1997-05-01

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Et al.

MOLECULAR AND CELLULAR BIOLOGY, May 1997, p. 2360–2371 Vol. 17, No. 5
0270-7306/97/$04.00+0

Role of p38 and JNK Mitogen-Activated Protein Kinases in the Activation of Ternary Complex Factors

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Received 3 September 1996/Returned for modification 4 November 1996/Accepted 31 January 1997

The transcription factors Elk-1 and SAP-1 bind together with serum response factor to the serum response element present in the c-*fos* promoter and mediate increased gene expression. The ERK, JNK, and p38 groups of mitogen-activated protein (MAP) kinases phosphorylate and activate Elk-1 in response to a variety of extracellular stimuli. In contrast, SAP-1 is activated by ERK and p38 MAP kinases but not by JNK. The proinflammatory cytokine interleukin-1 (IL-1) activates JNK and p38 MAP kinases and induces the transcriptional activity of Elk-1 and SAP-1. These effects of IL-1 appear to be mediated by Rho family GTPases. To examine the relative roles of the JNK and p38 MAP kinase pathways, we examined the effects of IL-1 on CHO and NIH 3T3 cells. Studies of NIH 3T3 cells demonstrated that both the JNK and p38 MAP kinases are required for IL-1-stimulated Elk-1 transcriptional activity, while only p38 MAP kinase contributes to IL-1-induced activation of SAP-1. In contrast, studies of CHO cells demonstrated that JNK (but not the p38 MAP kinase) is required for IL-1-stimulated Elk-1-dependent gene expression and that neither JNK nor p38 MAP kinase is required for IL-1 signaling to SAP-1. We conclude that (i) distinct MAP kinase signal transduction pathways mediate IL-1 signaling to ternary complex transcription factors (TCFs) in different cell types and (ii) individual TCFs show different responses to the JNK and p38 signaling pathways. The differential utilization of TCF proteins and MAP kinase signaling pathways represents a potential mechanism for the determination of cell-type-specific responses to extracellular stimuli.

Transcription of the prototypic immediate-early gene c-*fos* is rapidly induced in cells treated with a wide variety of extracellular stimuli, including growth factors, cytokines, UV radiation, and phorbol ester. The serum response element (SRE) is an important regulatory sequence located in the c-*fos* promoter (75) and is a target for a number of signal transduction pathways (7, 8, 81). The SRE is recognized by a complex of serum response factor (SRF) and a ternary complex factor (TCF) (76).

SRF is phosphorylated in vitro by casein kinase II, the ERK-activated S6 kinase p90^{rsk}, and a DNA-activated protein kinase (8). However, the role of these kinases in the transcriptional activation of SRF remains unclear. Calcium activates SRF-dependent transcription independently of TCF, by a mechanism involving Ca^{2+}/calmodulin-dependent protein kinases (51). A recent study has provided evidence that members of the Ras-related Rho family of GTPases are involved in signaling pathways that lead to SRF-mediated activation of the c-*fos* SRE independently of complex formation with TCFs (27). This family of GTPases includes Cdc42, Rac1, and RhoA. These GTPases play a critical role in regulating the actin cytoskeleton and other physiological processes (60). RhoA is important for SRF-mediated induction of the c-*fos* SRE by serum, lysophosphatidic acid, and aluminum fluoride (27). Activated mutants of Cdc42 and Rac1 have been proposed to stimulate SRF-mediated transcription independently of TCFs (27). The signaling mechanisms involved in these events await elucidation.

TCF proteins belong to a subgroup of the Ets domain family (33) that includes Elk-1 (28), SAP-1 (13), and SAP-2 (21, 44, 54). The primary sequences of these proteins show significant similarity in three distinct regions (76). The N-terminal 93 amino acids comprise the Ets domain, which mediates DNA binding (13, 32, 70). The more centrally located 21-amino-acid B box is required for ternary complex formation and has been demonstrated to mediate direct protein-protein contacts between Elk-1 and SRF (69). The COOH-terminal activation domain contains several conserved mitogen-activated protein (MAP) kinase phosphorylation sites (76). Phosphorylation of the TCF proteins Elk-1 and SAP-1 on sites within the C-terminal activation domain results in activated SRE-dependent gene expression (7, 8, 81).

MAP kinases are proline-directed Ser/Thr protein kinases that are regulated by extracellular signals including growth factors, mitogens, and cellular stress (14, 15, 81). The best-characterized MAP kinase subfamily includes ERK1 and ERK2, which are activated by growth factors via the conserved Ras/Raf-1/MEK pathway (15, 81). Recently, three distinct stress-activated MAP kinase subfamilies have been identified: JNK (also termed SAPK) (16, 22, 34, 38, 71), p38 (also termed CSBP) (24, 39), and BMK1 (also termed ERK5) (1, 40, 87). The JNKs were initially characterized by their ability to phosphorylate and activate the transcription factor c-Jun (16, 26, 38). The transcription factor ATF2 is also a target of JNK MAP kinase (23, 43, 77). p38 MAP kinase phosphorylates and activates ATF2 (57, 58) and CHOP (78). Components of the kinase cascade that leads to JNK activation have been identified and include the MEK homolog MKK4 (also termed SEK1 or JNKK) (17, 41, 64) and the MAP kinase kinase kinases MEKK1 (50, 84), MEKK2 (4), MEKK3 (4), Tpl-2 (63), SPRK/

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MLK3 (59, 74), and MUK (29). The MEK homologs MKK3 (17) and MKK6 (25, 52, 58, 72) activate p38 MAP kinase. A candidate MAP kinase kinase kinase for p38 MAP kinase is TAK1 (83), which activates both MKK3 and MKK6 (52). TAK1 is activated by TAK1-binding protein and has been proposed to mediate transforming growth factor β signal transduction (67).

It is established that Rho family GTPases are important for cytoskeletal regulation (60) and the transforming activity of oncogenic Ras (35, 36, 55, 56). Significantly, the full malignant transformation activity of Ras requires the coordinate activity of the Raf-1/MEK/ERK MAP kinase pathway and a pathway mediated by the Rho family GTPases Rac and Rho (36, 55, 56). Recently, the Rho family GTPases Cdc42 and Rac1 have been shown to stimulate the stress-activated MAP kinase pathways that lead to JNK and p38 MAP kinase activation (2, 11, 49, 53, 86).

A group of STE20-related serine/threonine kinases termed PAKs (p21-activated kinases) are direct targets for activation by Cdc42 and Rac1 (3, 5, 37, 45, 48) and appear to be intermediates between the GTPases and the MAP kinase cascades (3, 5, 86). Rho family GTPases and PAKs have been proposed to mediate the activation of JNK and p38 MAP kinases by the proinflammatory cytokine interleukin-1 (IL-1) (2, 86). Small GTPases also directly activate MAP kinase kinase kinases. For example, Ras binds to Raf-1 (81) and MEKK1 (61), and SPRK/MLK3 binds Cdc42 and Rac (6, 59, 74).

The TCF protein Elk-1 is phosphorylated by the ERK (18, 19, 31, 47) and JNK (9, 10, 20, 82, 88) groups of MAP kinases in response to growth factors, phorbol ester, IL-1, UV radiation, and treatment with the protein synthesis inhibitor anisomycin. ERK and JNK MAP kinases phosphorylate a similar set of sites within the Elk-1 activation domain (10, 20, 82), leading to increased DNA binding (66, 82), ternary complex formation (18–20, 82), and transcriptional activation (10, 18, 20, 31, 47, 82). Recently, it has been demonstrated that p38 MAP kinase phosphorylates Elk-1 in vitro and increases both Elk-1 transcriptional activity and SRE-dependent gene expression (58). SAP-1 activity is regulated by the ERK MAP kinase (30, 54, 70).

This study examines the role of the p38 and JNK MAP kinase signal transduction pathways in the regulation of the TCF transcription factors Elk-1 and SAP-1 in response to IL-1. We show that both Elk-1 and SAP-1 are phosphorylated by p38 MAP kinase, leading to increased DNA binding activity, ternary complex formation, and transcriptional activation. JNK MAP kinase phosphorylates and activates Elk-1 but not SAP-1. We used selective inhibitors of the JNK and p38 MAP kinase pathways to demonstrate that in CHO cells, the JNK signaling pathway is required to mediate IL-1-induced transcriptional activity of Elk-1 (but not SAP-1) and SRE-dependent gene expression. The IL-1-stimulated Elk-1 transcriptional activity in CHO cells was blocked by dominant-negative Rho family GTPases. Furthermore, we demonstrate that activated mutants of the Rho family GTPases increase Elk-1 activity and SRE-dependent gene expression in CHO cells and, in particular, that Cdc42-activated Elk-1-dependent reporter gene expression requires the JNK MAP kinase signal transduction pathway. Finally, in contrast to the results obtained for CHO cells, we show that in NIH 3T3 cells, IL-1-stimulated transcriptional activity of Elk-1 requires both the p38 and JNK MAP kinase signaling pathways, while p38 MAP kinase is essential for SAP-1-dependent gene expression.

These data implicate roles for both the JNK and p38 signaling pathways in mediating the transmission of extracellular signals to the TCF component of the ternary complex at the c-*fos* SRE. The relative importance of the JNK and p38 pathways is not conserved but depends on the cell type that is examined.

