MOLECULAR AND CELLULAR BIOLOGY, Mar. 1996, p. 1247–1255 Vol. 16, No. 3
0270-7306/96/$04.00+0

MKK3- and MKK6-Regulated Gene Expression Is Mediated by the p38 Mitogen-Activated Protein Kinase Signal Transduction Pathway

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Received 9 October 1995/Returned for modification 15 November 1995/Accepted 21 December 1995

The p38 mitogen-activated protein (MAP) kinase signal transduction pathway is activated by proinflammatory cytokines and environmental stress. The detection of p38 MAP kinase in the nucleus of activated cells suggests that p38 MAP kinase can mediate signaling to the nucleus. To test this hypothesis, we constructed expression vectors for activated MKK3 and MKK6, two MAP kinase kinases that phosphorylate and activate p38 MAP kinase. Expression of activated MKK3 and MKK6 in cultured cells caused a selective increase in p38 MAP kinase activity. Cotransfection experiments demonstrated that p38 MAP kinase activation causes increased reporter gene expression mediated by the transcription factors ATF2 and Elk-1. These data demonstrate that the nucleus is one target of the p38 MAP kinase signal transduction pathway.

Several mitogen-activated protein (MAP) kinase signal transduction pathways have been detected in mammalian cells (15). Three groups of MAP kinases have been molecularly cloned: ERK (7, 8), JNK (16, 22, 31, 34, 55), and p38 (27, 35, 47). These MAP kinases are activated by dual phosphorylation on Thr and Tyr within the motif Thr-Xaa-Tyr in subdomain VIII (15). The sequence of this dual phosphorylation motif differs for each MAP kinase group as follows: p38, Thr-Gly-Tyr; JNK, Thr-Pro-Tyr; and ERK, Thr-Glu-Tyr. Each MAP kinase group has a distinct substrate specificity and is regulated by a separate signal transduction pathway (15). Mammalian cells therefore contain multiple MAP kinase signal transduction pathways that mediate the effects of extracellular stimuli on a wide array of biological processes.

Detailed studies of the JNK and ERK groups of MAP kinase have led to significant insight into the physiological function of these signaling pathways (6, 13–15, 40, 45). In contrast, the role of the p38 MAP kinase signal transduction pathway is poorly understood (20, 27, 35, 44, 47). p38 MAP kinase is weakly activated by protein kinase C and receptor tyrosine kinases but is strongly activated by the treatment of cells with inflammatory cytokines (e.g., tumor necrosis factor and interleukin-1) and environmental stress (e.g., osmotic shock and UV radiation) (20, 27, 35, 44, 47). The contribution of the p38 MAP kinase pathway to the cellular response to these stimuli has not been established. However, recent studies have implicated p38 MAP kinase in the phosphorylation of the small heat shock protein Hsp27 (20, 47), in increased cytokine expression (35), and in programmed cell death (61). Furthermore, in vitro protein kinase assays demonstrate that p38 MAP kinase phosphorylates MAPKAP kinase-2 (20, 47) and the transcription factor ATF2 (17, 44).

The mechanism of p38 MAP kinase activation is mediated by dual phosphorylation on Thr and Tyr within the motif Thr-Gly-Tyr located in subdomain VIII (44). The p38 MAP kinase activator MKK3 has been molecularly cloned (17). MKK3 is a protein kinase that phosphorylates and activates p38 MAP kinase but does not phosphorylate the related JNK or ERK MAP kinases (17). MKK3 is therefore a specific activator of p38 MAP kinase that is independent of the JNK and ERK signaling pathways. A second MAP kinase kinase, MKK4, phosphorylates and activates both p38 MAP kinase and JNK in vitro (17, 36). Additional components of the p38 MAP kinase pathway have not been identified. However, the Rho family GTPases Rac1 and Cdc42 (5, 11, 41, 42, 62) and the STE20-related protein kinases PAK-1 (62), PAK-3 (5), and GC kinase (43) have been implicated in the p38 MAP kinase and JNK signaling pathways.

The purpose of this study was to examine the functional consequence of p38 MAP kinase activation in vivo. Cytokines and environmental stress activate several signal transduction pathways, including p38 MAP kinase. Identification of responses that are causally related to p38 MAP kinase activation is therefore difficult. Here we describe the molecular cloning of a novel activator of p38 MAP kinase (MKK6) that is related to MKK3. Dominant-active mutant forms of MKK3 and MKK6 were employed to selectively activate the p38 MAP kinase signal transduction pathway in cultured cells. This experimental approach allows the functional dissection of the p38 MAP kinase signal transduction pathway in vivo. We report that p38 MAP kinase can mediate signaling to the nucleus.

MATERIALS AND METHODS

Materials. Flag-MKK3 (17) was subcloned into pRc/RSV (Invitrogen, Inc.) at the *Hin*dIII and *Spe*I restriction sites to create the expression vector pRSV-Flag-MKK3. Dominant-active MKK3 [MKK3(Glu)] and dominant-negative MKK3 [MKK3(Ala)] were constructed by replacing Ser-189 and Thr-193 with Glu and Ala residues, respectively. The mutations were made by overlapping PCR (28), and the sequence was confirmed with a model 373A DNA sequencer (Applied Biosystems, Inc.). The expression vectors for epitope-tagged p38 (17, 44), epitope-tagged JNK1 (16), MEK1 with mutations of S-218 to E and S-222 to D (S218E/S222D MEK1) (38), and ΔN3/S218E/S222D MEK1 (where residues 32 to 51 deleted) (38) have been described previously. The expression vector for hemagglutinin (HA)-tagged ERK2 was provided by M. Weber. Bacterial expression of glutathione *S*-transferase (GST)-ATF2, GST-ERK2, GST–Elk-1, GST-Jun, GST-p38, and GST-JNK1 has been described previously (4, 16, 23, 60). The Flag peptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys was obtained from Chiron Corp.

Molecular cloning of MKK6. Human MKK6 cDNA clones were isolated from a skeletal muscle library (Stratagene, Inc.) by screening with an MKK3 probe at

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low stringency. Both of the largest clones obtained (1,699 and 2,942 bp) included the entire coding region of MKK6. However, the sequences of these clones differed in the 3′ noncoding region. The Flag epitope (-Asp-Tyr-Lys-Asp-Asp-Asp-Lys-; Immunex Corp.) was inserted between codons 1 and 2 of MKK6 by insertional overlapping PCR (28). A similar PCR procedure was used to replace Lys-82 with Ala to create MKK6(K82A) and to replace Ser-207 and Thr-211 with Glu to create MKK6(Glu). Mammalian MKK6 expression vectors were constructed by subcloning the MKK6 cDNA in the *Hin*dIII and *Xba*I sites of pCDNA3 (Invitrogen, Inc.). A bacterial MKK6 expression vector was constructed by subcloning the MKK6 cDNA in the *Eco*RI and *Xba*I sites of pGEX-3X (Pharmacia LKB Biotechnology, Inc.).

