p38-2, a Novel Mitogen-activated Protein Kinase with Distinct Properties*

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Mitogen-activated protein (MAP) kinases are involved in many cellular processes. Here we describe the cloning and characterization of a new MAP kinase, p38-2. p38-2 belongs to the p38 subfamily of MAP kinases and shares with it the TGY phosphorylation motif. The complete p38-2 cDNA was isolated by polymerase chain reaction. It encodes a 364-amino acid protein with 73% identity to p38. Two shorter isoforms missing the phosphorylation motif were identified. Analysis of various tissues demonstrated that p38-2 is differently expressed from p38. Highest expression levels were found in heart and skeletal muscle. Like p38, p38-2 is activated by stress-inducing signals and proinflammatory cytokines. The preferred upstream kinase is MEK6. Although p38-2 and p38 phosphorylate the same substrates, the site specificity of phosphorylation can differ as shown by two-dimensional phosphopeptide analysis of Sap-1a. Additionally, kinetic studies showed that p38-2 appears to be about 18 times more active than p38 on certain substrates such as ATF2. Both kinases are inhibited by a class of pyridinyl imidazoles. p38-2 phosphorylation of ATF2 and Sap-1a but not Elk1 results in increased transcriptional activity of these factors. A sequential kinetic mechanism of p38-2 is suggested by steady state kinetic analysis. In conclusion, p38-2 may be an important component of the stress response required for the homeostasis of a cell.

Several signaling cascades targeting different mitogen-activated protein kinases (MAPKs) have been identified over the last few years in yeast and vertebrates (1–9). The members of the MAPK family are proline-directed Ser/Thr kinases which themselves are activated upon phosphorylation on Thr and Tyr by dual specificity protein kinases, the MAPK kinases (MAPKKs). Specific protein kinase cascades (MAPKK→MAPK→MAPKK) constituted within the cytoplasm are stimulated by a variety of signals including growth factors, cytokines, ultraviolet light (UV), and other stress-inducing agents. Since these signals can affect cell proliferation, oncogenesis, development as well as differentiation, and the cell cycle, MAPKs may have a pivotal impact on these cellular processes.

The p38MAPK (cytokine-suppressive anti-inflammatory drug binding protein; CSBP1/2) was identified by homology to the yeast HOG1 MAPK and is activated by osmotic shock (10–12). Proinflammatory cytokines, lipopolysaccharide, and chemical stress such as H2O2 also can induce p38MAPK (10, 11, 13–19). An important role of p38 in cellular responses involving cytokine production and platelet aggregation was established from studies in which p38 was specifically inhibited by the pyridinyl imidazole derivative SB203580 (19–21).

Several substrate proteins for p38 have been identified, among them the transcription factors ATF2, CHOP-1, and Elk1 and the protein kinases MAPKAP K2/3 (14, 16, 22–24). Furthermore, a truncated splice variant of p38 with a distinct C terminus (Mxi2) phosphorylates the transcription factor Max (25). p38 itself is phosphorylated and thereby activated by the MAPKKs MKK3 (26), JNK2 (26, 27), and the recently discovered MEK6 (22, 28, 29). Furthermore, several candidates (MEKK1, Pak1, DLK, TAK1) for an upstream protein kinase (MAPKKK) for this cascade have been described (27, 30–33).

In an attempt to find novel members of the p38MAPK cascade, we cloned and characterized a new human MAPK, which we named p38-2. Analysis of various tissues demonstrated that p38-2 is differently expressed from p38. Like p38, p38-2 is activated by stress-inducing signals and cytokines. We show that MEK6 phosphorylates p38-2, suggesting its role as a specific MAPKK. Although p38-2 and p38 phosphorylate the same substrates, the site specificity of phosphorylation can differ, and p38-2 appears to be about 180 times more active on certain substrates such as ATF2.

EXPERIMENTAL PROCEDURES

cDNA Cloning—The expressed sequence tags (EST) subdivision of the National Center for Biotechnology Information (NCBI) GenBank data base was searched with the tblastn program and the human p38 (CSBP) amino acid sequence as query. The 154-bp EST sequence R72598 from a human breast cDNA library displayed the highest similarity score. A forward PCR primer (5′-GCGCCAGGCGGAGGAGGAGATGACC-3′) directed against the 3′ end of this sequence was designed with the help of the program Oligo version 4.0 (National Biosciences, Inc.). This gene-specific forward primer and the adaptor-specific primer from the Marathon cDNA Amplification Kit (CLONTECH) were used to PCR-amplify the 3′ portion of p38-2 from a skeletal muscle cDNA library (CLONTECH). 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). All PCR

1 The abbreviations used are: MAPKs, mitogen-activated protein kinases; MAPKK, MAP kinase kinase; MAPKKK, MAP kinase kinase kinase; MAPKAP, MAP kinase-activated protein; CSBP, cytokine-suppressive anti-inflammatory drug binding protein; JNK, c-Jun N-terminal kinase; JNKK, JNK kinase; MEKK, MAP/ERK kinase kinase; ERK, extracellular signal-regulated kinase; TAK1, TGFβ-activated kinase 1; EST, expressed sequence tags; NCBI, National Center for Biotechnology Information; HA, hemagglutinin; PCR, polymerase chain reaction; GST, glutathione S-transferase; kb, kilobase pair(s); bp base pair(s); PAGE, polyacrylamide gel electrophoresis; SRE, serum-response element; LUC, luciferase; TGFβ, transforming growth factor β.