MATERIALS AND METHODS

Materials. Plasmids J3HCdc42L61 and J3HCdc42N17, expressing hemagglutinin (HA)-tagged constitutively activated Cdc42 L61 and inactivated Cdc42 N17, were provided by R. A. Cerione (2). Plasmids pCDNA3/Rac1 L61, pCEV29/Rac1 N17, pCDNA3/RhoA L63, and pCEV29/RhoA N19, expressing activated and inactivated forms of Rac1 and RhoA, were provided by S. J. Gutkind (11). pCMV5/Ras L61, expressing activated Ras, was a gift from L. Kozma (University of Massachusetts Medical School). The vector pSG5 (Stratagene Inc.), which expresses wild-type mouse MAP kinase phosphatase 1 (MKP1; 3CH134), was provided by N. Tonks (73). Expression vectors for dominant-active MKK3 [MKK3(Glu)] (58), dominant-active MKK6 [MKK6(Glu)] (58), dominant-negative MKK4 (DN-MKK4) (82), MEKK1 (82), and activated MEK1 (ΔN3/S218E/S222D MEK1) (46) have been described elsewhere. The expression vector for HA-tagged ERK2 was provided by M. Weber (University of Virginia). HA-tagged JNK1 (16), Flag-tagged JNK1 and JNK2 isoforms (16, 22, 71), and Flag-tagged p38 (57) expression vectors have been described elsewhere. Vectors expressing the GAL4 DNA binding domain (62), GAL4–Elk-1 (82), GAL4–Elk-1 Ala-383 (82), GAL4–c-Jun (26), and GAL4–c-Jun Ala63/73 (26) have been described elsewhere. The GAL4–SAP-1 expression vector was constructed by subcloning a PCR fragment encoding SAP-1 residues 287 to 431 in the *Bam*HI and *Xba*I sites of pSG424 (62). The sequence of the construct was confirmed by using a model 373A DNA sequencer (Applied Biosystems Inc.). Bacterial expression of glutathione *S*-transferase (GST)-tagged full-length ATF2 (23), GST-ATF2 (residues 1 to 109) (23), GST–c-Jun (residues 1 to 79) (16), GST–Elk-1 (residues 307 to 428) (82), and GST–SAP-1 (residues 287 to 431) (82) has been described previously. Bacterial expression of hexahistidine (His_6)-tagged full-length Elk-1 and SAP-1 has been described elsewhere (82). Rabbit polyclonal antibodies that bind JNK and p38 MAP kinases have been described elsewhere (57). Polyclonal antibodies that bind ERK1, ERK2, and JNK1 were purchased from Santa Cruz Biotechnology. Mouse monoclonal M2 Flag antibody was from IBI-Kodak. Mouse monoclonal anti-HA antibody was a gift from J. Pouysségur (Centre National de la Recherche Scientifique, Nice, France). The p38 MAP kinase inhibitor VK19577 (also termed SB203580 [12, 39]) was provided by Vertex Pharmaceuticals Inc. and dissolved in dimethyl sulfoxide.

Tissue culture. Chinese hamster ovary (CHO) cells were maintained in Ham's F12 medium supplemented with 5% fetal bovine serum (Gibco-BRL). COS-7 cells and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10% calf serum, respectively. Plasmid DNA was transfected by the Lipofectamine method (Gibco-BRL). Cells were harvested 48 h after transfection following serum starvation for 12 h. Where indicated, CHO cells were treated with 10 ng of mouse IL-1α (Genzyme Corp.) per ml, 10 nM tetradecanoyl phorbol acetate (Sigma), or 40 J of UV-C per m^2.

Protein kinase assays. Cells were solubilized with Triton lysis buffer (20 mM Tris [pH 7.4], 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 0.5 mM dithiothreitol). The extracts were centrifuged at 14,000 × *g* for 15 min at 4°C. Endogenous ERK, JNK, and p38 were immunoprecipitated (3 h at 4°C) from extracts by using the appropriate polyclonal antibodies immobilized on protein A-Sepharose (Sigma). Epitope-tagged ERK2, JNK1, and p38 were immunoprecipitated with mouse monoclonal anti-HA or M2 Flag antibody immobilized on protein G-Sepharose (Pharmacia-LKB Biotechnology). The immunoprecipitates were washed three times with Triton lysis buffer and once with kinase buffer (25 mM HEPES [pH 7.4], 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM sodium orthovanadate). The kinase assays were initiated by the addition of 1 μg of substrate protein and 50 μM [γ-^{32}P]ATP (10 Ci/mmol) in a final volume of 40 μl. The reactions were terminated after 30 min at 25°C by the addition of Laemmli sample buffer. The phosphorylation of substrate proteins was examined after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by autoradiography.

Western blot analysis. HA-tagged JNK expression was detected by immunoblot analysis using a monoclonal anti-HA antibody. Immune complexes were detected by enhanced chemiluminescence as instructed by the manufacturer (Amersham International PLC).

Reporter gene expression. The activities of the GAL4 DNA binding domain, GAL4–Elk-1, GAL4–Elk-1 Ala-383, GAL4–SAP-1, GAL4–c-Jun, and GAL4–c-Jun Ala63/73 were measured in cotransfection assays in CHO cells with the reporter plasmid pG5E1bLuc (65), which contains five GAL4 DNA binding sites cloned upstream of a minimal promoter element and the firefly luciferase gene. SRE-dependent gene expression was measured in cotransfection assays with the reporter plasmid pSRE-Luc (65), which contains two copies of the c-*fos* SRE cloned upstream of a minimal promoter element and the firefly luciferase gene. Transfection efficiency was monitored by using a control plasmid expressing β-galactosidase (pCH110; Pharmacia-LKB Biotechnology Inc.). The luciferase and β-galactosidase activities were measured as described previously (65). The data are presented as the ratio of luciferase activity to β-galactosidase activity.

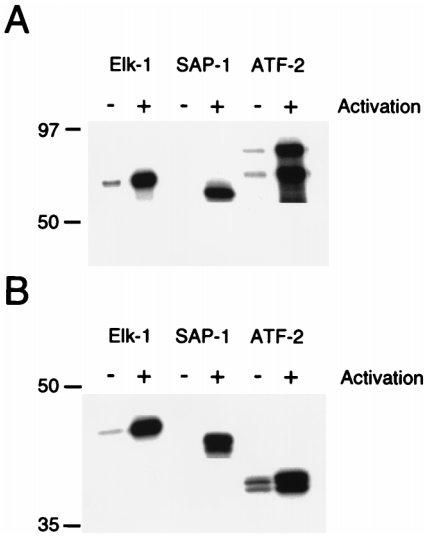

FIG. 1. TCFs are phosphorylated by p38 MAP kinase. The phosphorylation of Elk-1 and SAP-1 by p38 MAP kinase was examined by immune-complex protein kinase assay. Epitope-tagged p38 MAP kinase was immunopurified from unactivated (−) and activated COS-7 cells (40 J of UV-C per m^2) (+), using the Flag monoclonal antibody (M2). Kinase assays were performed with the following substrates: 0.5 μg of full-length proteins, His–Elk-1, His–SAP-1, and GST-ATF2 (A) and 1 μg of activation domains. GST–Elk-1 (residues 310 to 428), GST–SAP-1 (residues 287 to 431), and GST-ATF-2 (residues 1 to 109) (B). The assays were terminated after 30 min at 22°C, and the reaction products were examined by SDS-PAGE and autoradiography. Molecular masses are shown in kilodaltons. The full-length ATF-2 fusion protein with GST is expressed as a doublet (A).

DNA binding assays. Electrophoretic mobility shift analysis (EMSA) was performed with ^{32}P-labeled E74 and SRE double-stranded oligonucleotide probes and a 134-bp c-*fos* promoter fragment containing the SRE (SRE*) as described previously (82). Phosphorylation of His_6–Elk-1 and His_6–SAP-1 was performed with recombinant ERK2 and p38 MAP kinases activated in vitro by the MAP kinase kinases MEK1 and MKK6, respectively. MEK1 was isolated from epidermal growth factor-treated COS-7 cells with the rabbit polyclonal antibody 2880 (79). Epitope-tagged MKK6(Glu) was isolated from transfected COS-7 cells with the M2 Flag monoclonal antibody. Immune-complex kinase assays were performed with MEK1 and MKK6(Glu), 6 μg of bacterially expressed GST-ERK2 or GST-p38, and 0.5 μg of either His_6–Elk-1 or His_6–SAP-1 in 20 μl of kinase buffer supplemented with 50 μM ATP for 30 min at 22°C. The following amounts of phosphorylated Elk-1 and SAP-1 were used in DNA binding assays: 5 ng of Elk-1 and SAP-1 with the E74 probe, 6.25 ng of Elk-1 and 12 ng of SAP-1 with the SRE probe, and 2.5 ng of Elk-1 and 10 ng of SAP-1 with the SRE* probe. Binding reactions on SRE-containing sites also contained purified bacterially expressed $core^{SRF}$ (19). Control experiments were done without ATP or MAP kinase kinase or MAP kinase. Protein-DNA complexes were analyzed by PAGE (5% gel) in 0.5× Tris-borate-EDTA and visualized by autoradiography.

RESULTS

Phosphorylation of Elk-1 and SAP-1 by p38 MAP kinase. We have previously demonstrated that JNK MAP kinase efficiently phosphorylates Elk-1 in vitro, while SAP-1 is poorly phosphorylated (82). Moreover, the COOH-terminal activation domain of Elk-1 is a target for p38 MAP kinase (58). Here, we tested the ability of p38 MAP kinase to phosphorylate both full-length Elk-1 and SAP-1 and the COOH-terminal activation domains of these TCF proteins (Fig. 1). Both Elk-1 and SAP-1 are efficient substrates for p38 MAP kinase. The transcription factor ATF2, which is phosphorylated and activated by p38 MAP kinase (57, 58), was used as a positive control in these assays (Fig. 1). These data, together with our previous observations (58, 82), suggest that the p38 MAP kinase signal transduction pathway targets both Elk-1 and SAP-1, while the JNK signal transduction pathway selectively targets Elk-1.