The sequences of all plasmids were confirmed by automated sequencing with an Applied Biosystems model 373A machine.

Tissue culture. Chinese hamster ovary cells were maintained in Ham's F12 medium supplemented with 5% fetal bovine serum. COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (Gibco-BRL). Plasmid DNA (1 μg) was transfected by the Lipofectamine method (Gibco-BRL), and the cells were harvested after 48 h of incubation.

Protein kinase isolation. The cells were exposed to 40 J of UV-C radiation per m^2 (60 min) and solubilized with buffer A (20 mM Tris [pH 7.5], 10% glycerol, 1% Triton X-100, 0.137 M NaCl, 25 mM β-glycerophosphate, 2 mM EDTA, 0.5 mM dithiothreitol, 1 mM orthovanadate, 2 mM PP_i, 10 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride). The extracts were centrifuged at 100,000 × *g* for 15 min at 4°C. Epitope-tagged protein kinases were immunoprecipitated by incubation for 2 h at 4°C with the M2 Flag monoclonal antibody (IBI-Kodak) or HA monoclonal antibody (BAbCo) bound to protein G-Sepharose (Pharmacia LKB Biotechnology, Inc.). Endogenous p38 MAP kinase was immunoprecipitated with a rabbit polyclonal p38 antibody prebound to protein A-Sepharose (44). The immunoprecipitates were washed twice with buffer A and twice with kinase buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4], 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM sodium orthovanadate). Elution of Flag-tagged protein kinases from M2 immunoprecipitates was performed by incubation for 2 h at 4°C with 100 μg of Flag peptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys per ml.

Protein kinase assays. Recombinant protein kinases (100 ng), soluble immunopurified protein kinases, or protein kinase immunoprecipitates were employed for kinase assays. The reactions were initiated by the addition of 1 μg of substrate proteins (ATF2, Elk-1, c-Jun, ERK2, p38, or JNK1) and 50 μM [γ-^{32}P]ATP (10 Ci/mmol) in a final volume of 40 μl of kinase buffer. The reactions were terminated after 30 min at 25°C by addition of Laemmli sample buffer. The phosphorylation of the substrate proteins was examined after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by autoradiography.

Hybridization analysis. Northern (RNA) blot analysis was done with poly(A)$^+$ mRNA (2 μg) isolated from different human tissues (Clontech, Inc.). The mRNA was fractionated by denaturing agarose gel electrophoresis and transferred to a nylon membrane. The blot was probed with the MKK6 cDNA labeled by random priming with [α-^{32}P]dATP (Amersham International PLC).

Reporter gene expression. Cotransfection assays were performed with CHO cells. The luciferase reporter plasmids were pSRE-Luc (60), −79/+170*jun*-Luc (18), TNFα-Luc (provided by S. McKnight), CMV-Luc (53), SV40-Luc (53), HIV LTR-Luc (provided by W. Greene), HTLV-1 LTR-Luc (provided by W. Greene), and pRSV-Luc (53). The activities of the GAL4 DNA binding domain (49), GAL4/ATF2 (23), GAL4/ElkC (39), GAL4/Jun (31), and GAL4/VP16 (48) were measured in cotransfection assays with the reporter plasmid pG5E1bLuc (53). This reporter plasmid contains five GAL4 sites cloned upstream of a minimal promoter element and the firefly luciferase gene. Transfection efficiency was monitored by using a control plasmid that expresses β-galactosidase (pCH110; Pharmacia LKB Biotechnology, Inc.). The luciferase and β-galactosidase activity detected in cell extracts was measured (23, 24).

RESULTS

MKK3 causes increased p38 MAP kinase activity. The MKK3 protein kinase is activated by dual phosphorylation on Ser-189 and Thr-193 (17). The mechanism of MKK3 activation may be mediated by increased negative charge at the sites of phosphorylation. We therefore examined the effect of introducing a constitutive negative charge by replacing Ser-189 and Thr-193 with Glu. The wild-type and mutated MKK3 proteins were isolated from cells exposed to 0 and 40 J of UV-C per m^2. MKK3 protein kinase activity was measured in an immune complex assay using GST-p38 as a substrate (17). Figure 1A shows that UV irradiation caused increased activity of wild-type MKK3. In contrast, the MKK3(Glu) protein kinase was not regulated by UV irradiation. The similar protein kinase activities observed in assays of MKK3(Glu) and UV-stimulated wild-type MKK3 indicate that MKK3(Glu) is constitutively activated (Fig. 1A).

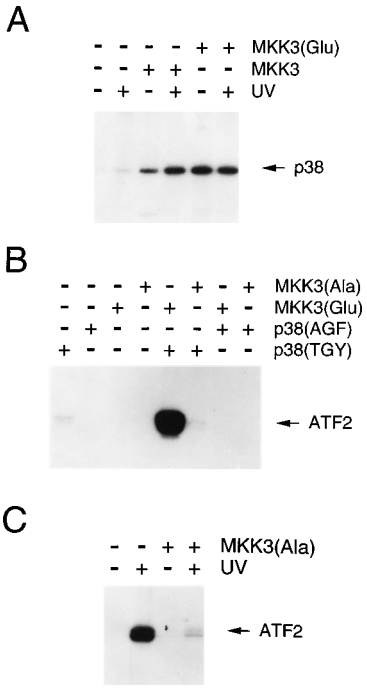

FIG. 1. Construction of a constitutively activated MKK3 protein kinase. The MKK3 protein kinase is activated by phosphorylation on Ser-189 and Ser-193 (17). Mutated MKK3 protein kinases were created by replacement of both phosphorylation sites with Glu or Ala. (A) Epitope-tagged wild-type MKK3 and S189E/T193E MKK3 [MKK3(Glu)] were expressed in COS cells. The cells were activated by exposure to 40 J of UV-C per m^2 and incubated for 1 h. The MKK3 protein kinase activity was measured in an immune complex kinase assay using GST-p38 MAP kinase as a substrate. (B) Constitutively activated MKK3(Glu) was expressed in COS cells together with epitope-tagged wild-type p38 MAP kinase (TGY) or mutated p38 MAP kinase (AGF). The mutated p38 MAP kinase is not a substrate for MKK3, because it lacks the sites of activating phosphorylation on Thr and Tyr (17). The p38 MAP kinase activity was measured in an immune complex kinase assay using GST-ATF2 as a substrate (17, 44). (C) Dominant-negative S189A/T193A MKK3 [MKK3(Ala)] was expressed in COS cells together with epitope-tagged p38 MAP kinase. The cells were exposed to 40 J of UV-C per m^2 and incubated for 1 h. The p38 MAP kinase activity was measured in an immune complex kinase assay using GST-ATF2 as a substrate.

