p38/MKP2 (11), and MKK4/SEK1/JNKK may function as an activator for SAPK/JNK, although it can also activate p38/MKP2 in vitro (11, 13, 14, 17, 18). Like classical MAPKs, these novel members of the MAPKK superfamily are thought to be activated by phosphorylation catalyzed by upstream serinethreonine kinases, members of the MAPKKK superfamily. In fact, it has been shown that MEKK can work as a direct activator for MKK4/SEK1 (17, 19). No reports have appeared, however, identifying an upstream kinase for MKK3.

Here we report cDNA cloning and characterization of a novel member of MAPKK, tentatively called MAPKK6, with 79% identity to MKK3, which specifically activates p38/MKP2. Our studies in vitro and in various cells further suggest that TAK1 (20), a recently identified, novel member of the MAPKKK family, can work as a direct activator for both MAPKK6 and MKK3. In addition, co-expression of TAK1 and p38/MKP2 resulted in activation of p38/MKP2, suggesting the existence of a novel kinase cascade consisting of TAK1, MAPKK6/MKK3, and p38/MKP2.

**MATERIALS AND METHODS**

**cDNA Cloning and Sequence Analysis—**Patient EY has a long history of Behcet disease and has an antiserum that reacts with nuclear and cytosolic proteins of HeLa-2 cells. EY serum was used for immunoscreening of 5 × 10^5 recombinants from a HeLa Zap cDNA library (Clontech). Three independent clones were isolated and subcloned in vivo into the pBluescript plasmid using the R408 helper phage. Their nucleotide sequences were determined by dye terminator sequencing with an Applied Biosystems model 373A machine.

**Hybridization Analysis—**Northern blots were performed using 2 μg of poly(A)^+^ RNAs isolated from different human tissues, fractionated by denaturing agarose gel electrophoresis, and transferred onto nylon membranes (Clontech). The blots were hybridized to a synthesized oligonucleotide probe (50 base pairs) designed from the sequence of the clone 4-3 and labeled with terminal desoxynucleotidyltransferase (TdT) and [α-32P]CTP (Amersham Corp.). The integrity of the mRNA was confirmed by hybridization to an actin probe.

Preparation of Recombinant Proteins—Human p38 (21) and human MKK3 (11) coding regions were amplified by the polymerase chain reaction, using human skeletal muscle cDNA (Clontech) as a template and the following oligonucleotide probes: 5'-GGCCGGATCCATGCTCTCAGAGGAGCCAC-3' and 5'-GGCCGGATCCATGCTCTCAGAGGAGCCAC-3'; and 5'-GGCCGGATCCATGCTCTCAGAGGAGCCAC-3' and 5'-GGCCGGATCCATGCTCTCAGAGGAGCCAC-3'. These authors contributed equally to this work.

§§ To whom correspondence should be addressed. Tel.: 81-52-744-2031 (M. H.) or 81-75-751-4019 (E. N.); Fax: 81-52-744-2041.

1 The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; SAPK, stress-activated protein kinase; JNK, c-jn UN terminal kinase; GST, glutathione S-transferase; MBP, myelin basic protein; ATF2, activating transcription factor 2; KN-*, kinase-negative; HA, hemagglutinin; PBS, phosphate-buffered saline; TGF β, transforming growth factor-β.
described previously (18). ATF2 was obtained as a kind gift from Drs. Suzanne J. Baker and Tom Curran (St. Jude Children’s Research Hospital).

Phosphorylation and Activation of MAPKK6 and MKK3 by TAK1—Yeast cells were transfected with plasmids containing either hemagglutinin (HA) epitope-tagged TAK1, HA-TAK1(K63W), or Gal4AD-HA-Raf-1 (20). For activation of TAK1, an activator of TAK1 (TAB1) was co-transfected with HA-TAK1. The cell lysates were subjected to immunoprecipitation with anti-HA monoclonal antibody.