Phosphorylation of Elk-1 and SAP-1 by p38 MAP kinase causes increased ternary complex formation and DNA binding activity. Phosphorylation of Elk-1 and SAP-1 by ERK MAP kinase, and phosphorylation of Elk-1 by JNK MAP kinase, causes an increase in ternary complex formation with SRF at the SRE and decreased ternary complex electrophoretic mobility on a c-*fos* promoter fragment (19, 66, 70, 82). To test whether p38 MAP kinase also regulates ternary complex formation by phosphorylation of Elk-1 and SAP-1, EMSA was performed. Experiments with an SRE oligonucleotide probe demonstrated that p38 MAP kinase phosphorylation of Elk-1 and SAP-1 caused an increase in ternary complex formation with $core^{SRF}$ (Fig. 2A, lane 8; Fig. 2B, lane 8), similar to that caused by ERK2 (Fig. 2A, lane 4; Fig. 2B, lane 4). Experiments using a 134-bp fragment of the c-*fos* promoter that includes the SRE demonstrated ternary complex formation in the absence of TCF phosphorylation (Fig. 2C, 3°I). Phosphorylation of Elk-1 and SAP-1 by p38 and ERK2 MAP kinases caused decreased electrophoretic mobility of the ternary complex (Fig. 2C, 3°II). These data indicate that p38 and ERK2 MAP kinases cause similar changes in ternary complex formation at the SRE.

Elk-1 binds to certain *ets*-like DNA sequences independently of SRF. This binding is regulated by ERK and JNK MAP kinases (66, 82). SAP-1 binding to similar sequences is regulated by ERK MAP kinase (70). We examined the effect of p38 MAP kinase phosphorylation of Elk-1 and SAP-1 on their ability to bind to a DNA probe containing the *Drosophila* E74 *ets*-like site. p38 MAP kinase caused enhanced DNA binding activity of Elk-1 and SAP-1 (Fig. 2A, lane 16; Fig. 2B, lane 16) comparably to ERK2 (Fig. 2A, lane 12; Fig. 2B, lane 12). These data demonstrate that p38 MAP kinase, like ERK MAP kinase, causes increased Elk-1 and SAP-1 DNA binding activity in the absence of SRF.

While ERK and p38 MAP kinase phosphorylation of Elk-1 and SAP-1 increases both ternary complex formation on the SRE oligonucleotide probe and E74-DNA binding activity (Fig. 2A and B), some ternary complex formation and E74-DNA binding was observed in the absence of phosphorylation in these assays. This is particularly evident for complexes formed with SAP-1 at the SRE (Fig. 2B, lanes 2 and 6). The likely explanation is that SAP-1 binds to the SRE with higher affinity than Elk-1 and forms more stable ternary complexes (70). In addition, the binding of MAP kinases to the TCFs (20, 85) may cause some enhancement of DNA binding and ternary complex formation in the absence of ATP by inducing conformational changes in the TCFs.

The drug VK19577 inhibits the activity of p38 MAP kinase and specific JNK MAP kinase isoforms in vitro. The drug VK19577 (also termed SB203580 [12, 39]) is a recently described inhibitor of p38 MAP kinase. To determine the usefulness of the drug VK19577 as a tool for examining the MAP kinase signal transduction pathways that target Elk-1 and SAP-1 in response to extracellular stimuli, we tested this drug for its ability to inhibit p38, ERK, and JNK MAP kinase activities in vitro. Nonactivated and activated epitope-tagged MAP kinases were immunoprecipitated from CHO cells and used in kinase assays with the substrate GST–Elk-1 (residues 310 to 428). A range of drug concentrations was included in the assays; 0.1 μM drug reduced p38 MAP kinase activity by more than 50%, 1 μM drug reduced activity to basal levels, and higher concentrations almost abolish activity (Fig. 3A). This inhibition of p38 is consistent with previous reports demonstrating the strong inhibitory effect of this drug on p38 MAP

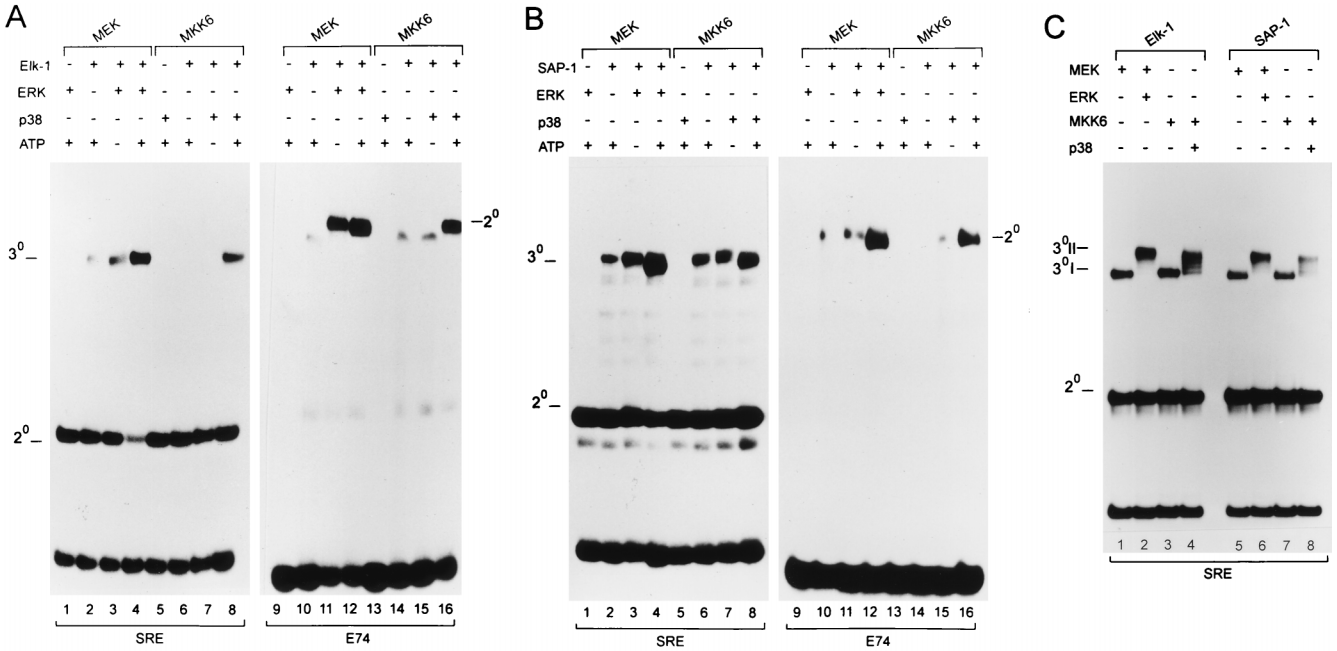

FIG. 2. Phosphorylation of Elk-1 and SAP-1 by p38 MAP kinase causes increased ternary complex formation and increased DNA binding activity. Elk-1 and SAP-1 were phosphorylated in vitro by bacterially expressed p38 and ERK2 which were activated by MKK6 and MEK, respectively, immunopurified from UV-irradiated or epidermal growth factor-treated COS-7 cells. (A) Ternary complex formation by Elk-1, SRF, and the SRE was measured by EMSA using a double-stranded ^{32}P-labeled oligonucleotide probe. The binary SRF-SRE complex (2°) and the ternary complex (3°) are indicated. DNA binding activity of Elk-1 was measured by EMSA using a double-stranded E74 probe that contains an *ets*-like binding site. The Elk-1–E74 complex is indicated (2°). (B) Ternary complex formation and DNA binding by SAP-1, using the oligonucleotide probes used for panel A. (C) Ternary complex formation by Elk-1 and SAP-1 with SRF and a 134-bp fragment of the c-*fos* promoter containing the SRE (SRE*). The binary SRF-SRE complex (2°) and the two ternary complexes (3°I and 3°II) are indicated.

kinase activity in vitro (12). ERK2 MAP kinase activity was only slightly reduced over the range of drug concentrations tested (Fig. 3A). However, the drug selectively inhibited the activity of JNK MAP kinase isoforms (Fig. 3B and C). The activities of the JNK2 isoforms JNK2β1 and JNK2β2 (22) were reduced to basal levels by 10 to 20 μM drug (Fig. 3C). The activities of the JNK2 isoforms JNK2α1 and JNK2α2 (22) were also reduced by increasing concentrations of drug but required a 10-fold-higher concentration of drug to be reduced to basal levels (Fig. 3C). The drug was a less potent inhibitor of JNK1 isoforms but displayed some selectivity for inhibition of JNK1α1 and JNK1α2 (22) compared with JNK1β1 and JNK1β2 (22) (Fig. 3B).

These data indicate that the drug VK19577 inhibits the activities of both p38 MAP kinase and particular JNK MAP kinase isoforms in vitro. However, at least 20-fold-higher concentrations of the drug were required to inhibit JNK isoforms compared with p38 MAP kinase. Careful titration of the drug is therefore necessary to observe specific inhibition of p38 MAP kinase activity.

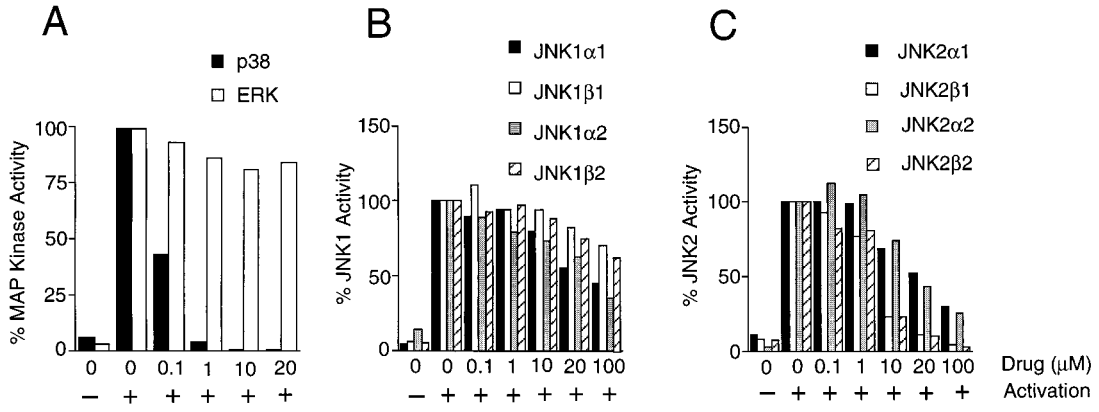

FIG. 3. Differential effects of the drug VK19577 on p38 (A), ERK (A), and JNK (B and C) MAP kinase activities in vitro. Epitope-tagged p38 (Flag), ERK2 (HA), and JNK MAP kinase isoforms (Flag) (22) were immunopurified from transiently transfected COS-7 cells activated with 40 J of UV-C per m^2 (p38 and JNK) or 10 nM tetradecanoyl phorbol acetate (ERK2). The kinases were incubated with 2 μg of GST–Elk-1 (residues 310 to 428) and 0, 0.1, 1, 10, 20, or 100 μM VK19577 (Drug) for 20 min at 22°C. The reaction products were examined by SDS-PAGE and autoradiography. The kinase activities were quantified with a PhosphorImager (Molecular Dynamics). The kinase activities immunopurified from activated cells not treated with drug are given a value of 100%. A similar profile of MAP kinase activities was observed in an independent experiment.