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amplifications were carried out in 0.2 ml of Perkin-Elmer thin wall MicroAmp tubes and a Perkin-Elmer model 2400 or 9600 thermocycler. The resulting 800-bp PCR fragment was ligated into pGEM-T (Promega) and sequenced (dye terminator cycle sequencing) with an ABI 373 Automated Sequencer (Applied Biosystems, Foster City, CA). We also sequenced the original p38 cDNA insert. The resulting 575-bp insert encoding the N terminus of p38-2 and ligating the resulting cDNA into SR3. GST-p38-2 was constructed by ligating a 1.1-kb DNA fragment encoding amino acid 1 through the stop codon of p38-2 with a serine to GST-p38-2 was constructed by ligating a 1.1-kb DNA fragment encoding amino acid 1 through the stop codon of p38-2 with a serine to alanine substitution in position 2 into pGEX-KG (34). 3xHA-MEK6 DDΔ-SRα3 was constructed by PCR mutagenesis of the wild type MEK6 expression vector (29) replacing the phosphorylation motif SVAK by SVAT. The following primers have been described previously: HA-JNK1 (35), HA-ERK1 (36), HA-TAK1, HA-TAK1ΔN, HA-TAK1-K63W (33), CMV-5MEKK1 (37), CMV-Elk1 2–428 (38), CMV- Sap-1a 268–431 (39), His-ERK1(K52R) (40), GST-c-Jun1–79 (41), GST-ATF2 (42), GST-ER (47), pEV3S (48), SRE 2-tk80-luc (49), pAG174 (49), GALA-ATF2p9–49 (49).

The pyridyl imidazole derivative, SB203580 (50), was prepared at Signal Pharmaceuticals.

**Northern Blot Analysis**—Northern blots were prepared using 2 μg of poly(A) RNA isolated from 16 different human tissues, fractionated by denaturing formaldehyde 1.2% agarose gel electrophoresis, and transferred onto a charge-modified nylon membrane (CLONTECH). The blots were hybridized to a probe 38 (850-bp cDNA fragment), p38-2 probe (900-bp p38-2 cDNA fragment), or p38-2 intron probe (oligonucleotide against first intron) using ExpressHyb (CLONTECH) according to the manufacturer’s instructions. Both cDNA probes were prepared by random prime labeling (Prime It II, Stratagene) of the cDNA with [γ-32P]dCTP (NEN Life Science Products). The oligonucleotide was end-labeled with [γ-32P]dATP (NEN Life Science Products).

For control purposes the blots were also hybridized to a radiolabeled β-actin probe.

**Transient Transfection and Extract Preparation**—Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 500 mg/literL-glutamine, and antibiotics. HeLa cells were transfected by the calcium phosphate coprecipitation method. HeLa cells were transfected in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 500 mg/literL-glutamine, and antibiotics. HeLa cells were transfected by the calcium phosphate coprecipitation method. HeLa cells were transfected by the calcium phosphate coprecipitation method.

**Reporter Gene Assays**—293 cells were transiently transfected by the calcium phosphate coprecipitation method with the CRE-tk80-luc luciferase reporter gene construct and either the empty expression vector pEV35 or the respective expression vector for Elk1 or Sap-1a as well as the indicated protein kinase vectors. Luciferase activity was determined 38 h after transfection and normalized to transfection efficiency of the respective protein kinase vectors. Luciferase activity was determined 38 h after transfection and normalized to transfection efficiency of the respective protein kinase vectors.

**Phosphopeptide Analysis**—In vitro phosphorylated GST-fusion proteins and purified by affinity chromatography on GSH-Sepharose 4B beads (Pharmacia Biotech Inc.) were digested as described previously (46). Kinase assays were performed as described previously (29).

**Phosphopeptide Analysis**—In vitro phosphorylated GST-fusion proteins were subjected to SDS-PAGE. The gel was dried and exposed to an x-ray film, and a gel slice containing the phosphorylated GST-fusion protein was cut out. The protein was extracted from the gel slice as described (53). After digestion with chymotrypsin, the resulting phosphopeptides were resolved on cellulose thin layer plates by electrophoresis in the first dimension in pH 1.9 buffer (88% w/v formic acid/glacial acetic acid/water, 50:15:65) and by ascending chromatography in 1-butanol/pyridine/glacial acetic acid/water (10:10:3:12) in the second dimension (53).