To test whether MKK3(Glu) causes constitutive p38 MAP kinase activation in vivo, we performed cotransfection studies using MKK3 and p38 MAP kinase. The p38 MAP kinase was isolated by immunoprecipitation, and the protein kinase activity in the immune complex with the substrate ATF2 was measured (44). Figure 1B shows that MKK3(Glu) caused marked activation of p38 MAP kinase. Control experiments demonstrated that MKK3(Glu) activated wild-type p38 MAP kinase (TGY) but not the phosphorylation-defective mutant (AGF) lacking the sites of activating Thr and Tyr phosphorylation. These data indicate that MKK3(Glu) causes increased p38 MAP kinase activity in vivo.

The constitutive activation of MKK3 caused by the replacement of Ser-189 and Thr-193 with Glu suggests that the replacement of these residues with Ala [producing MKK3(Ala)] would yield an inactive MKK3 protein kinase. Indeed, expression of MKK3(Ala) did not cause p38 MAP kinase activation (Fig. 1B). To test whether the Ala substitution acts as a dominant-interfering mutation, we investigated the effect of MKK3(Ala) on p38 MAP kinase activity in cotransfection experiments. Figure 1C shows that the expression of MKK3(Ala) caused a marked reduction in UV-stimulated p38 MAP kinase activity. Control experiments demonstrated that MKK3(Ala)

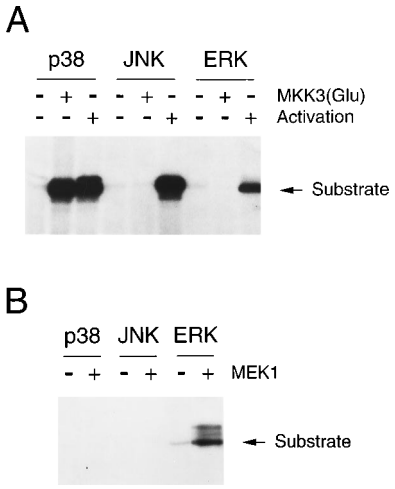

FIG. 2. Constitutively activated MKK3 stimulates p38 MAP kinase but not ERK or JNK. (A) COS cells were cotransfected with constitutively activated MKK3(Glu) and an epitope-tagged MAP kinase (p38 MAP kinase, ERK2, or JNK1). The cells were activated by exposure to 40 J of UV light per m^2 (p38 MAP kinase and JNK1) or 100 nM phorbol myristate acetate (ERK2). MAP kinase activity was measured in an immune complex kinase assay using ATF2 as a substrate for p38 MAP kinase and JNK. ERK protein kinase activity was measured by using the substrate GST-Myc. (B) Cells were cotransfected with constitutively activated ΔN3/S218E/S222D MEK1 (38) and an epitope-tagged MAP kinase (p38 MAP kinase, ERK2, or JNK1). The MAP kinase activity was measured in an immune complex kinase assay using the substrate GST-Myc (for ERK2) and GST-ATF2 (for p38 MAP kinase and JNK1).

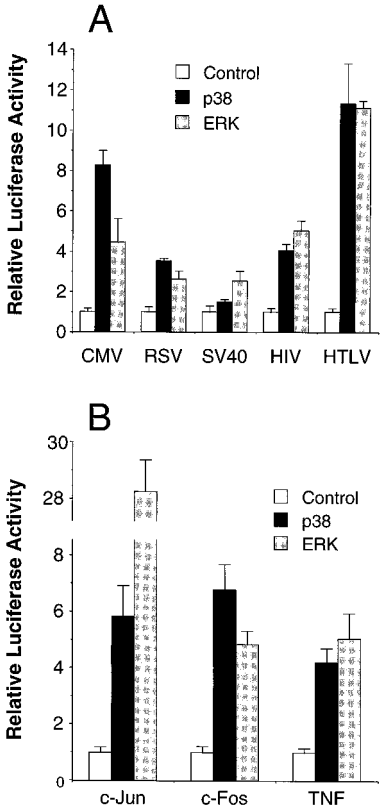

FIG. 3. Selective activation of viral, proto-oncogene, and cytokine promoters by the MKK3-p38 MAP kinase signal transduction pathway. CHO cells were cotransfected with luciferase reporter plasmids and an empty expression vector (Control) or expression vectors for MEK3(Glu) and p38 MAP kinase (p38) or activated MEK1 (S218E/S222D) (ERK). A β-galactosidase expression vector (pCH110) was employed to control for transfection efficiency. Cell extracts were prepared at 48 h following transfection, and the activity of β-galactosidase and luciferase was measured. The data are the means ± standard errors of the means of the ratios of luciferase activity to β-galactosidase activity (n = 3) and are presented as the activity relative to that of control cells. (A) Reporter plasmids with cytomegalovirus (CMV), Rous sarcoma virus (RSV), simian virus 40 (SV40), human immunodeficiency virus LTR (HIV), and HTLV-1 LTR (HTLV) promoters; (B) reporter plasmids with the tumor necrosis factor alpha promoter (TNF) or fragments of the c-*jun* (proximal AP-1 site) or c-*fos* (SRE) promoter.

also inhibited JNK activation, but it did not inhibit ERK activation (data not shown). The inhibition of both p38 and JNK suggests that MKK3(Ala) may function by sequestering upstream MAP kinase kinase kinases that activate the JNK and p38 MAP kinase signaling pathways.

Together, these data demonstrate that dominant-active and dominant-negative MKK3 mutants are created by the replacement of the sites of activating phosphorylation (Ser-189 and Thr-193) with Glu and Ala, respectively.

MKK3 causes selective activation of p38 MAP kinase. The MKK3 protein kinase phosphorylates and activates p38 MAP kinase by dual phosphorylation on Thr and Tyr (17, 44). In contrast, MKK3 does not phosphorylate JNK or ERK MAP kinases (17). This analysis indicates that the expression of constitutively activated MKK3 should cause selective activation of p38 MAP kinase in vivo. To test this hypothesis, we examined the effect of MKK3(Glu) on p38, JNK, and ERK in cotransfection assays. Figure 2A shows that MKK3(Glu) caused activation of p38 MAP kinase but not JNK or ERK. Control experiments demonstrated that activated MEK1 caused increased ERK activity but not increased p38 or JNK activity (Fig. 2B). These data indicate that MKK3 causes selective activation of the p38 MAP kinase signal transduction pathway.