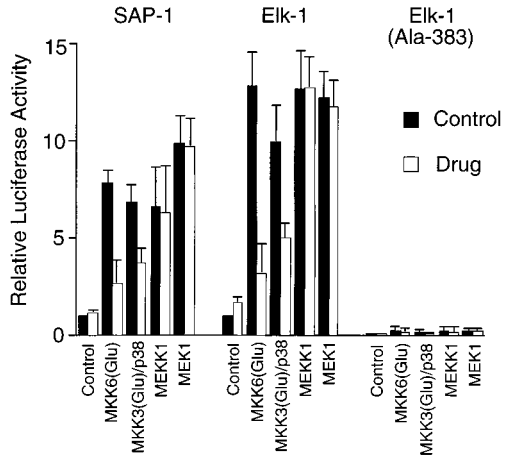

FIG. 4. The drug VK19577 inhibits activation of Elk-1 and SAP-1 by the p38 MAP kinase signal transduction pathway in vivo. CHO cells were transfected with the reporter plasmid pG5E1bLuc (0.25 μg), an expression vector for GAL4–Elk-1, GAL4–Elk-1 Ala-383, or GAL4–SAP-1 (0.25 μg of each), and a vector expressing either MKK3(Glu) (0.25 μg) and p38 (0.25 μg), MKK6(Glu) (0.1 μg), MEKK1 (0.1 μg), or activated MEK1 (0.2 μg). The β-galactosidase expression vector pCH110 was included as a control for transfection efficiency. The cells were transferred 12 h following transfection to serum-free medium for 6 h and then treated with 10 μM VK19577 (Drug) for 10 h or without drug (Control). Cell extracts were prepared, and the luciferase and β-galactosidase activities were measured. The relative luciferase activities (means ± standard deviations; $n = 3$) are presented.

The drug VK19577 inhibits activation of Elk-1 and SAP-1 by the p38 MAP kinase signal transduction pathway in vivo. To determine the specificity of the drug VK19577 in blocking the p38 MAP kinase signal transduction pathway in vivo, we examined its effect on GAL4-Elk-1- and GAL4–SAP-1-dependent reporter gene expression in CHO cells transfected with expression constructs for MEKK1, activated MEK1, activated MKK6 [MKK6(Glu)], and activated MKK3 [MKK3(Glu)]/p38 (Fig. 4). MEKK1, MEK1, and MKK6(Glu) have been demonstrated to activate JNK (50, 84), ERK (15, 81), and p38 (25, 52, 58, 72) MAP kinases, respectively. MKK3(Glu) does not appear to activate endogenous p38 but does activate p38 expressed in cotransfection experiments (58). GAL4-Elk-1- and GAL4-SAP-1-dependent reporter gene expression was increased by coexpression with MEKK1, activated MEK1, MKK6(Glu), and MKK3(Glu)/p38 (Fig. 4). The addition of 10 μM drug VK19577 to the culture medium inhibited MKK6(Glu)- and MKK3(Glu)/p38-stimulated reporter gene expression but had no effect on MEKK1- and MEK1-stimulated reporter gene expression (Fig. 4). Mutation of the MAP kinase phosphorylation site previously demonstrated to be critical for full Elk-1 transcriptional activity (Ser-383 replaced with Ala) resulted in the loss of MEKK1-, MEK1-, MKK6(Glu)-, and MKK3(Glu)/p38-stimulated Elk-1-dependent reporter gene expression (Fig. 4). These data demonstrate that 10 μM VK19577 blocks p38 MAP kinase signaling but not the JNK or ERK signal transduction pathways in vivo. Therefore, we used 10 μM VK19577 to selectively inhibit the p38 MAP kinase pathway in further experiments.

p38 MAP kinase is not essential for IL-1-induced Elk-1 and SAP-1 transcriptional activity in CHO cells. The proinflammatory cytokine IL-1 activates both the JNK and p38 MAP kinase signaling pathways (57). We have previously shown that IL-1 activates GAL4–Elk-1-dependent reporter gene expression in CHO cells (82). To determine whether p38 MAP kinase plays a critical role in mediating IL-1-stimulated TCF transcriptional activity, the effect of the drug VK19577 on IL-1-induced GAL4-Elk-1 and GAL4-SAP-1-dependent reporter gene expression was examined. Figure 5A shows that IL-1-induced Elk-1 and SAP-1 transcriptional activities were not inhibited by this drug in CHO cells (Fig. 5A). These data demonstrate that p38 MAP kinase is not essential for mediating IL-1 activation of the TCF proteins Elk-1 and SAP-1 in CHO cells.

We have demonstrated previously that a mutationally inactivated form of the JNK-activating kinase MKK4 (DN-MKK4) behaves as a dominant-negative inhibitor of the JNK pathway and blocks IL-1-induced GAL4–Elk-1-dependent reporter gene expression in CHO cells (82). The mechanism of inhibition

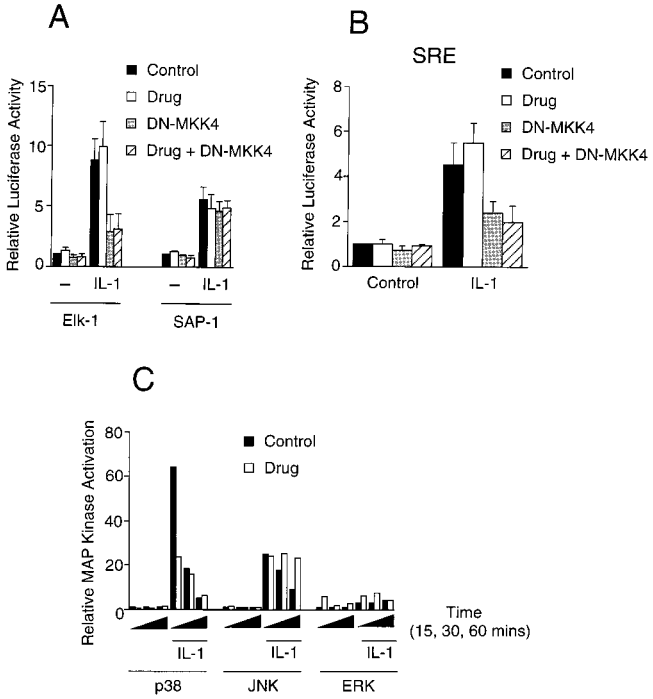

FIG. 5. p38 MAP kinase is not essential for IL-1-induced SRE-dependent gene expression and Elk-1 and SAP-1 transcriptional activity in CHO cells. (A) The p38 inhibitor drug VK19577 does not inhibit IL-1-induced Elk-1 and SAP-1 transcriptional activity in CHO cells. Cells were transfected with the reporter plasmid pG5E1bLuc (0.25 μg), the β-galactosidase plasmid pCH110 (0.1 μg), and a vector expressing either GAL4–Elk-1 or GAL4–SAP-1 (0.25 μg). Following incubation in serum-free medium for 12 h, the cells were treated with 10 ng of IL-1 per ml for 10 h with or without 10 μM VK19577 (Drug). The data are presented as relative luciferase activities (means ± standard deviations; $n = 3$). The effect of DN-MKK4 (0.5 μg of expression vector transfected) and DN-MKK4 in combination with 10 μM VK19577 (Drug) on GAL4–Elk-1- and GAL4–SAP-1-dependent reporter gene expression was examined in cells treated with IL-1. (B) The p38 inhibitor drug VK19577 does not inhibit IL-1-induced SRE-dependent reporter gene expression in CHO cells. Cells were transfected with the pSRE-Luc reporter plasmid (1 μg) and pCH110 (0.25 μg). The cells were incubated in serum-free medium for 12 h and then treated with 10 ng of IL-1 per ml for 10 h without or with 10 μM VK19577 (Drug). Cotransfection with the DN-MKK4 expression vector (0.5 μg) inhibited IL-1-induced SRE-dependent reporter gene expression. The data are presented as relative luciferase activities (means ± standard deviations; $n = 3$). (C) IL-1 activates p38 and JNK, but not ERK, MAP kinase activity in CHO cells. Cells were incubated in serum-free medium for 12 h and then treated without and with 10 ng of IL-1 per ml and 10 μM VK19577 (Drug) for 15, 30, and 60 min. The activities of endogenous p38, JNK, and ERK MAP kinases were measured in immune-complex kinase assays using the appropriate polyclonal antibodies and the respective substrates GST-ATF2 (residues 1 to 109), GST–c-Jun (residues 1 to 79), and GST–Elk-1 (residues 310 to 428). The kinase activities were quantified by PhosphorImager analysis, and the data are presented as relative MAP kinase activities compared to kinase activities immunopurified from untreated cells. A similar profile of MAP kinase activities was obtained in an independent experiment.

caused by DN-MKK4 probably involves the interaction with upstream (MAP kinase kinase kinases) or downstream (MAP kinases) components of the JNK MAP kinase signaling pathway. In transient transfection assays, DN-MKK4 had a small inhibitory effect on IL-1-induced GAL4-SAP-1-dependent reporter gene expression but significantly inhibited GAL4-Elk-1-dependent reporter gene expression. These data imply that IL-1 signaling to SAP-1 and Elk-1 occurs via different signaling pathways in CHO cells. The strong inhibitory effect of DN-MKK4 on IL-1-induced Elk-1 transcriptional activity (Fig. 5A) indicates a major role for the JNK MAP kinase signal transduction pathway. The lack of inhibition of IL-1-induced SAP-1 transcriptional activity by either DN-MKK4 or the p38 inhibitor drug, individually or combined (Fig. 5A), suggests that IL-1 signaling to SAP-1 is mediated by a novel kinase pathway in CHO cells.

