Cloning and Characterization of MEK6, a Novel Member of the Mitogen-activated Protein Kinase Kinase Cascade*

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Mitogen-activated protein kinases are members of a conserved cascade of kinases involved in many signal transduction pathways. They stimulate phosphorylation of transcription factors in response to extracellular signals such as growth factors, cytokines, ultraviolet light, and stress-inducing agents. A novel mitogen-activated protein kinase, MEK6, was cloned and characterized. The complete MEK6 cDNA was isolated by polymerase chain reaction. It encodes a 334-amino acid protein with 82% identity to MKK3. MEK6 is highly expressed in skeletal muscle like many other members of this family, but in contrast to MKK3 its expression in leukocytes is very low. MEK6 is a member of the p38 kinase cascade and efficiently phosphorylates p38 but not c-j un N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) family members in direct kinase assays. Coupled kinase assays demonstrated that MEK6 induces phosphorylation of ATF2 by p38 but does not phosphorylate ATF2 directly. MEK6 is strongly activated by UV, anisomycin, and osmotic shock but not by phorbol esters, nerve growth factor, and epidermal growth factor. This separates MEK6 from the ERK subgroup of protein kinases. MEK6 is only a poor substrate for MEKK, a mitogen-activated protein kinase kinase kinase that efficiently phosphorylates the related family member JNKK.

Protein phosphorylation plays a major role in many signal transduction pathways. Stress-activated or mitogen-activated protein kinases (MAPKs) are members of a conserved cascade of kinases that stimulate phosphorylation of transcription factors and other targets in response to extracellular signals such as growth factors, cytokines, ultraviolet light, and stress-inducing agents. In higher eukaryotes the physiological role of MAPK signaling has been correlated with cellular events such as proliferation, oncogenesis, development and differentiation, and cell cycle. Several MAPK cascades have been identified in yeast and vertebrates (for review see Refs. 1–10). Each cascade consists of several modules. Most of the mammalian MAPK modules have been identified by analogy with yeast protein kinase cascades. We are following the nomenclature of Seger and Krebs (10) and name the individual levels of kinases regardless of their pathway MAPK, MAPKK, and MAPKKK. The extracellular signal-regulated kinase (ERK) subgroup of MAPKs has been examined in detail (11–16). The c-j un N-terminal kinase (JNK) subgroup (also known as stress-activated protein kinase, or SAPK) was first described as a kinase cascade leading to the phosphorylation of J un (17–19). More recently the p38 MAPK pathway has been identified in analogy to the yeast HOG1 pathway (20, 21).

We were interested in identifying novel members of the MAPK family that use p38 as substrate. So far only two of these kinases have been identified in humans: MKK3 and JNKK (MKK4, SEK). JNKK is a substrate for MEKK1 (22–24) and phosphorylates and activates JNK1, JNK2, and p38 in vivo and in vitro (23, 25). In contrast, MKK3 phosphorylates and activates exclusively p38 (25); however, the upstream MAPKK for MKK3 is unknown, since MEKK1 is not an activator of p38 in vivo (22). In an attempt to find novel members of the p38 MAP kinase cascade we cloned and characterized a new human MAPKK, which we named MEK6. MEK6 differs from its closest homologue, MKK3, most significantly in the N-terminal and C-terminal amino acid regions. MEK6 efficiently phosphorylates p38 but not JNK or ERK. MEK6 is strongly activated by UV, anisomycin, and osmotic shock.