MKK3 causes increased reporter gene expression. We employed MKK3(Glu) to examine the functional consequence of p38 MAP kinase activation in vivo. In initial experiments, we examined the effect of activated MKK3 on the expression of a luciferase reporter gene. Control experiments using activated MEK1 to stimulate the ERK signal transduction pathway were also performed. Figure 3 shows that the expression of activated MEK1 increased luciferase expression in experiments using reporter plasmids that contained viral, proto-oncogene, and cytokine promoters. In contrast, MKK3(Glu) caused only a small increase in reporter gene expression (data not shown). This observation suggests that the ERK, but not the p38 MAP kinase, signal transduction pathway regulates reporter gene expression. However, control experiments demonstrated that while activated MEK1 caused increased activity of both endogenous and cotransfected ERK, MKK3(Glu) caused a marked increase in p38 MAP kinase activity only in cells that were cotransfected with p38 MAP kinase (Fig. 2). MKK3(Glu) caused only a small (twofold) increase in the activity of the endogenous p38 MAP kinase (data not shown). The lack of a marked effect of MKK3(Glu) on reporter gene expression may therefore be caused by the failure of MKK3(Glu) to cause a large increase in endogenous p38 MAP kinase activity. To test this hypothesis, we examined the effect of coexpression of MKK3(Glu) with p38 MAP kinase on reporter gene expression. We found that coexpression of MKK3(Glu) with p38 MAP kinase (Fig. 3), but not the expression of MKK3(Glu) or p38 MAP kinase alone (data not shown), caused increased reporter gene expression. This requirement for coexpression of p38 MAP kinase with MKK3(Glu) indicates that the effects of MKK3 on increased reporter gene expression are mediated by

p38 MAP kinase. However, we are not able to exclude the possibility that another (perhaps unidentified) MAP kinase also contributes to the effects of MKK3(Glu).

The increased reporter gene expression caused by activated MEK1 and MKK3 was observed in experiments using several different reporter plasmids that contain viral, proto-oncogene, and cytokine promoters (Fig. 3). However, small effects of these activated MAP kinase kinases were observed in experiments using reporter plasmids with Rous sarcoma virus and simian virus 40 promoters. Larger increases in luciferase expression were found in experiments using reporter plasmids with cytomegalovirus, human immunodeficiency virus long terminal repeat (LTR), human T-cell leukemia virus type 1 (HTLV-1) LTR, and tumor necrosis factor alpha promoters or fragments of the c-*jun* (proximal AP-1 site) and c-*fos* (SRE) promoters. Together, these studies demonstrate that the p38 MAP kinase signal transduction pathway, like the ERK signal transduction pathway, leads to increased gene expression.

The largest effects of MKK3(Glu) on reporter gene expression were observed in experiments using the HTLV-1 LTR promoter, the c-*fos* SRE, and the c-*jun* proximal AP-1 site (Fig. 3). The mechanism that accounts for the increased gene expression is unclear. We therefore examined the effect of MKK3(Glu) on reporter gene expression in a simpler experimental model employing GAL4 fusion proteins. Cotransfection assays using a reporter plasmid with GAL4 DNA binding sites cloned upstream of a luciferase gene were performed. While the level of luciferase expression detected in experiments using the GAL4 DNA binding domain was extremely low, a high level of luciferase activity was found in cells transfected with GAL4/VP16 (Fig. 4). Expression of MKK3(Glu) caused a small increase in GAL4/VP16-dependent luciferase expression (Fig. 4).

MKK3(Glu)-stimulated reporter gene expression was also examined in experiments using the GAL4 DNA binding domain fused to the activation domains of the ATF2, c-Jun, and Elk-1 transcription factors. MKK3(Glu) increased ATF2- and Elk-1-dependent reporter gene expression but caused only a small increase in Jun-dependent gene expression (Fig. 4). In contrast, activated MEK1 increased Elk-1- and c-Jun-dependent gene expression but not ATF2-dependent gene expression (Fig. 4). Control experiments demonstrated that the increased gene expression was reduced by mutation of the phosphorylation sites Thr-69 and Thr-71 (ATF2), Ser-63 and Ser-73 (c-Jun), and Ser-383 (Elk-1) (Fig. 4). The activation of ATF2 and Elk-1 by MKK3(Glu) suggests that these transcription factors are targets of the p38 MAP kinase signal transduction pathway. Indeed, p38 MAP kinase phosphorylates ATF2 and Elk-1 but does not phosphorylate c-Jun (Fig. 4E).

These data demonstrate that the transcription factors ATF2 and Elk-1 can account, in part, for transcriptional regulation by the MKK3-p38 MAP kinase signal transduction pathway. However, an important caveat must be placed on this conclusion because the activation of ATF2 and Elk-1 by MKK3(Glu) requires the overexpression of p38 MAP kinase (Fig. 3). For example, the overexpressed p38 MAP kinase may phosphorylate proteins that are not physiological substrates of p38 MAP kinase. Further experiments are therefore required to examine the effect of p38 MAP kinase activation in the absence of p38 MAP kinase overexpression. These studies require the use of a novel p38 MAP kinase activator. A candidate activator is MKK6.

Molecular cloning of MKK6. The MAP kinase kinase MKK6 was identified by screening a human skeletal muscle cDNA library. Sequence analysis demonstrated the presence of an open reading frame that encodes a protein kinase related