**Evaluation of p38-2**—The p38-2 reaction velocities were determined by quantifying the amount of 32P incorporation into GST-ATF2. GST-p38-2 activity was monitored as a function of both GST-ATF2 concentration (0.31, 0.62, 1.25, 2.5, and 5.0 μM) and ATP concentration (0.05, 0.5, 2.5, and 5.0 μM). Enzymatic reactions (0.1 ml) were carried out in wells of a 96-well assay plate (Corning) for 1 h at room temperature. Reactions were terminated and the addition of trichloroacetic acid (150 μl) for 15 min (25 °C). The subsequent 20-min incubation with trichloroacetic acid at 4 °C precipitated the proteins from solution. The trichloroacetic acid-mediated precipitate was then collected on 96-well glass fiber plates (Packard) and washed 10 × with approximately 0.3 ml per well of phosphate-buffered saline, pH 7.4, using a Packard Filtermate 190. Scintillation fluid (0.05 ml, MicroScent, Packard) was added to each well, and the plate was analyzed for 32P using a Packard TopCount scintillation counter. Reactions contained 20 μl of recombinant p38-2 (0.25 μg/ml) in a dilution buffer that contained 20 mM HEPES, pH 7.6, 0.2 mM EDTA, 2.5 mM MgCl2, 0.004% Triton X-100, 2 mM dithiothreitol, 5 μg/ml leupeptin, 20 μM β-glycerolphosphate, 0.1 mM sodium vanadate, 25 μl of ATP solution (in distilled, deionized water), 10 μl of recombinant GST-ATF2 (in 20 mM HEPES, pH 7.6, 50 mM NaCl, 0.1 mM EDTA, 2.5 mM MgCl2, 0.5% Triton X-100, 2 mM dithiothreitol). A typical control reaction in the absence of GST-ATF2 that contained 722,808 cpm would result in a background of 584 cpm. The 32P-labeled GST-ATF2 typically ranged from 15,712 to 84,410 cpm which was significantly greater than the background and ensured accurate velocity values. Double reciprocal analysis was used to assess the kinetic mechanism. The data were fit to the equation for a sequential mechanism by nonlinear least squares method of Cleland (54) to obtain kinetic constants. The assay for p38-2 activity was a discontinuous assay with data taken after 1 h of room temperature reaction. The reaction time course was initiated by the addition of a kinase reaction for the conditions used in the kinetic experiments. There is a linear relationship between enzyme activity and enzyme concentration for p38-2 concentrations from 6.1 to 49 nM. Less than 10% ATP was turned over in the course of the assay.

**Kinetic Comparison of p38 and p38-2**—The apparent kinetic constants for recombinant p38 and p38-2 were determined by the assay method described in the previous section. Data were taken in the linear portion of the reaction time course. Less than 10% ATP was turned over by p38 in the course of the assay. The final concentrations of GST-p38 and GST-p38-2 were 25 and 0.075 mg/ml, respectively. The GST-ATF2 concentration was varied (0.156, 0.313, 0.625, 1.25, and 2.50 μM). A common solution of GST-p38 and GST-p38-2 was used for both p38 and p38-2 reactions. The ATP concentration in the kinase buffer was held constant at 15 μM. Reactions were initiated with the addition of a common kinase buffer that delivered 0.5 μM of [γ-32P]ATP (15 μM). After 1 h at room temperature, reactions (0.1 ml) were terminated and proteins were precipitated by the addition of 150 μl of 12.5% trichloroacetic acid (20 min incubation at 4 °C). Kinetic constants were derived from a nonlinear least squares fit to the Michaelis-Menten equation in the manner outlined by Cleland (54).

**RESULTS**

**Isolation of p38-2 cDNA**—We performed BLAST homology searches of the EST subdivision of NCBI GenBank data bank to identify EST sequences that encode peptides related to human p38MAPK (CSBP). A 154-bp EST fragment with the accession number R72589 that encoded a peptide related to p38 was identified. The corresponding cDNA clone was obtained from Research Genetics (clone ID 156272) and its sequence determined. The 900-bp cDNA fragment contained the putative 5′ end of a novel gene. Although the cDNA fragment had an in-frame stop codon, the region before and after this stop codon encoded peptides with significant homology to p38. A forward PCR primer was designed to amplify the missing 3′ portion of the potential new gene from an adapter-ligated skeletal muscle cDNA library. A population of PCR fragments
was obtained and subcloned into pGEM-T. Sequencing revealed several identical PCR clones with open reading frames followed by a stretch of about a 300-bp untranslated region and a poly(A)-tail. We combined the cDNA insert of R72598 with one of the PCR clones to obtain a cDNA of maximum length. A GenBank BLAST search revealed no identical sequences to this cDNA, and we named the respective gene p38-2, based on its similarity to p38.