Fig. 1. Primary structure and expression of MAPKK6. A, the amino acid sequence of MAPKK6 was deduced from the nucleotide sequence of clone 4-3 isolated from a human HeLa cell library (see “Materials and Methods”). The sequence of MAPKK6 was aligned and compared with those of five human MAPKKs (MKK1 (MEK1), MKK2 (MEK2), MKK3, MKK4, and MEK5). The protein sequences are presented in single-letter code. The sites of activating phosphorylation of MAPKK are indicated by asterisks.

B, the expression of MAPKK6 was examined by Northern blot analysis of poly(A) mRNA isolated from various human tissues, probed with 32P-labeled MAPKK and actin cDNAs. kb, kilobase pair(s).

C, cytosolic localization of MAPKK6 was shown by indirect immunofluorescence (magnification, ×400). The HA-tagged MAPKK6 clone was introduced into Mv1 Lu cells, and the expressed protein was stained with anti-HA monoclonal antibody.
**Results and Discussion**

One of 3 clones isolated by screening a human HeLa cell cDNA library with patient EY autoantiserum encoded a novel protein kinase. The cDNA contains an open reading frame encoding a protein of 334 amino acids with a calculated molecular mass of 37 kDa (Fig. 1A), and a kinase-negative TAK1 (TAK1(K63W)) and a kinase-negative TAK1 (TAK1(K63W)) and a kinase-negative TAK1 (TAK1(K63W)) recombinant KN–MPK2 was assayed as described under "Materials and Methods." After electrophoresis, phosphorylation of the His-MAPKK6 (A), the GST-MKK3 (C), or the KN–MPK2 (B and D) was detected by autoradiography. Upper panels, autoradiography; lower panels, Coomassie Blue staining. Closed arrowheads (A and B), His-MAPKK6; closed arrowheads (C and D), GST-MKK3; open arrowheads (B and D), KN–MPK2.

**FIG. 3. Phosphorylation and activation of MAPKK6 (A and B) and MKK3 (C and D) by TAK1.** HA-TAK1 (lane 2), TAK1(K63W) (lane 3), and Raf-1 (lane 4) expressed in yeast cells were immunoprecipitated with monoclonal antibody 12CA5 and assayed for their ability to phosphorylate (A and C) or activate (B and D) recombinant His-MAPKK6 (A and B) or GST-MKK3 (C and D). In lane 1 in A–D, a control (without immunoprecipitation) is shown. In B and D, the ability of His-MAPKK6 and GST-MKK3 to phosphorylate recombinant KN–MPK2 was assayed as described under "Materials and Methods." After electrophoresis, phosphorylation of the His-MAPKK6 (A), the GST-MKK3 (C), or the KN–MPK2 (B and D) was detected by autoradiography. Upper panels, autoradiography; lower panels, Coomassie Blue staining. Closed arrowheads (A and B), His-MAPKK6; closed arrowheads (C and D), GST-MKK3; open arrowheads (B and D), KN–MPK2.

**FIG. 2. Substrate specificity of MAPKK6.** The ability of MAPKK6 to activate MAPK, SAPK, and p38 was measured as described under "Materials and Methods." His-tagged MAPKK6 was incubated with His-MAPK, His-SAPK, or His-p38 in the presence of ATP and Mg2+.

Then the activities of MAPK, SAPK, and p38 were measured by using MBP, c-Jun, and ATF2 as substrates, respectively. After SDS-polyacrylamide gel electrophoresis, the radioactivity of the MBP, c-Jun, and ATF2 bands was detected by autoradiography (lower panel and data not shown) and quantified using an image analyzer (Fuji BAS2000) (results shown in upper panel). The radioactivity incorporated into p38 was also detected by autoradiography and shown here.