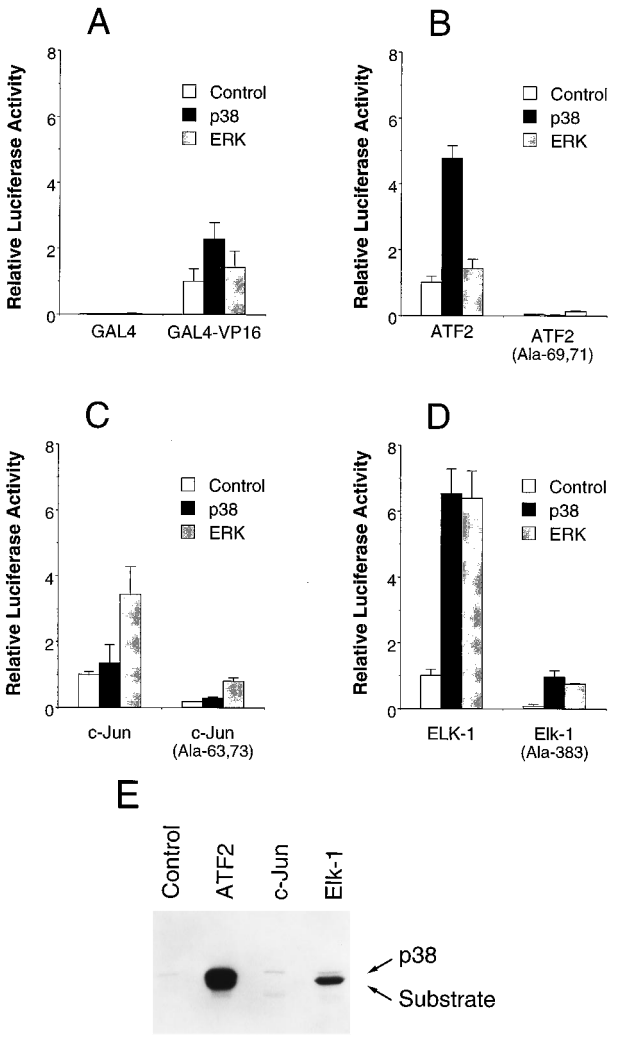

FIG. 4. ATF2 and Elk-1 mediate transcriptional regulation by the MKK3-p38 MAP kinase signal transduction pathway. (A) CHO cells were cotransfected with the β-galactosidase expression vector pCH110, the reporter plasmid pG5E1bLuc, and an expression vector for the GAL4 DNA binding domain (residues 1 to 147) or the GAL4 DNA binding domain fused to the acidic activation domain of VP16. The graph presents the effects of the expression of S218E/S222D MEK1 or MKK3(Glu) and p38 MAP kinase. Cell extracts were prepared at 48 h following transfection, and the activity of β-galactosidase and luciferase was measured. The data are presented as the means ± standard errors of the means of the ratios of luciferase activity to β-galactosidase activity ($n = 3$). (B to D) The transcriptional activities of GAL4-ATF2 and GAL4-ATF2(T69A/T71A) (B), GAL4–Elk-1 and GAL4–Elk-1(S383A) (C), and GAL4–c-Jun and GAL4–c-Jun(S63A/S73A) (D) were measured in cotransfection assays using the reporter plasmid pG5E1bLuc. The data are the means ± standard errors of the means of the ratios of luciferase activity to β-galactosidase activity ($n = 3$) and are presented as the activity relative to that of control cells transfected with GAL4-ATF2, GAL4–Elk-1, and GAL4–c-Jun, respectively. (E) Epitope-tagged p38 MAP kinase was immunopurified from activated cells (40 J of UV-C per m^2) by immunoprecipitation with the Flag monoclonal antibody M2 and elution of p38 MAP kinase by incubation with 100 μg of the Flag peptide MDYKDDDDK per ml for 1 h at 4°C. Kinase assays were performed with 1 μg of substrate (GST-ATF2, GST-Jun, or GST–Elk-1) and 50 μM [γ-^{32}P]ATP. Control experiments were performed by using GST as a substrate. The assays were terminated after 20 min at 22°C, and the reaction products were examined by SDS-PAGE and autoradiography.

to the MAP kinase kinase group (Fig. 5A). In-frame termination codons present in the 5′ and 3′ noncoding regions indicated that the MKK6 clones contained the complete coding sequence. Two groups of clones with identical coding regions but distinct 3′ noncoding regions were identified. Comparison

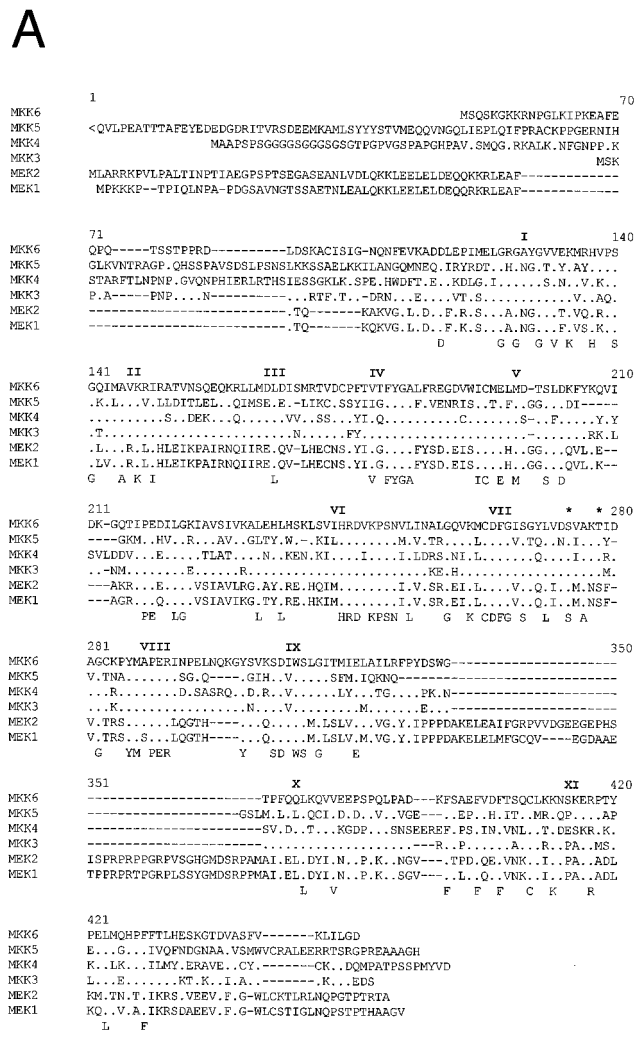

FIG. 5. Primary structure of human MKK protein kinases. (A) The primary sequence of MKK6 was deduced from the sequences of cDNA clones isolated from a human skeletal muscle library. In-frame stop codons are located in the 5′ and 3′ noncoding regions of the MKK6 cDNAs. The sequence of MKK6 was compared with those of the human MAP kinase kinases MEK1 (51, 63), MEK2 (63), MKK3 (17), MKK4 (17, 36), and MKK5 (65) with the PILE-UP program (version 7.2; Wisconsin Genetics Computer Group). The protein sequences are presented in single-letter code. The MKK5 sequence is truncated at the NH_2 terminus (<). Gaps introduced into the sequences to optimize the alignment are illustrated with dashes. Identical residues are indicated with periods. The sites of activating phosphorylation of MEK (3, 64) are indicated with asterisks. The sequences of two MKK6 cDNA clones (1,699 and 2,942 bp) with identical coding regions and alternative 3′ untranslated regions have been deposited in GenBank under accession numbers U39656 and U39657. (B) The relationships between members of the human MAP kinase kinase group are presented as a dendrogram created by the unweighted pair-group method using arithmetic averages (PILE-UP program). The human MAP kinase kinases MEK1, MEK2, MKK3, MKK4, MKK5, and MKK6 are presented.

of MKK6 with other human MAP kinase kinases demonstrated that MKK6 is most similar to the p38 MAP kinase activator MKK3 (Fig. 5B).

The expression of MKK6 was examined by Northern blot analysis of mRNA isolated from different human tissues. A small transcript (approximately 1.7 kb) and a large transcript (approximately 12 kb) were detected (data not shown). The isolation of MKK6 cDNAs with alternative 3′ untranslated regions may account for the different mRNA transcripts detected by hybridization analysis. The highest level of expression was detected in skeletal muscle (data not shown).

MKK6 activates p38 MAP kinase in vitro. The substrate specificity of MKK6 was examined by investigating the phosphorylation of MAP kinases (p38, JNK, and ERK) by MKK6 in an in vitro protein kinase assay. MKK6 phosphorylated p38 MAP kinase but not JNK1 or ERK2 (Fig. 6A). A low level of MKK6 phosphorylation by activated p38 MAP kinase was also detected (Fig. 6A). These data demonstrate that the substrate specificity of MKK6 is similar to that of MKK3, a MAP kinase kinase that selectively activates p38 MAP kinase (17). However, the specific activity of the MKK6 protein kinase was approximately 300-fold greater than that of MKK3 (data not shown).

The sites of p38 MAP kinase phosphorylation by MKK6 were examined by mutational analysis. Replacement of the p38 MAP kinase dual phosphorylation motif Thr-Gly-Tyr with the phosphorylation-defective motif Ala-Gly-Phe (17, 44) blocked the phosphorylation of p38 MAP kinase by MKK6 (Fig. 6B). These data indicate that MKK6 phosphorylates p38 MAP kinase on Thr-180 and Tyr-182, the sites of phosphorylation that activate p38 MAP kinase (44).