EXPERIMENTAL PROCEDURES

cDNA Cloning—The Expressed Sequence Tags (EST) subdivision of the National Center for Biotechnology Information (NCBI) GenBank™ databank was searched with the tblast program and the human MKK3 amino acid sequence as query using the BLAST e-mail server. The 223-bp EST sequence F00521 displayed the highest similarity score. A reverse PCR primer (5’-CACATCTTCATTGACCAGAGCA-3’) directed against this sequence was designed with the help of the program Oligo, version 4.0 (National Biosciences, Inc.). Poly(A) RNA was prepared from unstimulated Jurkat T cells using the MicroFast Track Kit (Invitrogen). One μg of this RNA was used to generate an adaptor-ligated cDNA library that can be used for 5’ and 3’ rapid amplification of cDNA ends ( Marathon cDNA Amplification Kit, Clontech). The adaptor-specific primer from the kit and the gene specific reverse primer were used to PCR-amplify the 5’ portion of MEK6. PCR amplification was performed with a combination of Taq and Pwo polymerases (Expand Long Template PCR system, Boehringer Mannheim) in the presence of TaqStart antibody (Clontech). This mixture is designed to produce high yield of long PCR fragments and to provide proofreading function. All PCR amplifications were carried out in 0.2-ml Perkin-Elmer thin-wall MicroAmp tubes and a Perkin-Elmer model 2400 or 9600 thermocycler. The resulting 0.8-kb PCR fragment was ligated into pGEM-T (Promega) and sequenced (dye terminator cycle sequencing) with an ABI 373 Automated Sequencer. The sequence information from the 5’ end of the partial MEK6 cDNA was used to design a forward PCR primer (5’TGTGCTCCCCCTCCCCCATACAG-GAAA-3’) for 3’ rapid amplification of cDNA ends. The gene-specific forward primer and the gene-specific primer were used to PCR-amplify the complete MEK6 cDNA from an adaptor-ligated MOLT-4 cDNA library. This library was generated using 1 μg of MOLT-4
poly(A)+ RNA (Clontech) and the Marathon cDNA Amplification Kit (Clontech). The 1.6-kb PCR fragment was ligated into pGEM-T (Promega), and three clones were sequenced several times on both strands with an ABI 373 automated sequencer. The BLAST program was used to search the NCBI GenBankTM data base for related cDNAs. The Bestfit program from the Wisconsin Genetics Computer Group (Madison, WI) was used for calculating the amino acid identities between MKK3 and MEK6. The Macvector program (Kodak-I/BI) was used for aligning the amino acids of MKK3 and MEK6.

Human p38 (GenBankTM accession number U10871) was cloned by PCR amplification of a Jurkat cDNA library with primers against the S’ end (5’-CACAACATGCTGAGGAGAG-3’) and 3’ end (5’-CCGATCTCCATCTC-3’) of the published human p38 sequence. Each strand of the PCR fragment was sequenced several times with an ABI 373 automated sequencer.

Plasmids—3xHA-MEK6-Srα3 was constructed by replacing serine in position 2 of MEK6 with alanine, adding sequence encoding three copies of a 10-amino acid hemagglutinin (HA) epitope to the N terminus of MEK6 and ligating the resulting cDNA into Srα3. 3xHA-MEK6-Srα3 was constructed by adding sequence encoding three copies of the HA epitope to the N terminus of mouse 3xNKK initiated at amino acid 35 (22) and ligating the resulting cDNA into Srα3. Both plasmids expressed proteins of the expected size as verified with the Tnt SP6 Coupled Reticulocyte Lysate System (Promega). GST-MEK6 was constructed by ligating a 1.3-kb DNA fragment encoding amino acid 1 through the stop codon of MEK6 with a serine to alanine substitution of amino acid 2 into pGEX-KG (26). Similarly, GST-p38 and GST-JNK2 were constructed by ligating the respective cDNA fragments encoding amino acid 1 through the stop codon into pGEX-KG.

RESULTS

Isolation of MEK6 cDNA—We performed BLAST homology searches of the EST subdivision of the NCBI GenBankTM data bank with the tblastn program to identify EST sequences that encode peptides related to human MKK3. We identified a 223-bp EST fragment with the accession number F00521 that was related to MKK3 at the amino acid level but showed significant differences at the nucleotide level. A reverse PCR primer was used to amplify the 5’ portion of the potential new gene from an adaptor-ligated Jurkat cDNA library. A popula-

![Fig. 1. Primary structure of MEK6.](http://www.jbc.org/)

![Fig. 2. Tissue distribution of MKK3 and MEK6.](http://www.jbc.org/)
tions during the PCR amplification procedure. A GenBank™ BLAST search revealed no similar sequences. Since the most recently published MAPKK was called MEK5 (33, 34), we named this new gene MEK6. The 1.6-kb cDNA encodes a potential protein of 334 amino acids with a calculated molecular weight of 37,500. MEK6 has 82% amino acid identity with its closest homologue, MKK3. All relevant kinase subdomains are conserved. The most divergent regions are the N-terminal region with an additional 18 amino acids and the C-terminal region (Fig. 1).