Closer inspection of the sequence surrounding the internal stop codon and alignment of the encoded peptide with p38 revealed an 86-bp intron with typical splice junction consensus sequences. This suggests that the poly(A) selected mRNA

Fig. 1. Primary structure of p38-2. A, primary amino acid sequences of full-length p38-2 and isoforms 1 and 2. * indicates the threonine and tyrosine in the TGY dual phosphorylation motif. The accession number for the p38-2 sequence is U92268. B, the MacVector program (Oxford Molecular Group) was used to perform a Clustal alignment of the amino acid sequences of human p38-2, p38β, p38, Mxi2, and ERK6. Identical amino acid residues are darkly shaded, and conservative changes are lightly shaded.
preparation used for creation of the cDNA library contained unspliced mRNA. Therefore, we reamplified the intron area from a different skeletal muscle cDNA library. About 50% of the PCR clones had no intron, and about 25% had the previously described intron at amino acid position 102/103, and 25% had a different intron at amino acid position 149/150. From these data we conclude that p38-2 potentially exists in several isoforms. The 1.3-kb cDNA without introns encodes a protein of 364 amino acids with a calculated molecular mass of 41.3 kDa, and the cDNAs with intron 1 or intron 2 encode shorter proteins of 102 and 155 amino acids, respectively (Fig. 1A). Both shorter isoforms are missing the phosphorylation motif. p38-2 has 73% amino acid identity and 86% similarity with its closest homologue, p38. p38-2 is a member of the p38 subgroup of MAPK. A Clustal alignment of all five human p38 family members (p38, p38β, p55, p58, Mxi2, and ERK5) is shown in Fig. 1B. Relevant kinase subdomains are conserved as indicated by the shaded areas; all five kinases unlike other known MAPK have the TGY phosphorylation motif in the activation loop that is recognized by a MAPKK and have the same length of linker loop 12.

**Tissue Distribution of p38-2**—The expression patterns of human p38 and p38-2 were examined by Northern blot analysis of RNA isolated from various human tissues. p38 is widely expressed as a 4.3-kb mRNA in adult human tissues with highest levels in skeletal muscle (Fig. 2A). In contrast, p38-2 is expressed as a 4.5-kb mRNA at very high levels in heart followed by skeletal muscle and at lower levels in various other tissues (Fig. 2B). We obtained an identical pattern of tissue distribution when we used as a probe a short oligonucleotide directed against the first intron of p38-2 (data not shown). This suggests that the poly(A)" RNA preparation used for the Northern blot as well as the previously described cDNA library contained unspliced p38-2 mRNA species. All tissue samples expressed similar levels of β-actin mRNA (data not shown).

**Substrate Specificity of p38-2**—To determine whether p38-2 is a functional protein kinase, either a GST-p38-2 fusion protein produced in bacteria or HA-tagged p38-2 immunoprecipitated from non-stimulated transiently transfected 293 cells was employed in in vitro kinase assays with various substrates (Fig. 3, A and B). Recombinant p38-2 is active even without stimulation. The high basal level of activity may be due to strong autophosphorylation of the threonine and tyrosine in the TGY motif of the kinase domain as determined with phospho-specific antibodies (data not shown). p38-2 strongly phosphorylated the Ets family members Elk1 and Sap-1α, the bZIP protein ATF2, and very weakly c-Jun. In contrast, the NF-κB family members p65 and p50, IκBα, C/EBPβ, and estrogen receptor were not targeted by p38-2 kinase. Similar substrate specificity has been observed for p38 (23).

However, phosphorylation of a transcription factor does not necessarily lead to its activation. Therefore, we tested whether phosphorylation of the Ets transcription factor family members Elk1 and Sap-1α, which are involved in the regulation of the c-fos proto-oncogene via the serum response element (SRE) (38), leads to activation of c-fos SRE-dependent gene transcription. To that end, 293 cells were transfected with a luciferase reporter gene driven by two copies of the c-fos SRE, expression vectors for Elk1, Sap-1α, or the empty vector pEV3S and in vitro kinase assays with recombinant p38-2 or p38 in vitro. As shown in Fig. 3C, Sap-1α-dependent transcription was activated by p38-2 in a dose-dependent manner, whereas Elk1 could not activate transcription. A similar behavior has been observed with p38 (44). These results suggest that although the activity of p38-2 can be monitored in vitro with different substrates, this phosphorylation does not always lead to activation of the downstream target.

We next compared the phosphorylation of Sap-1α by p38-2 and p38 in more detail. To that end, GST-Sap-1α was phosphorylated in vitro by recombinant p38-2 and p38 and cleaved with chymotrypsin, and the resulting phosphopeptides were separated in two dimensions on cellulose thin layer plates (Fig. 4A). Although both MAPKs led to the generation of an identical phosphopeptide pattern, the intensity of the spots was different: whereas p38-2 phosphorylated peptides a–c approximately equally as well as peptides 1–5, p38 preferentially phosphorylated the peptides corresponding to spots 1–5. Mutational analysis has revealed that spots 1–5 are due to phosphorylation at serines 381 and 387 (44). These data suggest that serines 381 and 387 may be more critical for the activation of Sap-1α by p38 than by p38-2. To test this hypothesis, different potential MAPK phosphorylation sites in Sap-1α were mutated. The activity of the mutants was compared with that of the wild type molecule in transiently transfected 293 cells with the c-fos SRE.