The activation of p38 MAP kinase by MKK6 was examined by measurement of p38 MAP kinase activity with the substrate ATF2 (17, 44). This analysis demonstrated that MKK6 caused p38 MAP kinase activation (Fig. 6C). In contrast, MKK6 did not activate the phosphorylation-defective (Ala-Gly-Phe) p38 MAP kinase (Fig. 6C). Together, these data demonstrate that MKK6 functions as a potent and selective activator of p38 MAP kinase in vitro.

MKK6 activates p38 MAP kinase in vivo. MKK6 protein kinase activity was measured in an immune complex assay using p38 MAP kinase as a substrate (17). Exposure of cells to UV radiation caused increased MKK6 protein kinase activity (Fig. 7A). Inclusion of the p38 MAP kinase substrate ATF2 in the kinase assays demonstrated that the UV-activated MKK6 caused increased p38 MAP kinase activity (Fig. 6A). This analysis suggests that MKK6 may activate p38 MAP kinase in vivo.

MAP kinase kinases are activated by dual phosphorylation within subdomain VIII (3, 9, 12, 29, 38, 52, 64). These phosphorylation sites are conserved in MKK6 (Ser-207 and Thr-211). The mechanism of MKK6 activation may therefore be mediated by increased negative charge on Ser-207 and Thr-211. To test this hypothesis, we examined the effect of introducing a constitutive negative charge by replacing Ser-207 and

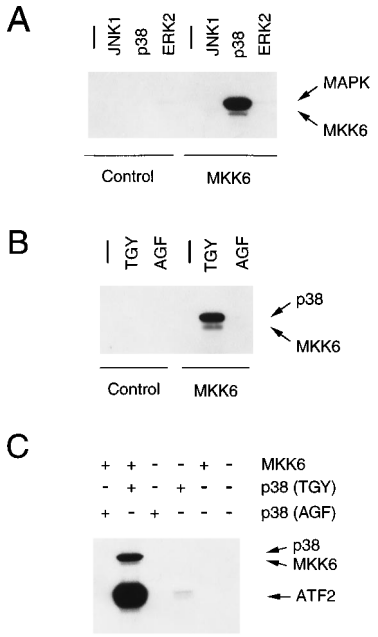

FIG. 6. MKK6 phosphorylates and activates p38 MAP kinase in vitro. (A) Recombinant GST-MKK6 was incubated with [γ-^{32}P]ATP and buffer (—), GST-JNK1 (JNK1), GST-p38 MAP kinase (p38), or GST-ERK2 (ERK2). The phosphorylation reaction was terminated by addition of Laemmli sample buffer, and the phosphorylated proteins were detected after SDS-PAGE by autoradiography. (B) GST-MKK6 was incubated with [γ-^{32}P]ATP and buffer (—), wild-type GST-p38 MAP kinase (TGY), or mutated GST-p38 MAP kinase (AGF). In the mutated p38 MAP kinase, Thr-180 and Tyr-182 were replaced with Ala and Phe, respectively. The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. (C) GST-MKK6 was incubated with [γ-^{32}P]ATP and buffer, wild-type GST-p38 MAP kinase (TGY), or mutated GST-p38 MAP kinase (AGF). The p38 MAP kinase substrate ATF2 was included in each incubation (44). The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography.

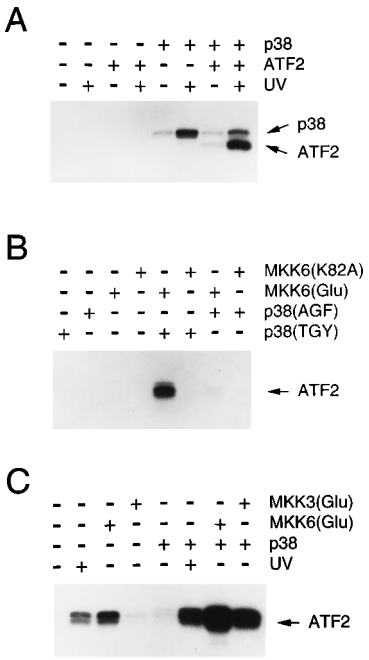

FIG. 7. MKK6 phosphorylates and activates p38 MAP kinase in vivo. (A) Cells expressing epitope-tagged MKK6 were exposed in the absence and presence of 40 J of UV-C per m^2. MKK6 was isolated by immunoprecipitation and used for protein kinase assays with p38 MAP kinase as a substrate. The transcription factor ATF2 was included in the incubations as a substrate for p38 MAP kinase (17, 44). The phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP and was terminated by the addition of Laemmli sample buffer. The phosphorylated proteins were visualized after SDS-PAGE by autoradiography. (B) Constitutively activated MKK6(Glu) and kinase-negative MKK6(K82A) were expressed together with epitope-tagged wild-type p38 MAP kinase (TGY) or mutated p38 MAP kinase (AGF). The mutated p38 MAP kinase is not a substrate for MKK6, because it lacks the sites of activating phosphorylation on Thr and Tyr (17). The p38 MAP kinase activity was measured in an immune complex kinase assay using ATF2 as a substrate (17, 44). (C) MKK3(Glu), MKK6(Glu), and p38 MAP kinase were expressed in cells that were activated by exposure to 40 J of UV light per m^2. p38 MAP kinase activity was measured in an immune complex kinase assay with the substrate ATF2. Transfected and endogenous p38 MAP kinase was assayed in an immune complex kinase assay with a polyclonal p38 antibody (44).

Thr-211 with Glu. We coexpressed MKK6(Glu) with p38 MAP kinase in cultured cells. The p38 MAP kinase activity was measured in an immune complex kinase assay using the transcription factor ATF2 as a substrate. MKK6(Glu) caused activation of wild-type (Thr-Gly-Tyr) p38 MAP kinase but not activation of the phosphorylation-defective (Ala-Gly-Phe) mutant (Fig. 7B). In contrast, kinase-negative MKK6 did not activate p38 MAP kinase (Fig. 7B). Together, these data demonstrate that MKK6(Glu) is an activator of p38 MAP kinase in vivo.

The activation of transfected p38 MAP kinase by MKK6(Glu) (Fig. 7B) suggests that the expression of MKK6(Glu) may activate the endogenous p38 MAP kinase in cultured cells. To test this hypothesis, we compared the effects of MKK6(Glu) on endogenous and transfected p38 MAP kinase. MKK6(Glu) was found to efficiently activate both endogenous and overexpressed p38 MAP kinase (Fig. 7C). In contrast, a constitutively activated mutant of the p38 MAP kinase activator MKK3 [MKK3(Glu)] that markedly stimulates overexpressed p38 MAP kinase (Fig. 2) caused only a small increase (twofold) in the activity of endogenous p38 MAP kinase (Fig. 7C). These data demonstrate that constitutively activated MKK6 (and not MKK3) is an effective activator of endogenous p38 MAP kinase in vivo. We therefore employed MKK6(Glu) in further studies to examine the function of the p38 MAP kinase signal transduction pathway.