Elk-1 and SAP-1 are potential components of ternary complexes formed with SRF at the SRE. To determine whether the lack of inhibition of IL-1 signaling to Elk-1 or SAP-1 by the drug VK19577 in CHO cells correlated with a similar lack of inhibition of IL-1 signaling to ternary complexes at the SRE, the effect of the drug on IL-1 signaling to tandem c-*fos* SREs cloned upstream of a reporter gene was examined. The drug did not inhibit SRE-dependent reporter gene expression (Fig. 5B). However, coexpression with DN-MKK4 did inhibit SRE-dependent reporter gene expression (Fig. 5B). These data imply that JNK, but not p38 MAP kinase, is required for IL-1 signaling to ternary complexes at the SRE in CHO cells.

To confirm which MAP kinases were activated by IL-1 in CHO cells and also to examine the effect of the p38 inhibitor drug VK19577 on the activation of the MAP kinases (as opposed to the effect on their kinase activities), CHO cells were treated with or without IL-1 and the drug over a period of 60 min, and endogenous p38, JNK, and ERK MAP kinases were immunoprecipitated, washed to remove the drug VK19577, and assayed for kinase activity (Fig. 5C). p38 and JNK MAP kinases were strongly activated by IL-1, while there was weak activation of ERK MAP kinase (Fig. 5C). Inhibition of both basal and IL-1-stimulated p38 MAP kinase activation was observed (Fig. 5C). The drug VK19577 therefore blocks the p38 MAP kinase signaling pathway at two distinct steps: (i) by inhibiting p38 MAP kinase activation (Fig. 5C) and (ii) by inhibiting p38 MAP kinase activity (Fig. 3A). JNK activation was not inhibited by the drug. Indeed, the duration of JNK activation by IL-1 was increased by treatment with the drug (Fig. 5C). The drug also caused a small increase in ERK activation (Fig. 5C). The drug therefore displays opposing effects on IL-1-stimulated p38 and JNK activation. It inhibits the activation of p38 MAP kinase but prolongs the activation of JNK MAP kinase.

p38 MAP kinase is required for IL-1-induced Elk-1 and SAP-1 transcriptional activity in NIH 3T3 cells. We have demonstrated that in CHO cells, IL-1 signaling to the TCF protein Elk-1 is primarily mediated by the JNK MAP kinase pathway (Fig. 5). However, IL-1 may signal through different protein kinase pathways in different cell types. For example, in COS-7 cells, IL-1 treatment causes significant activation of ERK MAP kinase, which is known to regulate TCF transcriptional activity (80). We therefore investigated IL-1 signaling pathways targeting TCFs in a different cell type. We chose NIH 3T3 cells for these studies because this cell line has been extensively characterized by examination of signaling pathways that converge at the SRE (76). IL-1-stimulated GAL4–Elk-1- and GAL4–SAP-1-dependent reporter gene expression was inhibited by the drug VK19577 in NIH 3T3 cells (Fig. 6A). This observation indicates that p38 MAP kinase is an important component of

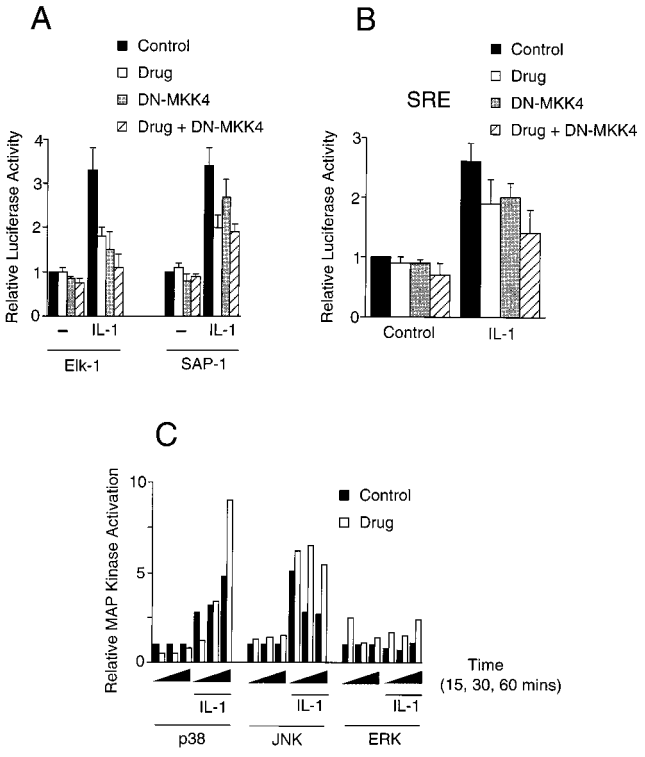

FIG. 6. p38 MAP kinase is required for IL-1-induced Elk-1 and SAP-1 transcriptional activity in NIH 3T3 cells. (A) Cells were transfected with the reporter plasmid pG5E1bLuc (0.25 μg), pCH110 (0.25 μg), and a vector expressing either GAL4–Elk-1 or GAL4–SAP-1 (0.25 μg). Following incubation in serum-free medium for 12 h, the cells were treated with 10 ng of IL-1 per ml for 10 h with or without 10 μM VK19577 (Drug). Cotransfection of a vector expressing DN-MKK4 (0.5 μg) was also examined. The data are presented as relative luciferase activities (means ± standard deviations; $n = 3$). (B) The pSRE-Luc reporter plasmid (1 μg) was cotransfected with or without a vector expressing DN-MKK4 (0.5 μg). The cells were incubated in serum-free medium for 12 h and then treated with 10 ng of IL-1 per ml for 10 h without or with 10 μM VK19577 (Drug). (C) IL-1 activates JNK and p38 MAP kinase activities in NIH 3T3 cells. Cells were incubated in serum-free medium for 12 h and then treated without and with 10 ng of IL-1 per ml and 10 μM VK19577 (Drug). The activities of endogenous p38, JNK, and ERK MAP kinases were measured by immune-complex kinase assays using the appropriate polyclonal antibodies and the respective substrates GST-ATF2 (residues 1 to 109), GST–c-Jun (residues 1 to 79), and GST–Elk-1 (residues 310 to 428). The kinase activities were quantified by PhosphorImager analysis, and the data are presented as relative MAP kinase activities compared with untreated cells. A similar profile of MAP kinase activities was obtained in an independent experiment.

signaling pathways that target the TCF proteins Elk-1 and SAP-1 in response to IL-1 treatment of NIH 3T3 cells.

Coexpression of DN-MKK4 significantly inhibited GAL4–Elk-1-dependent reporter gene expression but had only a small inhibitory effect on GAL4–SAP-1-dependent reporter gene expression. This result is consistent with JNK MAP kinase being required for IL-1 signaling to Elk-1 but not SAP-1. The inclusion of both DN-MKK4 and the drug VK19577 resulted in a further decrease in GAL4–Elk-1 transcriptional activity (Fig. 6A). Therefore, in NIH 3T3 cells, both JNK and p38 MAP kinases are required for IL-1 signaling to Elk-1. Similar experiments using the c-*fos* SRE reporter gene demonstrated that both the drug VK19577 and DN-MKK4 cause some inhibition of IL-1-induced reporter gene expression (Fig. 6B), indicating that the JNK and p38 MAP kinase signaling pathways contribute to the regulation of the c-*fos* SRE in NIH 3T3 cells.

Control experiments were performed to examine the effect of the drug VK19577 on IL-1-stimulated MAP kinase activa-