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3 The intron 2 sequence is GTAGGTCCAGCAGGGTGAAGGTCGGTGCCAGCAAGGCCTCAGGCCTCAGCTGTCACGGCTCGCGTGACCCTGCAG.
luciferase reporter construct (Fig. 4B). Mutation of serines 381/387 reduced the transactivation potential of Sap-1a upon stimulation with both p38 and p38-2. But consistent with our in vitro phosphopeptide analysis, the serine 381/387 to alanine mutation had a more severe effect upon p38 stimulation than upon p38-2, relative luciferase activity was reduced to 10 and 30%, respectively. As a control we also tested Sap-1a alanine mutants at other sites previously shown to be targeted by MAPKs (44). Mutation of the MAPK sites at positions 420/425 did not affect the transactivation potential of Sap-1a, whereas mutation of threonines 361/366 to alanine affected Sap-1a activity to the same extent upon both p38-2 and p38MAPK stimulation. Combined mutation of all six aforementioned putative phosphorylation sites (6xA) resulted in an inactive Sap-1a molecule upon p38 and p38-2 stimulation.

Kinetic Characterization of p38 and p38-2—Interestingly, p38-2 expressed in bacteria or in mammalian cells is always more active than p38. A more detailed titration analysis of recombinant GST-p38-2 and GST-p38 revealed about 100 times higher kinase activity of p38-2 toward the substrate ATF2 (data not shown). This prompted us to carry out a kinetic analysis of both kinases using ATF2 as substrate.

The kinetic mechanism of GST-p38-2 was investigated by varying the concentrations of both ATP and GST-ATF2 in a single experiment. Both double-reciprocal plots of 1/v versus 1/[GST-ATF2] at fixed ATP concentrations (Fig. 5A) and 1/v versus 1/[ATP] at fixed GST-ATF2 concentrations (Fig. 5B) exhibited an intersecting pattern consistent with a sequential reaction mechanism. A sequential mechanism would proceed through a ternary complex of p38-2, ATP, and GST-ATF2 before a chemical step. Clearly, the double-reciprocal plots do not have a family of parallel lines, the hallmark of a ping-pong type mechanism. Initial velocity data were subjected to a nonlinear, least squares fit to the general rate equation of a Bi Bi mechanism excluding product inhibition terms (reactions had less than 10% of the ATP turned over) (see Table I) (57, 58). Note that the $K_{i,\text{ATP}}$ (“inhibition constant” for ATP) and $K_{i,\text{GST-ATF2}}$ (“inhibition constant” for GST-ATF2) values are similar to the $K_m$ values, which would be expected for a kinetic mechanism that is not ordered. This similarity is consistent with a rapid equilibrium, random mechanism, but it is not proof of a kinetic mechanism.

Equal amounts of GST-p38 and GST-p38-2 proteins expressed in bacteria and processed to similar purity were employed for kinase activity studies. Comparison of GST-p38 and
GST-p38-2 kinase activity at a fixed concentration of ATP (15 μM) and variable GST-ATF2 concentrations revealed that there was a modest but significant difference in the apparent $K_{m}^{\text{GST-ATF2}}$ values: 3.9 ± 0.3 μM for p38-2 and 9.2 ± 1.6 μM for p38 (Fig. 5C). This indicates an approximate 2-fold higher affinity of p38-2 for its substrate GST-ATF2. The major difference in the kinetic parameters resides in the $k_{\text{cat}}$ values: 14.3 ± 0.6 min$^{-1}$ for p38-2 and 0.079 ± 0.011 min$^{-1}$ for p38.

p38-2 Is a Stress-activated Kinase—Next, we examined whether p38-2 like p38 is activated by stress-inducing signals. COS cells were transiently transfected with a vector encoding epitope-tagged p38-2 (3xHA-p38-2). Immune complex kinase assays with ATF2 as substrate demonstrated an up to 4-fold increase in p38-2 kinase activity when cells were treated with interleukin-1β, NaCl, UV light, or anisomycin (Fig. 6, lanes 5–13). Stimulators of the ERK cascade including phorbol 12-myristate 13-acetate and growth factors did not activate p38-2. These results indicate that p38-2 is a member of the family of stress-activated kinases.

We were then interested in identifying components of the upstream activator cascade. MEK6 and TAK1 have been described to activate p38 (22, 28, 29, 32). Cotransfection experiments in COS cells yielded similar results for p38-2. MEK6 increased the kinase activity of p38-2 by 5.5-fold, and TAK1 increased p38-2 activity by 4.2-fold (Fig. 6, lanes 2 and 4). In contrast, MEKK1, a specific activator of JNK, activated p38-2 2.2-fold only (Fig. 6, lane 3). To exclude that changes of p38-2 kinase activity are caused by different levels of expression of p38-2 in response to treatment of cells with stimulators, we performed Western blot analysis with an anti-HA antibody. p38-2 was present at equal levels in all cell lysates (data not shown).