MKK6 causes increased ATF2-dependent gene expression. The transcription factor ATF2 is phosphorylated in vitro by p38 MAP kinase on Thr-69 and Thr-71 (17, 44). Phosphorylation of ATF2 on these sites causes increased transcriptional activation potential (23, 37, 58). To test whether ATF2 is phosphorylated and activated by p38 MAP kinase in vivo, we examined the effect of expression of MKK6(Glu) in cotransfection assays (Fig. 8A). Increased ATF2-dependent gene expression was observed in cells transfected with MKK6(Glu). In contrast, MKK3(Glu) did not increase ATF2-dependent gene expression in the absence of overexpression of p38 MAP kinase (Fig. 8A). These data are consistent with the potent activation of p38 MAP kinase caused by MKK6(Glu) and with the ineffective activation of endogenous p38 MAP kinase caused by MKK3(Glu) (Fig. 7C).

The specificity of MKK6-stimulated ATF2-dependent gene expression was examined in cotransfection assays (Fig. 8B). MKK6(Glu) caused a marked increase in ATF2-dependent reporter gene expression in experiments using wild-type (Thr-69, Thr-71) ATF2. In contrast, the effect of MKK6(Glu) was markedly reduced in experiments using phosphorylation-defective (Ala-69, Ala-71) ATF2. Control experiments demonstrated that wild-type MKK6 caused only a small increase in reporter gene expression and that kinase-negative MKK6 (K82A) did not increase ATF2-dependent reporter gene expression. Together, these data confirm the hypothesis that transcription factors are targets of the p38 MAP kinase signal

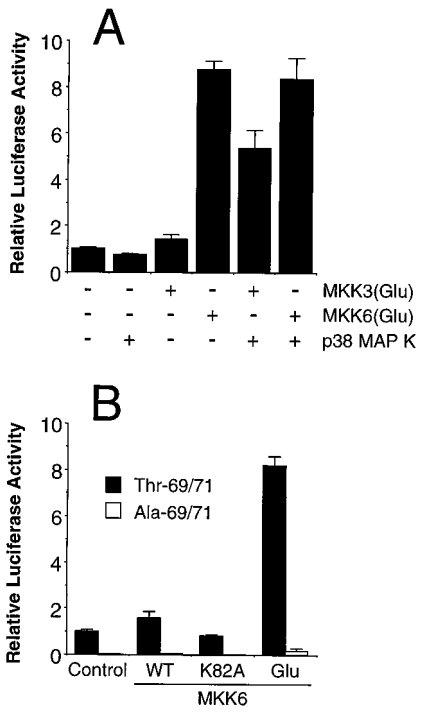

FIG. 8. Increased gene expression mediated by the MKK6-p38 MAP kinase signal transduction pathway. (A) ATF2-dependent gene expression was examined in cotransfection assays using a luciferase reporter gene. Transfection efficiency was monitored by measurement of β-galactosidase expression. The relative luciferase activities (means ± standard deviations; $n = 3$) detected following transfection of the empty expression vector, MKK3(Glu), MKK6(Glu), and p38 MAP (p38 MAP K) kinase are presented. (B) Cells were transfected with an empty expression vector (Control), MKK6 (WT), MKK6(Glu), and MKK6(K82A). ATF2-dependent reporter gene expression was measured by using wild-type ATF2 (Thr-69/71) and mutated, phosphorylation-defective ATF2 (Ala-69/71). The data are presented as relative luciferase activities (means ± standard deviations; $n = 3$).

transduction pathway (44) and establish the utility of MKK6(Glu) as a reagent that can be employed to dissect the function of the p38 MAP kinase signal transduction pathway in vivo.

DISCUSSION

Selective activation of the p38 MAP kinase signal transduction pathway. The p38 MAP kinase signal transduction pathway is activated by proinflammatory cytokines and environmental stress. These stimuli also activate other signal transduction pathways. The identification of cellular responses that are causally related to p38 MAP kinase activation is therefore difficult. Methods that allow the selective activation of the p38 MAP kinase pathway are required. Here we report the use of MKK3 and MKK6 as specific activators of p38 MAP kinase. MKK3 is activated by phosphorylation on Ser-189 and Thr-193 (17). Similarly, MKK6 is activated by dual phosphorylation on Ser-207 and Thr-211 (Fig. 5). Constitutively activated MKK3 and MKK6 mutants were constructed by replacing these phosphorylation sites with Glu residues. The constitutive activation probably results from the presence of negative charges (Glu) at the sites of activating phosphorylation. A similar approach has been employed to create constitutively activated MEK1 to increase ERK protein kinase activity in vivo (9, 12, 29, 38, 52).

The constitutively activated MAP kinase kinases MKK3 (Glu) and MKK6(Glu) may be generally applicable to studies of the p38 MAP kinase signal transduction pathway in vivo. A possible limitation of this approach is that p38 MAP kinase activation may lead to increased cytokine expression (35) and autocrine activation of additional signaling pathways in some cells. However, for CHO cells and COS cells, the expression of MKK3(Glu) or MKK6(Glu) was found to markedly activate the p38 MAP kinase signal transduction pathway (Fig. 2 and 7).

Regulation of gene expression by p38 MAP kinase. To examine the role of the p38 MAP kinase signaling pathway, we investigated the effect of MKK3(Glu) and MKK6(Glu) on luciferase expression by using reporter plasmids containing viral, proto-oncogene, and cytokine promoters. These studies demonstrated that the p38 MAP kinase signaling pathway increases gene expression. This increase in reporter gene expression caused by p38 MAP kinase activators may be accounted for by a general effect of p38 MAP kinase on transcription or by posttranscriptional actions of p38 MAP kinase (e.g., mRNA processing, mRNA export, mRNA stability, and translation). However, reporter gene expression from some promoters (e.g., the simian virus 40 promoter) was not significantly increased, while marked increases in reporter gene expression were observed in experiments using other promoters (e.g., the HTLV-1 LTR promoter). This differential effect of p38 MAP kinase activators in increasing luciferase expression in experiments using different promoters indicates that the p38 MAP kinase signal transduction pathway causes a selective increase in gene expression.

To identify specific transcription factors that may mediate the effects of the p38 MAP kinase signaling pathway, we examined the effect of p38 MAP kinase activators [MKK3(Glu) and MKK6(Glu)] in cotransfection experiments using GAL4 fusion proteins. Small increases in reporter gene expression were observed in experiments using GAL4/VP16 and GAL4/Jun. These small increases may be mediated by posttranscriptional effects of the p38 MAP kinase signaling pathway. In contrast, p38 MAP kinase activators caused a larger increase in reporter gene expression in experiments using GAL4/ATF2 and GAL4/Elk.