The expression of human MKK3 and MEK6 was examined by Northern blot analysis of RNA isolated from various human tissues. MKK3 is widely expressed in many adult human tissues with highest levels in skeletal muscle and leukocytes (Fig. 2A). In contrast, MEK6 is predominantly expressed in skeletal muscle and at lower levels in heart and pancreas (Fig. 2B). All tissue samples expressed similar levels of β-actin mRNA (data not shown).

Substrate Specificity of MEK6—To characterize the kinase activity of MEK6, we subcloned the cDNA into a bacterial GST fusion protein expression vector. We investigated the substrate specificity of MEK6 in an in vitro kinase assay with bacterially expressed MAPKK substrates (JNK2, p38, and ERK1(K52R)) as described under “Experimental Procedures.” MEK6 efficiently phosphorylated p38 but none of the other substrates (Fig. 3A, compare lanes 1–4 with lanes 5–8). To determine whether phosphorylation of p38 is an activating event we analyzed the phosphorylation of recombinant ATF2 (a substrate for p38) in a coupled in vitro kinase assay. MEK6 did not cause increased phosphorylation of Jun either directly or in combination with JNK2 (Fig. 3B, lanes 1–5); ATF2, however, was strongly phosphorylated by p38 that had been activated by MEK6 (Fig. 3B, lane 5). ATF2 was not directly phosphorylated by MEK6. These data establish that MEK6 is a functional MAPKK in vitro and that p38 is a substrate for MEK6.

MEK6 Is Activated by Anisomycin and UV—Next, we examined whether in vivo activated MEK6 can phosphorylate and activate p38 in an immune complex kinase assay. HeLa cells were transiently transfected with epitope-tagged MEK6 and treated with anisomycin (50 ng/ml) or UV (254 nm; 120 J/m²) for the times indicated. Cell lysates were prepared and used in an immune complex kinase assay with GST-p38 substrate as described under “Experimental Procedures.” Reactions were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The positions of protein molecular mass markers in kDa are shown on the left. MEK6 activity was quantitated with a PhosphorImager and ImageQuant software and is shown in a bar graph. The presence of equal amounts of MEK6 in all kinase reactions was confirmed by Western blot analysis of the cell lysates with an anti-HA antibody (data not shown).
MEK6 was isolated by immunoprecipitation with an anti-HA monoclonal antibody (12CA5). In an initial experiment we investigated the time course of MEK6 activation by anisomycin and UV treatment of transfected cells. MEK6 activation by anisomycin as measured by its ability to phosphorylate p38 was observed as early as 10 min after treatment (Fig. 4). The activation was transient and peaked at 40 min after treatment. In contrast, activation by UV was delayed by about 10–15 min and reached a plateau between 60 and 120 min (Fig. 4). Analysis of the UV dose response of MEK6 in HeLa cells revealed that doses up to 120 J/m² yielded increasing activity of MEK6 (Fig. 5).

To determine whether the increase in p38 phosphorylation by activated MEK6 augments p38 kinase activity, a coupled immune complex kinase assay was performed. Epitope-tagged MEK6 was isolated by anisomycin-treated HeLa cells and subjected to two subsequent kinase reactions using recombinant p38, ATF2, and GST alone. In support of our in vitro results, anisomycin treatment caused increased phosphorylation of ATF2 only when MEK6 and p38 were present (Fig. 6, compare lanes 5 and 6 with lanes 7 and 8). Similar results have been found with MEK6 activated by UV treatment of cells (data not shown). No inducible phosphorylation of p38 or ATF2 was observed in HeLa cells transfected with the empty expression vector SRα3 (Fig. 6, compare lanes 5 and 6 with lanes 13 and 14). This clearly indicates that the inducible phosphorylation of ATF2 depends on a kinase cascade comprised of MEK6 and p38. Interestingly, p38 also phosphorylated weakly a protein with a mobility slightly faster than ATF2 (indicated by an asterisk in Fig. 6). This phosphorylation event was slightly augmented by anisomycin in the presence of MEK6 (Fig. 6, compare lanes 3 and 4 with lanes 11 and 12).