In a similar experiment we analyzed the effect of MEK6 and TAK1 on p38-2 in 293 cells. p38-2 kinase activity was measured in an immune complex kinase assay with Elk1 as a substrate (Fig. 7A, upper panel). TAK1 wild type increased the phosphorylation of Elk1 3.6-fold above the level obtained with a kinase-defective TAK1-K63W mutant (33) (Fig. 7A, compare lanes 4 and 5). TAK1ΔN, an N-terminally truncated version of TAK1 missing the first 22 amino acids, was slightly less active (2.2-fold). No phosphorylation of Elk1 was detected in the absence of p38-2 (Fig. 7A, lanes 1–3). Western blots confirmed that the expression of TAK1 did not change p38-2 protein levels (Fig. 7A, lower panel). In a parallel study we investigated the effect of MEK6 and TAK1 with the SRE-luciferase reporter system. Confirming the in vitro kinase studies shown in Fig. 6, wild type MEK6 and to a greater extent the constitutive active mutant MEK6(DD) increased the effect of p38-2 on the SRE-luciferase reporter (Fig. 7B). Additionally, TAK1 and TAK1ΔN but not TAK1-K63W stimulated p38-2. Since a detailed analysis of the TAK1 MAPKKK has not been performed, we investigated which MAPK pathways were activated by TAK1. To that end, TAK1 was coexpressed with HA-tagged ERK-1, JNK-1, and p38-2 in 293 cells, and the activity of the different MAPKs was assessed after immunoprecipitation in an in vitro kinase assay (Fig. 7C). Similar to p38-2, ERK-1 was only 3-fold stimulated by TAK1, but JNK-1 was more than 15-fold stimulated. Thus, TAK1 may activate all three known MAPK pathways in mammals but appears to be most efficient as a MAPKK in the JNK pathway.

Pyridinyl Imidazole Inhibits p38 and p38-2—A specific inhibitor of p38 with no effect on ERK and JNK was described by Lee and co-workers (12). SB203580, a pyridinyl imidazole derivative, efficiently blocks the kinase activity of p38 and also interferes with p38-2 kinase activity using ATF2 as substrate. As shown in Fig. 8A, SB203580 blocked phosphorylation of ATP2 by p38 as well as by p38-2 with an IC$_{50}$ of around 1 μM for both kinases.

To evaluate the specificity of this compound in vivo, we employed a transcription factor based assay that depends on the phosphorylation of ATP2 at positions 69 and 71. Since ATP2 is a target for the JNK and p38 cascades, we used rather selective upstream activators for each cascade, constitutively active MEKK1 and MEK6, respectively (29). As shown in Fig. 8B, MEKK1 as well as MEK6(DD) increased the activity of phosphorylation of p38-2, a New MAP Kinase Family Member
GAL4-ATF2 about 8-fold. In accordance with our in vitro data, addition of SB203580 to the cells decreased stimulation of ATF2 activity by MEK6 but not by MEKK1 in a dose-dependent manner. SB203580 had no effect on the expression of MEK6 as confirmed by Western blot analysis (data not shown).

DISCUSSION

In this report we describe the cloning and features of a novel member of the MAPK family, p38-2. This protein kinase shares 73% amino acid identity and 86% similarity with mammalian p38 and especially displays the same dual phosphorylation motif TGY, which groups p38-2 into the p38 MAPK subfamily. Interestingly, p38-2 exists in at least three isoforms due to unspliced mRNA species that contain introns providing in-frame stop codons. The mRNAs with introns were found in several independent RNA preparations. Furthermore, the signal strength of Northern blot analyses with an intron 1 probe was similar in intensity to that of a p38-2 cDNA probe (data not shown). Reverse transcriptase-PCR confirmed that in some cell lines up to 50% of the p38-2 mRNA has introns (data not shown). This suggests that mRNA species with introns are quite prominent and therefore are not likely to be caused by a contamination with nuclear RNA. The tissue distribution of isoform 1 mRNA is identical to the intron-less mRNA (data not shown). Assuming similar transcription and protein stability, the wild type and truncated ratios (50:25:25) could yield significant amounts of each isoform. We are currently investigating the effect of p38-2 isoforms on the MEK6/p38-2 signaling cascade. Since isoform 1 as well as 2 lack the TGY phosphorylation motif, they are unlikely to perform an active part in signaling. Since isoform 1 as well as 2 lack the TGY phosphorylation motif, they are unlikely to perform an active part in signaling cascade. Since isoform 1 as well as 2 lack the TGY phosphorylation motif, they are unlikely to perform an active part in signaling cascade. Since isoform 1 as well as 2 lack the TGY phosphorylation motif, they are unlikely to perform an active part in signaling cascade. Since isoform 1 as well as 2 lack the TGY phosphorylation motif, they are unlikely to perform an active part in signaling cascade. Since isoform 1 as well as 2 lack the TGY phosphorylation motif, they are unlikely to perform an active part in signaling cascade. Since isoform 1 as well as 2 lack the TGY phosphorylation motif, they are unlikely to perform an active part in signaling cascade. Since isoform 1 as well as 2 lack the TGY phosphorylation motif, they are unlikely to perform an active part in signaling cascade. Since isoform 1 as well as 2 lack the TGY phosphorylation motif, they are unlikely to perform an active part in signaling cascade. Since isoform 1 as well as 2 lack the TGY phosphorylation motif, they are unlikely to perform an active part in signaling cascade. Since isoform 1 as well as 2 lack the TGY phosphorylation motif, they are unlikely to perform an active part in signaling cascade. Since isoform 1 as well as 2 lack the TGY phosphorylation motif, they are unlikely to perform an active part in signaling cascade. 