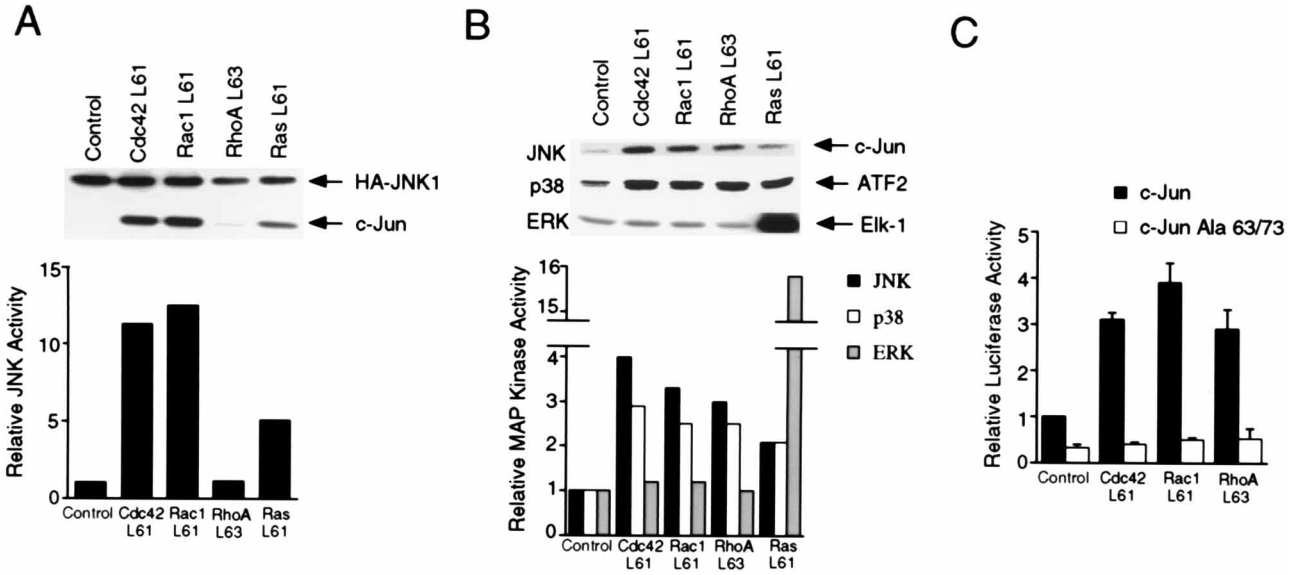

FIG. 7. MAP kinase activation by Ras and Rho family GTPases. (A) Activation of transfected JNK by Rho family GTPases. CHO cells were transfected with vectors expressing HA-JNK1 (0.5 μg) and either activated Rho family GTPases (0.5 μg) or activated Ras (0.1 μg). The expression levels of HA-JNK1 were monitored by immunoblot analysis of cell lysates with an anti-HA monoclonal antibody (upper panel). HA-JNK1 activity was measured by immune-complex kinase assay using GST–c-Jun as a substrate (lower panel). The kinase activities were quantitated with a PhosphorImager (Molecular Dynamics), and the data are presented as fold stimulation compared to cells transfected with HA-JNK1 plasmid alone (Control). The experiment was repeated twice with similar results. (B) Activation of endogenous MAP kinases by Rho family GTPases. CHO cells were transfected with plasmids expressing activated Rho family GTPases (0.5 μg) or activated Ras (0.1 μg). Endogenous JNK, p38, and ERK activities were measured by immune-complex kinase assays using the substrates GST–c-Jun (residues 1 to 79), GST-ATF2 (residues 1 to 109), and GST–Elk-1 (residues 310 to 428), respectively. Kinase activities were quantitated with a PhosphorImager (Molecular Dynamics), and the results are presented as fold stimulation compared to cells transfected with the empty expression vector (Control). The experiment was repeated twice with similar results. (C) Rho family GTPases increase c-Jun transcriptional activity. Luciferase reporter gene expression was measured in extracts prepared from CHO cells transfected with the pG5E1bLuc reporter plasmid (0.25 μg), the β-galactosidase expression plasmid pCH110 (0.1 μg), vectors expressing activated Rho family GTPases (0.5 μg), and either GAL4–c-Jun or GAL4–c-Jun Ala63/73 (0.25 μg). The data are presented as relative luciferase activities (means ± standard deviations; $n = 3$).

tion in NIH 3T3 cells. Cells treated with the drug were incubated with or without IL-1, and the endogenous p38, JNK, and ERK MAP kinases were immunopurified and assayed for protein kinase activity. IL-1 activated both p38 and JNK MAP kinases but not ERK MAP kinase (Fig. 6C). Addition of the drug VK19577 inhibited p38 activation by IL-1 at 15 min but appeared to increase IL-1-induced p38 activation following further incubation (Fig. 6C). The reason for this biphasic activation response is unclear. The enhancement of IL-1-induced p38 activation caused by the drug VK19577 is negated by the inhibition of p38 kinase activity caused by the drug. Similar to its effect on CHO cells (Fig. 5C), the drug increased the duration of JNK activation and also caused a small activation of ERK MAP kinase.

These observations provide evidence that IL-1 signaling to the TCF proteins Elk-1 and SAP-1 can be mediated by distinct MAP kinase pathways in different cell types, for example, CHO cells and NIH 3T3 cells.

Rho family GTPases regulate the activation of Elk-1- and SRE-dependent gene expression via MAP kinase pathways in CHO cells. Several recent studies have implicated members of the Rho family of GTPases as components of the JNK and p38 MAP kinase signaling pathways (2, 11, 49, 53, 86). In addition, it has been suggested that Rho family GTPases may mediate IL-1 signaling events (2). To determine which MAP kinases are activated by Rho family GTPases in CHO cells, we performed a series of immune-complex kinase assays. Initially the abilities of constitutively activated GTPases to stimulate transfected epitope-tagged JNK1 protein kinase activity were tested. Activated forms of Cdc42, Rac1, RhoA, and Ras were coexpressed with HA-tagged JNK1 in CHO cells. The HA-JNK1 was immunopurified from the cells by using an anti-HA monoclonal antibody and tested for kinase activity toward the substrate GST–c-Jun (Fig. 7A). Activated Cdc42, Rac1, and Ras increased HA-JNK1 activity, but RhoA did not. Immunoblots of cell extracts prepared from the transfected cells indicate that HA-JNK1 is expressed at similar levels when cotransfected with each of the GTPases (Fig. 7A). The same experiment was performed in COS-7 and NIH 3T3 cells with similar results (80). These data confirm the results of previous studies performed using COS, NIH 3T3, and HeLa cells (2, 11, 49, 53, 86).

A potential flaw in the cotransfection assays used to measure MAP kinase activation by Rho family GTPases (Fig. 7A and references 2, 11, 49, 53, and 86) is that the MAP kinases are overexpressed. As MAP kinase activation is proposed to occur within signaling modules that ensure specificity via protein-protein interactions (14, 81), the regulation of overexpressed MAP kinases may differ from regulation of MAP kinases expressed at physiological levels. We therefore performed a more thorough series of experiments to assess the abilities of Rho family GTPases to activate endogenous MAP kinases. The Rho family GTPases were expressed in CHO cells, and the endogenous ERK, JNK, and p38 MAP kinases were immunopurified with the appropriate polyclonal antibodies and used in kinase assays with the substrates GST–Elk-1 (residues 310 to 428), GST–c-Jun (residues 1 to 79), and GST-ATF2 (residues 1 to 109), respectively. Activated Cdc42, Rac1, Ras, and RhoA caused activation of endogenous JNK and p38 MAP kinases, while only activated Ras increased endogenous ERK MAP kinase activity (Fig. 7B). As only 10 to 20% of the cells are transfected with activated GTPases and we are measuring endogenous MAP kinase activities from the entire cell population, the levels of MAP kinase activation that we observed are likely to be a large underestimate of the true MAP kinase

activation. These experiments demonstrate that RhoA activates the endogenous JNK and p38 MAP kinase signaling pathways in CHO cells (Fig. 7B). This effect of RhoA is in contrast to observations made for overexpressed JNK and p38 MAP kinases (Fig. 7A and references 11, 49, 53, and 86).

To test whether JNK activation by Rho family GTPases is functionally coupled to a downstream signal transduction target, we examined the effects of expressing activated Rho family GTPases on transcriptional activation by c-Jun. We found that Cdc42, Rac1, and RhoA activated GAL4–c-Jun-dependent reporter gene expression (Fig. 7C). Mutation of the JNK MAP kinase phosphorylation sites (Ser-63 and Ser-73) on c-Jun by replacement with Ala blocked Rho family GTPase stimulation of GAL4–c-Jun transcriptional activity (Fig. 7C). These data demonstrate that the JNK MAP kinase signal transduction pathway may mediate Cdc42, Rac1, and RhoA regulation of c-Jun transcriptional activity in CHO cells and support the conclusion that RhoA, like other Rho family GTPases, activates the JNK signaling pathway (Fig. 7B). This conclusion contrasts with inferences drawn from experiments using overexpressed JNK (Fig. 7A and references 11, 49, and 53).

SRE-dependent gene expression is increased following MAP kinase phosphorylation and activation of TCF proteins within the ternary complex (7, 8, 81). However, it has been reported that activated forms of Cdc42, Rac1, and RhoA increase SRF-dependent transcriptional activity independently of ERK, JNK, and p38 MAP kinases and that these GTPases are not relevant to the regulation of Elk-1 transcriptional activity (27). To determine whether Cdc42, Rac1, and RhoA are components of MAP kinase signaling pathways leading to SRE-dependent gene expression in CHO cells, plasmids expressing activated forms of these GTPases were cotransfected together with the pSRE-Luc reporter plasmid. All three Rho family GTPases activated SRE-dependent gene expression (Fig. 8A). Coexpression of MKP1 in these experiments led to inhibition of SRE-dependent gene expression caused by activated Rho family GTPases (Fig. 8A). These data indicate that MAP kinase pathways contribute to the activation of SRE-dependent gene expression stimulated by Rho family GTPases in CHO cells.

To examine whether Rho family GTPases can regulate Elk-1 transcriptional activity via MAP kinase pathways in CHO cells, we coexpressed activated forms of the Rho family GTPases together with MKP1, GAL4–Elk-1, and the reporter plasmid pG5E1bLuc. MKP1 has been shown to dephosphorylate, and thereby inactivate, the ERK, JNK, and p38 MAP kinases (22, 42, 57, 73). Activated Cdc42, Rac1, and RhoA stimulated luciferase reporter gene expression (Fig. 8B). Coexpression of MKP1 markedly reduced the effect of the Rho family GTPases to activate GAL4–Elk-1-dependent luciferase expression (Fig. 8B). These data demonstrate that Rho family GTPase signaling to Elk-1 requires MAP kinase activation. Mutation of a MAP kinase phosphorylation site (Ser-383) known to be critical for MAP kinase-stimulated Elk-1 transcriptional activity (10, 18, 20, 31, 47, 58, 82) greatly reduced Rho family GTPase stimulation of Elk-1 transcriptional activity (Fig. 8B). Together, these data indicate that Rho family GTPases are components of MAP kinase pathways leading to increased Elk-1 transcriptional activity.