Immunoprecipitation with anti-HA monoclonal antibody (12CA5) as described previously (20). The immunoprecipitated Raf-1 was capable of activating Xenopus MAPKK (data not shown). The immunoprecipitates were incubated with MAPKK6 or MKK3 (20 μg/ml) and 100 μM [γ-32P]ATP (1 μCi) in the absence (for detecting the ability to phospho-
ylate MAPKks) or presence (for detecting the ability to activate MAPKKs) of KN–MPK2 (100 μg/ml) in a solution containing 20 mM Tris-Cl, pH 7.5, 2 mM EGTA, and 10 mM MgCl2 for 30 min at 30°C. To detect the ability to activate MAPKK6, the immunoprecipitate was first incubated with MAPKK6 (20 μg/ml) in a solution containing 100 μM ATP, 20 mM Tris-Cl, pH 7.5, 2 mM EGTA, and 10 mM MgCl2 for 20 min at 30°C, then KN–MPK2 (final concentration, 100 μg/ml) and 1 μCi of [γ-32P]ATP were added, and the reaction mixture was incubated for a further 10 min at 30°C. The reaction was stopped by the addition of Laemmli’s sample buffer. The radioactivity incorporated into recombinant MAPKK6, MKK3, and KN–MPK2 was detected by autoradiography.

**Plasmids—** An HA tag was introduced into the BglII-EcoRI sites of a mammalian expression vector pSRα456 (23) by ligating with the digo-
nucleotides 5′-GATCCGCCCGCACCATGTCACCATACGCTCCCAG-
ATTACGCTCCGAGATCTG-3′ and 5′-AAATTCGATCCTCCGGGAGG
AGGTCAATCTGGACGTCTATGTTACTGTTGCCC-3′ yielding pSRα-HA. cDNAs encoding MKK3 or p38 were subcloned into the BglII site of pSRα-HA. MAPK6 cDNA was subcloned into the SalI/HindIII sites of another N-terminal HA epitope-tagged expression vector pME-HA constructed by T. Okajima. An N-terminal truncated active TAK1 (TAK1(N)) and a kinase-negative TAK1 (TAK1(K63W)) were expressed in the pEcoF vector (20).

**Cell Cultures and Transfection—** COS7 cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Mv1Lu cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum. When needed, cells were transfected using LipofectAMINE according to the manufacturer’s instructions (Life Technologies Inc.). For preparing lysates, cells were washed once with ice-cold Hepes-buffered saline and lysed in a

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3 T. Okajima, S. Okuno, H. Fujisawa, and M. Hagiwara, unpublished data.

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37% identity to human MKK1 (25), 34% identity to human MKK2 (26), 52% identity to human MKK4 (11), and 38% identity to human MEK5 (15) (Fig. 1A).

Thus, this novel kinase appears to belong to the MAPKK superfamily and has been tentatively named MAPKK6. Northern blot analysis showed that MAPKK6 is expressed most highly in skeletal muscle and moderately in thymus, small intestine, and pancreas (Fig. 1B). When an HA-tagged MAPKK6 was expressed in cells, the distribution was throughout the cytoplasm (Fig. 1C). Thus nuclear staining of HEp-2 cells may be attributable to the other clones (nudecoprin (NUP358) and an unidentified human cDNA clone 42678) simultaneously isolated by this immunoscreening with EY serum (data not shown).

To examine whether MAPKK6 is able to activate members of the MAPK superfamily, MAPKK6 was expressed as a His-tagged protein in bacteria and purified. Subgroups of the MAPK superfamily (Xenopus classical MAPK, rat SAPKα, and human p38) were also expressed in bacteria and purified. When the purified MAPKK6 was incubated with the MAP, SAPK, or p38 in the presence of ATP, only p38 was heavily phosphorylated (Fig. 2, lower panel); the level of phosphorylation of MAP or SAPK was below 1% of that of p38 (data not shown). Thus, only the activity of p38 was specifically and strongly stimulated by incubation with MAPKK6 (Fig. 2, upper panel, and data not shown). In these experiments, MBP, c-J un, and ATF2 were used as substrates for MAPK, SAPK, and p38, respectively (Fig. 2). The results clearly indicated that MAPKK6 is a specific activator for the p38/MPK2 subgroup of the MAPK superfamily. As MKK3 was earlier shown to be a specific activator of p38 (11), MAPKK6 may be most closely related to MKK3 in terms of both structure and substrate specificity.