Although these data establish that transcription factors (e.g., ATF2 and Elk-1) are targets of the p38 MAP kinase signal transduction pathway, they do not exclude the possibility that p38 MAP kinase may also control gene expression by posttranscriptional regulation of specific gene transcripts. For example, p38 MAP kinase may regulate tumor necrosis factor expression (35) by regulating transcription, mRNA stability, and translation (26, 32, 33, 54). Further studies are required to identify the molecular basis of these possible actions of p38 MAP kinase.

Transcription factor Elk-1 is regulated by the p38 MAP kinase signal transduction pathway. Elk-1 is an ETS domain transcription factor that binds together with serum response factor to the SRE. Elk-1 contains a COOH terminal transcriptional activation domain that is phosphorylated by the ERK (21, 30, 39) and JNK (60) groups of MAP kinase. Expression of activated MEK1 caused increased Elk-1-dependent reporter gene expression (Fig. 4). Similar increases in Elk-1 transcriptional activity were observed in cells transfected with MKK3(Glu) (Fig. 4) and MKK6(Glu) (data not shown). Furthermore, similar effects of ERK and p38 MAP kinase activators were observed in experiments using a reporter plasmid containing the c-*fos* SRE (Fig. 3). Mutation of the Elk-1 phosphorylation site Ser-383 reduced the increases in luciferase expression caused by both ERK and p38 MAP kinase activators (Fig. 4). Together, these data indicate that Elk-1 is a target of all three groups of MAP kinase (p38, JNK, and ERK). Elk-1 therefore serves to integrate signals from multiple MAP kinase signal transduction pathways in response to extracellular stimuli (Fig. 9). However, the effect of SRE activation is likely to

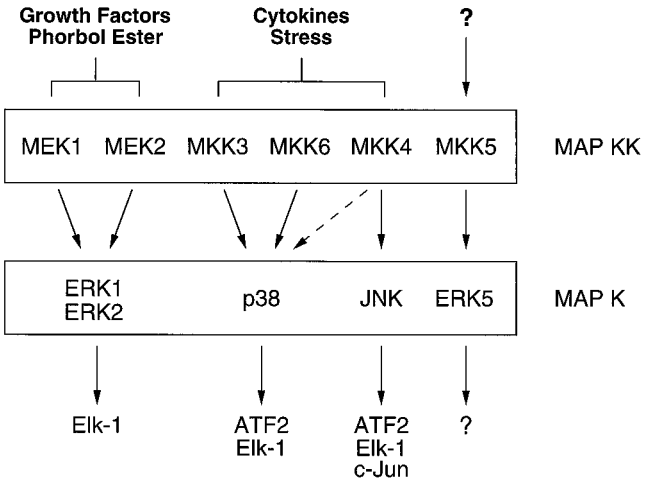

FIG. 9. Schematic representation of mammalian MAP kinase (MAP K) signal transduction pathways. The ERK signaling pathway is activated by tyrosine kinases and protein kinase C (6, 10, 14). The MAP kinase kinases (MAP KK) MEK1 and MEK1 activate the ERK1 and ERK2 group of MAP kinases (2). The ERK1 and ERK2 MAP kinases phosphorylate and activate the transcription factor Elk-1 (21, 39). The p38 MAP kinase (15) phosphorylates and activates the transcription factors ATF2 (17, 44) and Elk-1 (Fig. 4). The MAP kinase kinases MKK3 (17), MKK4 (17, 36), and MKK6 (Fig. 5) phosphorylate and activate p38 MAP kinase. The JNK group of MAP kinases (15) are activated by the MAP kinase kinase MKK4 (17, 36, 50). The JNK protein kinases phosphorylate and activate the transcription factors ATF2 (23), c-Jun (16), and Elk-1 (60). The MAP kinase ERK5 (65) is phosphorylated and activated by MKK5 (19, 65). Targets of the ERK5 MAP kinase pathway have not been identified (65).

differ for each MAP kinase pathway because the SRE functions with other promoter elements (which may be differentially regulated by p38, JNK, and ERK) to control gene expression (46, 56).

Transcription factor ATF2 is regulated by the p38 MAP kinase signal transduction pathway. The ATF2 transcription factor is phosphorylated by p38 MAP kinase on Thr-69 and Thr-71 (44). Phosphorylation of ATF2 on these sites causes increased transcriptional activity in vivo (23, 37, 58). The phosphorylation of ATF2 by p38 MAP kinase may therefore account for the increases in ATF2-dependent reporter gene expression caused by the p38 MAP kinase activators MKK3(Glu) and MKK6(Glu) (Fig. 4). The activation of the HTLV-1 LTR (which binds ATF2 [59]) is consistent with this hypothesis (Fig. 3). Furthermore, the activation of ATF2 may account, in part, for the increases in reporter gene expression caused by p38 MAP kinase activators in experiments using a fragment (bp −73 to +170) of the c-*jun* promoter (Fig. 3) that binds ATF2 as a heterodimer with c-Jun (57, 58).

Control experiments demonstrated that the expression of activated MEK1 (which stimulates the ERK group of MAP kinases) did not increase ATF2-dependent reporter gene expression (Fig. 4). ATF2 is therefore not a target of the ERK signal transduction pathway (Fig. 9). However, ATF2 is activated by the JNK signal transduction pathway (23, 37, 58). Thus, both the p38 and JNK signaling pathways contribute to the regulation of ATF2 transcriptional activity in cells exposed to proinflammatory cytokines and environmental stress. It is likely that the consequence of ATF2 activation by each of these pathways is different. For example, the p38 MAP kinase activates ATF2 (Fig. 4), while JNK phosphorylates and activates both ATF2 (23, 37) and c-Jun (1, 16, 31, 55). The ATF2–c-Jun heterodimer (25) is therefore predicted to be differentially regulated by the p38 and JNK signal transduction pathways.

Signaling of p38 MAP kinase in the nucleus. Activation of p38 MAP kinase causes a selective increase in gene expression (Fig. 4). The p38 MAP kinase pathway may increase gene expression by a general effect on transcription, by the activation of specific transcription factors, or by posttranscriptional regulation (mRNA processing, nuclear export, mRNA stability, translation, and protein stability). Autocrine activation of cells in response to p38 MAP kinase-stimulated cytokine expression (35) may contribute to these regulatory processes. The results of this study indicate that part of the genetic response to p38 MAP kinase activation is mediated by the direct activation of transcription factors. Immunofluorescence microscopic analysis demonstrates that p38 MAP kinase is present in the nucleus and the cytoplasm (44). The nuclear targets of p38 MAP kinase include the transcription factors ATF2 and Elk-1 (Fig. 9).

ACKNOWLEDGMENTS

We thank N. Ahn, W. Greene, M. Karin, S. McKnight, A. Sharrocks, R. Treisman, R. Ulevitch, and M. Weber for providing reagents; I.-H. Wu for technical assistance; and M. Roberts for secretarial assistance.

R.J.D. is an Investigator of the Howard Hughes Medical Institute.

This work was supported by grants CA58396 and CA65861 from the National Cancer Institute.

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