To determine whether the Rho family GTPases Cdc42, Rac1, and RhoA play a role in the signaling pathways that mediate IL-1-induced Elk-1 transcriptional activity, we transfected CHO cells with plasmids expressing inactivated forms of Cdc42, Rac1, and RhoA GTPases, GAL4–Elk-1, and the reporter plasmid pG5E1bLuc. We subsequently treated the cells with IL-1 and measured the reporter luciferase expression (Fig. 8C). The inactivated Cdc42, Rac1, and RhoA mutants blocked IL-1 signaling to Elk-1, indicating their potential involvement in IL-1-stimulated Elk-1 transcriptional activity. However, the inactivated Rho-GTPases may bind to endogenous factors, such as guanine nucleotide exchange factors, which are common to other small GTP binding proteins, and thereby inhibit their action. Further studies are required to provide direct evidence for a role of Rho family GTPases in IL-1 signal transduction.

The JNK MAP kinase signaling pathway mediates Cdc42 GTPase regulation of Elk-1 transcriptional activity in CHO cells. Rho family GTPases are established to activate JNK and p38 MAP kinases in CHO cells (Fig. 7B), leading to transcriptional activation of Elk-1 (Fig. 8B). We therefore used the selective inhibitors of the p38 and JNK MAP kinase pathway to differentiate between the respective roles of JNK and p38 MAP kinases in the regulation of Elk-1 transcriptional activity by the Rho family GTPase Cdc42. DN-MKK4 inhibited Cdc42-mediated activation of the transcriptional activity of both Elk-1 and the JNK substrate c-Jun (Fig. 8D). In control experiments using activated Ras, DN-MKK4 did not block Ras signaling to Elk-1 (mediated by the ERK pathway) but did block Ras signaling to c-Jun (mediated by the JNK pathway) (Fig. 8E). Incubation of transfected cells with the p38 inhibitor drug VK19577 did not result in inhibition of Cdc42-mediated activation of Elk-1 or c-Jun. Control studies demonstrate that this drug did inhibit p38 MAP kinase signaling under these conditions (Fig. 4). Together, these data indicate that in CHO cells, Cdc42-regulated Elk-1 transcriptional activity is mediated primarily through the JNK MAP kinase signal transduction pathway and that Rho family GTPases such as Cdc42 may be a link between activated IL-1 receptors and the JNK MAP kinase cascade.

DISCUSSION

Elk-1 and SAP-1 are activated by distinct groups of MAP kinases. The c-*fos* SRE is activated by a wide array of stimuli mediated by pathways leading to the TCF and SRF components of the ternary complex (7, 8). The TCF protein Elk-1 is a target for the MAP kinases ERK (18, 19, 31), JNK (10, 20, 82, 88), and p38 (58). The related TCF protein SAP-1 is targeted by ERK MAP kinase (30, 54, 58, 70). Here we demonstrate that both Elk-1 and SAP-1 are phosphorylated by p38 MAP kinase (Fig. 1), leading to increased ternary complex formation (Fig. 2), DNA binding activity (Fig. 2), and transcriptional activation (Fig. 4). The transcription factors Elk-1 and SAP-1 therefore serve to integrate signals from multiple MAP kinase pathways in response to extracellular stimuli (Fig. 1, 2, and 4 and references 10, 18 to 20, 31, 47, 58, 82, and 88).

SAP-1 is a poor substrate for JNK MAP kinase in vitro (82). The selective targeting of Elk-1 compared to SAP-1 by JNK MAP kinase may allow for differential responses of the SRE to particular stimuli depending on the composition of the ternary complex. Interestingly, Elk-1 and SAP-1 display different specificities of DNA binding to various *ets*-related sites in vitro (68, 70). Promoters may therefore be targeted by different TCF proteins depending on their DNA binding specificities and the MAP kinases that phosphorylate them.

IL-1 and Cdc42 GTPase signaling to Elk-1 requires the JNK MAP kinase pathway in CHO cells. While both the p38 and JNK pathways are capable of targeting TCF proteins, it is unclear which pathways are utilized by particular extracellular stimuli. We used the recently described p38 inhibitor VK19577 (also termed SB203580) as a tool to elucidate the pathways involved in IL-1 signaling to TCFs. This drug inhibits both p38

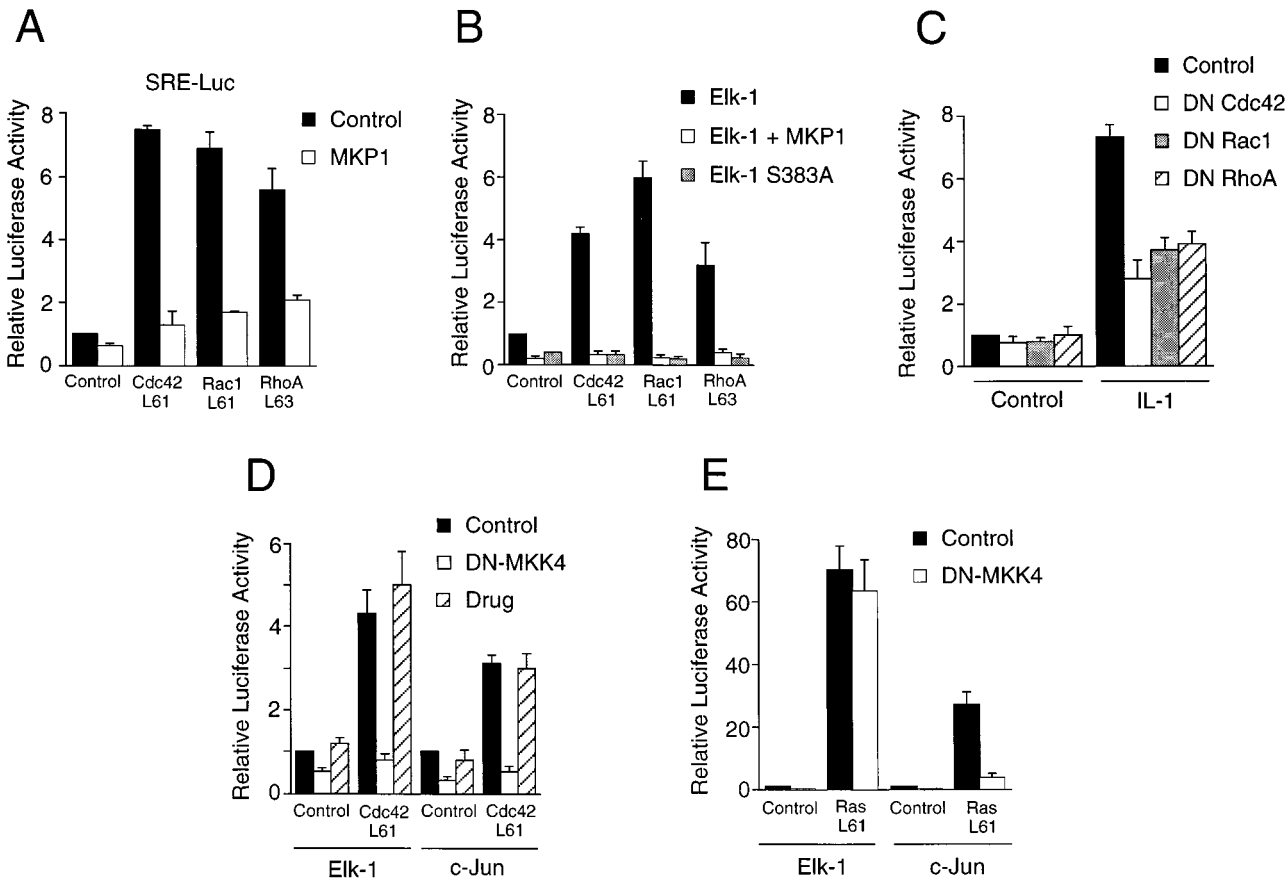

FIG. 8. Rho family GTPases signal to Elk-1 and the SRE in CHO cells. (A) Rho family GTPases regulate SRE-dependent gene expression via MAP kinases in CHO cells. Cells were transfected with the pSRE-Luc reporter plasmid (1 μg) together with plasmids expressing activated Rho family GTPases (0.5 μg). The effect of MKP1 expression (0.5 μg of expression vector transfected) was examined. Transfection efficiency was monitored by using the β-galactosidase expression vector pCH110 (0.1 μg). The relative luciferase activities (means ± standard deviations; $n = 3$) are presented. (B) Rho family GTPases regulate activation of Elk-1 via MAP kinase pathways in CHO cells. The effect of expression of MKP1 (0.5 μg of expression vector transfected) and activated Rho family GTPases (0.5 μg of expression vectors transfected), and the mutation of a critical MAP kinase phosphorylation site in Elk-1 (Ser-383 to Ala), on GAL4–Elk-1-mediated luciferase gene expression from the reporter plasmid pG5E1bLuc was examined. The data are presented as relative luciferase activities (means ± standard deviations; $n = 3$). (C) Dominant-negative mutants of Cdc42, Rac1, and RhoA inhibit IL-1-stimulated gene expression mediated by Elk-1. CHO cells were transfected with the pG5E1bLuc reporter plasmid (0.25 μg), an expression vector for GAL4-Elk-1 (0.25 μg), and an expression vector for either inactivated Cdc42 (DN-Cdc42), inactivated Rac1 (DN-Rac1), or inactivated RhoA (DN-RhoA) (0.5 μg of each). The β-galactosidase expression vector pCH110 was included as a control for transfection efficiency. The cells were transferred 24 h following transfection to serum-free medium for a further 12 h. The cells were then treated with or without 10 ng of IL-1 per ml in serum-free medium for 10 h. Cell extracts were prepared, and the luciferase and β-galactosidase activities were measured. The data are presented as relative luciferase activities (means ± standard deviations; $n = 3$). (D) Requirement of JNK, but not p38 MAP kinase, for Cdc42-stimulated Elk-1 transcriptional activity in CHO cells. CHO cells were cotransfected with a β-galactosidase expression vector (pCH110), the reporter plasmid pG5E1bLuc (0.25 μg), and expression vectors for either GAL4-Elk-1 or GAL4-cJun (0.25 μg). Where indicated, plasmids expressing activated Cdc42 GTPase (Cdc42 L61) (0.5 μg) and DN-MKK4 (0.5 μg) were included in the transfections, or the cells were treated with 10 μM p38 inhibitor VK19577 (Drug). Cell extracts were prepared 48 h following transfection, and the luciferase and β-galactosidase activities were measured. The data are presented as relative luciferase activities (means ± standard deviations; $n = 3$). (E) CHO cells were transfected as in panel D except that a vector expressing activated Ras GTPase (Ras L61) (0.1 μg) was used instead of activated Cdc42 GTPase.