While this work was under preparation a nearly identical kinase, p38β (Genbank® accession number U53442), was identified by Jiang and co-workers (55). This protein kinase has three substitutions and an insert of eight amino acids was identified by Jiang and co-workers (55). This protein kinase, p38β, is also known as CSBP, RK, p38-2, SAPK3, m, and KEK6 (also known as SAPK3).

A comparison of p38 and p38-2 mRNA expression revealed that both protein kinases are rather widely expressed. However, both kinases displayed a great variance in the degree of expression depending on the tissue analyzed, and also p38-2 and p38 were differently expressed. Expression levels in heart and testis are significantly higher for p38-2, and expression levels in placenta and ovary are significantly lower. The expression pattern of Mxi2 is similar to that of p38 (25). In contrast, ERK6 has been described to be restricted to skeletal muscle (56), which is puzzling in view of the wide tissue distribution of its rat homologue SAPK3 as well as human SAPK3 (59, 60).

Similarities between p38-2 and p38 prompted us to investigate their substrate specificity. p38-2 as well as p38 efficiently phosphorylate ATF2, Elk1, and Sap-1a. However, c-Jun, the preferred substrate for JNK, is only weakly phosphorylated by p38-2. This suggests that the substrate selectivity of p38-2 is very similar to p38 although we cannot exclude that there are other substrates that distinguish between these two MAPK. The substrate specificity of SAPK3 overlaps but is distinct from p38 and p38-2. SAPK3 does not phosphorylate MAPKAP K2 (59). Strikingly, the site preference for individual phosphorylation sites within one target protein can differ, as shown with Sap-1a. Phosphopeptide analyses revealed that p38 as well as p38-2 have overlapping phosphorylation sites in Sap-1a. However, p38 prefers serines 381/387 in Sap-1a relative to p38-2. Consequently, mutation of serines 381/387 affected activation of Sap-1a-mediated transcription by p38 in vivo significantly more than that by p38-2. The phosphopeptides a–c that are strongly recognized by p38-2 have not been mapped. It would be interesting to compare these two phosphopeptide patterns with the pattern created by SAPK3, which also has been described to phosphorylate Sap-1a. These studies open the question how does differential phosphorylation of a substrate affect its activity? Would it be possible to design inhibitors that block phosphorylation of a substrate by one kinase but not by the other? Do MAPK differentially phosphorylate their substrates dependent on the stimulator used?

In addition, we found that phosphorylation of Elk1 by p38-2, in contrast to Sap-1a and ATF2, does not lead to an increase in Elk1-mediated transcription, a phenomenon that has also been observed with p38 (44). This suggests that Elk1 is not phosphorylated at sites critical for its transcriptional activity and stresses the fact that phosphorylation of a transcription factor does not necessarily lead to its activation.

In addition to the differential phosphorylation of Sap-1a by p38 and p38-2, we observed that ATF2 is a much better substrate for p38-2 than p38. p38β is also more active than p38 using GST-ATF2 as substrate (55). Investigation of the kinetic mechanism of p38 and p38-2 using ATP as substrate revealed a modest 2-fold higher substrate affinity and more than 180-fold higher catalytic activity of p38-2. The concentration of kinase used in our experiments was 18–600 nM, which is the

### Table I

Kinetic parameters of p38–2

| Substrate | $K_m$ (μM) | $K_m$ (μM) | $K_m$ (μM) | $K_m$ (μM) |
|-----------|------------|------------|------------|------------|
| GST-ATF2  | 1.6 ± 0.4  | 10.8 ± 2.6 | 2.5 ± 0.4  | 9.3 ± 3.0  |
| ATP       | 5.8 ± 1.8  |            |            |            |