MEK6 Is Activated by Stress-inducing Agents—MAPK cascades in mammalian cells respond to a variety of extracellular stimuli. To investigate the pattern of MEK6 regulation, cells were transiently transfected with expression vector for 3×HA-MEK6 and treated with various stimulators of the MAPK pathway. In HeLa cells, strongest inducers of MEK6 were UV, anisomycin, and NaCl followed by weak induction with interleukin-1β (Fig. 7A). NGF and EGF, two strong inducers of the ERK pathway, did not activate MEK6, although we noted the inducible phosphorylation of two lower molecular weight bands (see "Discussion"). Similar experiments were performed in COS cells, demonstrating a strong induction of MEK6 by UV and to a lesser extent by anisomycin (Fig. 7B). To exclude the possibility that changes of MEK6 kinase activity are caused by different levels of expression of MEK6 in response to treatment of cells with stimulators we performed Western blot analysis. MEK6 was present at equal levels in all cell lysates as determined by Western blot analysis with an anti-HA antibody (data not shown).

MEK6 Is Not a Physiological Substrate for MEKK—MEKK has been described as a MAPKKK leading to the phosphorylation and activation of JNKK (22–24). In an initial experiment with 1000 ng of cotransfected expression vector for MEKK we observed stimulation of MEK6 activity in COS cells but not in HeLa cells (Fig. 7, A and B). This prompted us to examine more carefully whether MEKK is able to activate MEK6. COS cells were transfected with increasing amounts of expression vector encoding MEKK in the presence of a constant amount of expression vector encoding MEK6 in HeLa cells transfected with as little as 125 ng of the MEKK expression vector. Comparable amounts of MEK6 activation, however, were not observed until 1000 ng of the MEKK expression vector were cotransfected. These data suggest that MEKK is not a physiological activator of the MEK6 and p38 kinase cascade.

DISCUSSION

In this report, we describe the cloning and characterization of a novel member of the MAPKK family of dual specificity protein kinases, which we named MEK6. MEK6 was first identified in a BLAST homology search of the EST subdivision of NCBI GenBank™ as a 223-bp partial cDNA fragment. The full-length cDNA was obtained by PCR amplification of Jurkat and MOLT-4 cDNA libraries with gene-specific primers. We minimized the chance of introducing errors in the amplified sequence by using a DNA polymerase mixture with proofreading function. Further, we sequenced three independent clones that were identical. The cDNA has a long open reading frame that is preceded by about 250 bp of sequence with stop codons in all three reading frames. The size determination of in vitro translated protein indicates that translation starts at the identified start codon of MEK6 cDNA. Further, Northern blot analysis revealed that the size of MEK6 mRNA corresponds to the size of MEK6 cDNA. This suggests that we cloned the full-length cDNA of MEK6. MEK6 has significant homology on the amino acid level to MKK3 (82% amino acid identity) but is not significantly related to MKK3 at the DNA level. This clearly demonstrates that MEK6 is encoded by a novel gene. MEK6 differs from MKK3 most significantly in the C terminus and N

Fig. 6. Coupled kinase assay in HeLa cells. HeLa cells were transiently transfected with epitope-tagged MEK6 (lanes 1–8) or the empty expression vector SRα3 (lanes 9 and 16) and treated for 45 min with anisomycin (An., 50 ng/ml) or left untreated (ctrl) as indicated. Cell lysates were prepared and used in a coupled immune complex kinase assay as described under "Experimental Procedures" and the legend to Fig. 3. The positions of protein molecular mass markers in kDa are shown on the left. The positions of p38, ATF2, and an unknown protein (*) are indicated on the right.
terminus, which has an additional 18 amino acids. All relevant kinase subdomains, the ATP acceptor site, and phosphorylation sites are conserved.