We have recently identified TAK1, a novel member of the MAPKKK family, that may function in the transforming growth factor-β (TGF-β) signaling pathway (20). Since TAK1 does not activate classical MAPK, we considered the possibility that MKK3 and MAPKK6 could be its natural substrates. To address this possibility, activated TAK1 was expressed in yeast, purified by immunoprecipitation, and incubated with bacterially expressed MAPKK6 in the presence of [γ-32P]ATP. MAPKK6 was found to be heavily phosphorylated (Fig. 3A, lane 2). When the incubation was performed in the absence of TAK1 (Fig. 3A, lane 1) or with a kinase-inactive mutant of TAK1 instead of the activated TAK1 (Fig. 3A, lane 3), no phosphorylation of MAPKK6 was observed, indicating that the catalysis was by TAK1 itself. When Raf-1 (Fig. 3A, lane 4) or MEKK1 (data not shown) was used as the kinase, no phosphorylation of MAPKK6 was detected, although both Raf-1 and MEKK1 could efficiently phosphorylate classical MAPKK and/or MEKK4. Therefore, MAPKK6 was phosphorylated by TAK1 but not by Raf-1 or MEKK1, the resultant form demonstrating a much higher kinase activity toward the kinase-inactive form of p38/MPK2 than unphosphorylated MAPKK6 (Fig. 3B). Thus, MAPKK6 was established to be functionally activated by TAK1-mediated phosphorylation. The same kind of experiments with MKK3 revealed similar TAK1-mediated phosphorylation (Fig. 3C) and activation (Fig. 3D). We conclude, from these results, that TAK1 is a direct activator of both MAPKK6 and MKK3 in vitro. As far as we know, this is the first identification of an upstream kinase for MKK3.

To examine whether TAK1 can work as an activator in cells, MAPKK6 or MKK3 was expressed together with active TAK1 or kinase-inactive TAK1 in COS7 cells. The expressed MAPKK6 or MKK3 was greatly activated by co-expression of active TAK1 (Fig. 4A) but not by co-expression of inactive TAK1 (Fig. 4A and data not shown). These results taken together suggest that TAK1 can work as a direct activator of MAPKK6 and MKK3, two specific activators of the p38/MPK2 subgroup of the MAPK superfamily. Consistent with this suggestion, co-expression of active TAK1 and p38 in COS7 cells resulted in activation of p38 (Fig. 4A). Thus, this study established the existence of a kinase cascade consisting of TAK1, MAPKK6/MKK3, and p38. Since TAK1 was earlier shown to be activated by treatment with TGF-β (20), we also examined whether MAPKK6 or MKK3 was affected by this growth factor. Both MAPKK6 (Fig. 4B) and MKK3 (data not shown) were indeed activated, but the extent was not as large as that activated with exposure to hyperosmolality (Fig. 4B). This might be interpreted as suggesting the existence of an as yet unidentified member of the MAPKK superfamily, which is a better substrate for TAK1 than MAPKK6 or MKK3. Conversely, it is possible that there are as yet unidentified members of the MAPKK family lying upstream of MAPKK6 and MKK3.

In summary, this study identified a novel kinase cascade consisting of TAK1, MAPKK6/MKK3, and p38, by identifying a novel MAPKK member, MAPKK6, and revealing activation of both MAPKK6 and MKK3 by TAK1, a newly identified MAPKK. Further work is needed to elucidate the functions and regulatory mechanisms of this kinase cascade in vivo.

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Addendum—While this manuscript was under review, Raingeaud et al. (27) and Han et al. (28) reported cDNA cloning of MKK6 and MKK6b, respectively, which are identical to MAPKK6 in this paper.
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