**FIG. 6. Stimulators of p38-2 in vivo.** COS cells were transiently transfected with epitope-tagged p38-2 and cotransfected with expression vectors for constitutive active MEK6 (MEK6DD), MEKK1, and TAK1 (lanes 3 and 4) or treated for 45 min with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma), epidermal growth factor (50 ng/ml; Life Technologies, Inc.), nerve growth factor (50 ng/ml; Life Technologies, Inc.), TGFβ (20 ng/ml; Life Technologies, Inc.), interleukin-1β (10 ng/ml; R&D Systems), tumor necrosis factor-α (10 ng/ml; R&D Systems), NaCl (200 mM; Sigma), UV light (254 nm; 120 J/m²), anisomycin (50 ng/ml; Sigma) (lanes 5–12). Cell lysates were used in an immune complex kinase assay with GST-ATF2 substrate as described under “Experimental Procedures.” The position of protein molecular mass markers in kDa is shown on the left. The presence of equal amounts of p38-2 in all kinase reactions was confirmed by Western blot analysis (data not shown). The relative phosphorylation of GST-ATF2 substrate in lanes 1–13 is 1.0, 5.5, 2.2, 4.2, 1.4, 0.6, 0.6, 1.1, 3.3, 0.6, 3.2, 2.5, and 4.0.

*DISCUSSION*
physiological range of MAPK family members in the cell (30–2800 nM) (61). As the $k_{cat}$ values for p38 were less than 5 min$^{-1}$, which is considered low and problematic (58), p38 appears to be a very inefficient kinase, and it is possible that its true substrate has yet to be identified. This effect could be caused by a less efficient turnover of GST-ATF2 by p38 or a lower fraction of active p38 in the bacterial fusion protein preparation. However, the latter is unlikely since several independent preparations of bacterial GST-p38 and GST-p38-2 proteins yielded similar results. Furthermore, Coomassie staining of purified GST-p38 and GST-p38-2 showed similar yield and purity (data not shown). Although our data likely reflect true differences in the catalytic activity of GST-p38 and GST-p38-2, we cannot exclude that upon activation in vivo by MAPKK, the catalytic activities of p38 and p38-2 may not be so dramatically different. However, preliminary data demonstrated that p38-2 activated in vivo by cotransfected, constitutively active MEK6 is about 30 times more active than a similarly activated p38 (data not shown). We also discovered that p38-2 but not p38 prepared in bacteria is phosphorylated. It is therefore possible that p38-2 activates itself by autophosphorylation. Autophosphorylation has been described for many MAPK. More work is necessary to distinguish between autophosphorylation and phosphorylation by a bacterial kinase. Generation of p38-2 mutants of the ATP binding site or the phospho-acceptor sites should help to answer these questions in future studies.

Consistent with its classification as a member of the p38MAPK subfamily, p38-2 was activated in vivo by stress-inducing signals. Osmotic shock, UV light, anisomycin, and interleukin-1β strongly increased p38-2 activity, whereas TGFβ and tumor necrosis factor-α were more modest activators. This profile of stimulation of p38-2 is reminiscent of p38. The upstream protein kinase MEK6 is a very efficient activator of p38 in vivo (22, 29). We show here that MEK6(DD), a constitutively active mutant of MEK6, also increased p38-2 activity in vivo. Furthermore, we and others (27, 29) have previously shown that MEKK1, an activator of JNKK, can cross-talk to the MKK3/MEK6 cascade, but a careful titration analysis showed that much higher amounts of MEKK1 are necessary for activation of MEK6 compared with JNKK (29). In support of these observations MEKK1 was found to be significantly less active than MEK6. In summary these and other studies showed that at least four members of the p38 family (p38, p38-2, p38β, and ERK6) are activated by MEK6.
three major MAPK cascades, p38-2, JNK, and ERK-1, revealed that JNK is by far the best target for TAK1. Our findings are in agreement with the suggestion that JNK is a downstream target for TAK1 (33). Many more studies will be needed to sort out which of the described kinases from the MAPKKK level (DLK, MEKK1, MLK3, MUK, Pak1, TAK1, and Tpl2) leads to physiological activation of MEK6 and MKK3.

Co- and workers (55) previously showed that p38 is inhibited by the pyridyl imidazole derivative SB203580. This compound is highly selective for p38 and does not interfere with closely related kinases such as JNK and ERK. Our studies showed that p38-2 is also a target for this inhibitor with an IC50 identical to p38. Studies by Jiang et al. (55) showed that an analogue of SB203580, SB202190, inhibits p38 equally well. Interestingly, SAPK3, the rat homologue of ERK6, is not inhibited by SB203580 at concentrations up to 100 μM (59).

SB203580 also interferes with p38/p38-2 activity in vivo (Fig. 8). Therefore, some of the biological effects attributed to p38 may be mediated by p38-2 and p38b. The selective activation of GAL4-ATF2 by low concentrations of MEKK1 is likely to affect only the activation of the JNKK→JNK→ATF2 cascade. These data support our conclusion that MEKK1 is the physiological activator of the JNK but not the p38 cascade. On the other hand, activation of GAL4-ATF2 by MEK6 via p38/p38-2 was efficiently blocked by SB203580. Further studies in vivo with this compound are required to unravel the redundancy as well as specificity of these kinases. All p38 family members phosphorylate a number of proteins in vitro. However, not all phosphorylation events lead to an increase in transcriptional activity of the substrates. Furthermore, substrate specificity in vitro may vary in vivo.

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