Many members of the MAPK cascades are expressed at high levels in skeletal muscle (18, 25, 33), which prompted us to compare the expression of MEK6 and its closest homologue MKK3 in 16 different adult human tissues. We observed very high expression of MEK6 mRNA in skeletal muscle, consistent with the high levels of other MAPK family members, but surprising was the absence of MEK6 mRNA in spleen, thymus, prostate, ovary, small intestine, colon, and leukocytes. Analysis of the same blot showed that MKK3 is widely expressed, and all 16 tissues expressed equal amounts of β-actin mRNA. We assume that MEK6 is expressed in these tissues at levels that are below the detection limit of Northern blot analysis. The fact that we isolated MEK6 cDNA from human T cells implies that activated T cells can express MEK6. Interestingly, some of the tissues expressed an MEK6-related mRNA of about 4.2 kb. This band was not observed when we used a MEK6-specific probe that was directed against the 3’ end of MEK6 cDNA.

Similarities between MEK6 and MKK3 prompted us to investigate whether MEK6 is able to utilize p38 as substrate. In vitro MEK6 efficiently phosphorylated p38 but not ERK and JNK, although in parallel experiments the phosphorylation of JNK by JNKK was observed (data not shown). This indicates that MEK6, like MKK3, has substrate selectivity for the p38 subgroup of MAPK. Activation of p38 requires phosphorylation of Thr180 and Tyr182 (20). We subjected p38 that has been phosphorylated by MEK6 to a second kinase reaction with ATF2 as substrate. MEK6 induced phosphorylation of ATF2 by p38 but did not directly phosphorylate ATF2. These experiments were carried out with MEK6 prepared in bacteria as GST fusion protein and with epitope-tagged MEK6 isolated from HeLa cells after stimulation with anisomycin or UV.

**Fig. 7.** Stimulators of MEK6 in vivo. HeLa (A) or COS cells (B) were transiently transfected with epitope-tagged MEK6 (lanes 1–12) or the empty expression vector SRα3 (lanes 13–16) and treated for 45 min with interleukin-1β (IL-1β, 10 ng/ml; R & D Systems), tumor necrosis factor-α (TNF-α, 10 ng/ml; R & D Systems), EGF (50 ng/ml; Life Technologies, Inc.), NGF (50 ng/ml; Life Technologies, Inc.), phorbol 12-myristate 13-acetate (PMA, 50 ng/ml; Sigma), anisomycin (50 ng/ml; Sigma), cycloheximide (C. X. 50 ng/ml; Sigma), arsenite (Arsen., 200 μM; Sigma), NaCl (200 mM; Sigma), or UV (254 nm; 120 J/m2) or cotransfected with 1000 ng CMV5-MEKK as indicated. Cell lysates were used in an immune complex kinase assay with GST-p38 substrate as described under “Experimental Procedures.” The positions of protein molecular mass markers in kDa are shown on the left. MEK6 activity was quantitated with a PhosphorImager and ImageQuant software. The presence of equal amounts of MEK6 in all kinase reactions was confirmed by Western blot analysis (data not shown).
MEK6 is strongly activated by stress-inducing and DNA-damaging agents, anisomycin, UV, and also osmotic shock. Phorbol esters, NGF, and EGF, strong stimulators of the ERK pathway in the same cell lines analyzed (11, 12, 14), did not stimulate MEK6. Similarly, cycloheximide, a stimulator of p54 kinase (JNK2) (35) and of the ERK pathway (36), did not significantly activate MEK6. Anisomycin and cycloheximide are known to be effective protein synthesis inhibitors, yet the described effects on the MAPK pathways occur at concentrations that only marginally affect protein synthesis. Interestingly, we noted in our in vivo kinase assays and therefore is most likely a contamination of the immunoprecipitation.

A time course experiment revealed that the induction of MEK6 by UV lagged behind the anisomycin induction by about 10–15 min. Further, in contrast to the transient activation by anisomycin, UV-induced MEK6 stayed active for at least 120 min. The slight reduction of MEK6 activity at the 90-min time point was not observed in other experiments. This time course experiment suggests that different pathways are used for anisomycin-induced MEK6 and p38. A schematic presentation of the MAPK pathways in mammals is presented in Fig. 9.

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