MAP kinase and particular JNK isoform protein kinase activities in vitro (Fig. 3). However, careful titration allows specific inhibition of p38 protein kinase activity (Fig. 3). In CHO cells, the drug inhibits activation of Elk-1 and SAP-1 by the p38 MAP kinase signaling pathway (mediated by MKK3 and MKK6) but does not inhibit Elk-1 and SAP-1 activation by alternative MAP kinase pathways (mediated by MEK1 and MEKK1) (Fig. 4). MEK1 is a component of the ERK MAP kinase signaling pathway (15, 81), while MEKK1 is a component of the JNK MAP kinase signaling pathway (50, 84). Therefore, under controlled experimental conditions, the p38 inhibitor drug VK19577 does not appear to inhibit ERK or JNK signaling in vivo. Interestingly, expression of MEKK1 increases SAP-1-dependent reporter gene expression in these assays (Fig. 4). As SAP-1 is a poor substrate for JNK MAP kinase (82), this result suggests that MEKK1 activates a novel signaling pathway that targets SAP-1.

IL-1 increases JNK and p38 MAP kinase activity (Fig. 5C and reference 57) and the transcriptional activity of Elk-1 and SAP-1 in CHO cells (Fig. 5A and reference 82). Rho family GTPases also regulate the JNK and p38 MAP kinase signaling pathways (Fig. 7 and references 2, 11, 49, 53, and 86). However, a previous study of NIH 3T3 cells indicated that Rho family GTPases activate SRF (and not Elk-1), leading to increased SRE-dependent gene expression, and that the ERK, JNK, and p38 MAP kinases are not involved in mediating this effect (27). Here we demonstrate that in CHO cells the Rho family GTPases are involved in signaling pathways leading to increased SRE-dependent gene expression mediated by Elk-1 (Fig. 8). Activated forms of Cdc42, Rac1, and RhoA cause

increased Elk-1 transcriptional activation (Fig. 8B) and SRE-dependent gene expression (Fig. 8A). The MAP kinase phosphatase MKP1 markedly inhibits Rho family GTPase-mediated Elk-1 activation, suggesting that Rho family members signal to Elk-1 exclusively via MAP kinase pathways (Fig. 8B). However, SRE-dependent transcription is only partially inhibited by MKP1 (Fig. 8A), indicating that a component of the activation of SRE-dependent gene expression may not involve MAP kinases. This pathway may be accounted for by signals from Rho family GTPases that activate SRF (27). The apparent discrepancy between our results demonstrating a previously unreported link between Rho family GTPases and TCFs and those recently described (27) could be due to the different cell lines used in these experiments. Distinct signaling mechanisms may be utilized by Rho family GTPases in different cell lines.

To address the respective roles of JNK and p38 MAP kinases in IL-1- and Rho family GTPase-mediated Elk-1 transcriptional activity, we examined the effects of the p38 inhibitor drug VK19577 and the expression of DN-MKK4 on IL-1- and Cdc42-induced GAL4-Elk-1-dependent reporter gene expression in CHO cells. DN-MKK4 inhibited IL-1-stimulated (Fig. 5A) and Cdc42-stimulated (Fig. 8D) Elk-1-dependent transcriptional activity whereas the drug VK19577 did not, indicating that the JNK group of MAP kinases is required for IL-1- and Cdc42-stimulated Elk-1 activation. Taken together with the data demonstrating that inactivated forms of Cdc42, Rac1, and RhoA block IL-1-induced activation of Elk-1 in CHO cells (Fig. 8C), these results suggest that IL-1 signaling to Elk-1 in CHO cells may be mediated by Cdc42 (and/or other Rho family GTPases) and the JNK MAP kinase signal transduction pathway.

Neither the p38 inhibitor drug VK19577 nor DN-MKK4 inhibited the IL-1-induced transcriptional activity of SAP-1 in CHO cells (Fig. 5A). These data indicate that the p38 and JNK MAP kinase signaling pathways are not essential for mediating the effects of IL-1 on SAP-1 transcriptional activity. This IL-1 signaling to SAP-1 therefore occurs via a novel signaling pathway in CHO cells. This novel pathway may involve MEKK1, since MEKK1 induces SAP-1 transcriptional activity (Fig. 4).

c-*fos* SRE-dependent reporter gene expression was inhibited in CHO cells by coexpression of DN-MKK4 but not by treatment with the drug VK19577. This result suggests that Elk-1, rather than SAP-1, may be a major component of ternary complexes formed at the c-*fos* SRE in CHO cells. However, other ternary complex factors and SRE or SRF binding proteins may also participate in transcriptional regulation of the c-*fos* SRE.

The p38 and JNK MAP kinase signaling pathways are required for IL-1 signaling to Elk-1 in NIH 3T3 cells. Considering that IL-1 activates both JNK and p38 MAP kinases and that TCF proteins are targets of both the JNK and p38 MAP kinase signal transduction pathways, it seemed plausible that the signaling pathways utilized by IL-1 to activate TCFs may depend on cell type. To test this hypothesis, we examined NIH 3T3 cells, which have been extensively used in studies of signal transduction pathways converging at the SRE (76). In direct contrast to CHO cells, the p38 inhibitor drug VK19577 inhibits IL-1-induced Elk-1 and SAP-1 transcriptional activities in NIH 3T3 cells (Fig. 6A). Therefore, the p38 MAP kinase signaling pathway is required for IL-1-induced activation of these TCF proteins in NIH 3T3 cells. DN-MKK4 blocks IL-1 signaling to Elk-1 (but not SAP-1), indicating that in this cell type, both the p38 and JNK MAP kinase signaling pathways are required to mediate full transcriptional activation of Elk-1 by IL-1. The JNK and p38 MAP kinase signaling pathways may therefore be selectively utilized in different cell types to activate TCF proteins in response to treatment with IL-1.

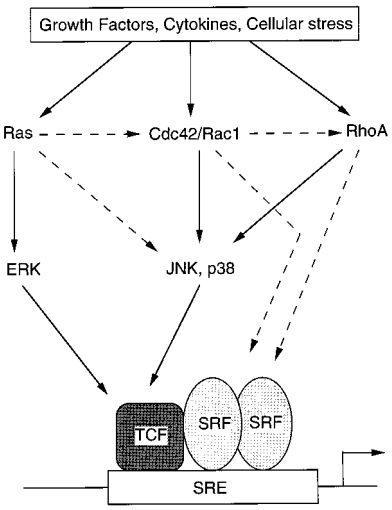

FIG. 9. Rho family GTPase-mediated signal transduction to the c-*fos* SRE. A complex of SRF and TCF binds to the c-*fos* SRE and mediates increased gene expression in response to extracellular stimuli (7, 8). The Rho family GTPases Cdc42, Rac1, and RhoA mediate JNK and p38 MAP kinase activation. Ras can also partially activate JNK and p38, possibly through Rac1 and RhoA (35, 36, 55, 56) or MEKK1 (61). Ras strongly activates the ERK MAP kinase pathway (15). Activated ERK, JNK, and p38 MAP kinases phosphorylate TCF/Elk-1, leading to increased transcriptional activation. Cdc42/Rac1 and RhoA are also components of signaling pathways that may regulate SRF transcriptional activity (27). Rho family GTPases are therefore involved in distinct signal transduction pathways which regulate SRE-dependent transcription through TCF/Elk-1 and SRF.

Convergence of MAP kinase signaling pathways at the SRE. The c-*fos* SRE is a site of integration of multiple signal transduction pathways in response to a variety of extracellular stimuli. A component of this regulation involves the TCF proteins Elk-1 and SAP-1 that are activated by MAP kinases. Elk-1 and SAP-1 can be regulated by the ERK and p38 MAP kinase signal transduction pathways, while JNK MAP kinase selectively targets Elk-1. ERK, JNK, and p38 MAP kinases phosphorylate sites within the COOH-terminal activation domain of these TCFs, leading to enhanced SRE-dependent transcription. The potential signaling routes to the SRE mediated by MAP kinases are depicted in Fig. 9. In response to growth factors, Ras activates a pathway that leads to the ERK group of MAP kinases via Raf-1 and MEK. Ras may also have a role in JNK MAP kinase activation (16), either directly upstream of MEKK1 (61) or by activating Rac1 and RhoA. Cdc42, Rac1, and RhoA activate the JNK and p38 MAP kinase pathways leading to the regulation of TCF transcriptional activity. In addition, RhoA regulates SRF transcriptional activity by a pathway that appears to be independent of Cdc42 and Rac1 regulation of SRF activity (27). The components of the pathways that activate SRF remain to be elucidated.

Both JNK and p38 MAP kinase pathways contribute to IL-1 signaling to TCFs. We have provided evidence that both JNK and p38 MAP kinase signal transduction pathways can target TCF proteins and the SRE in response to treatment with IL-1. Induction of Elk-1 transcriptional activity by IL-1 can be mediated by the JNK and/or p38 MAP kinase signaling pathways depending on the cell type. This differential utilization of MAP kinase signaling pathways represents one potential mechanism for the determination of cell-type-specific responses to extracellular stimuli. A goal for future studies will be to define the molecular basis of the cell-type-specific targeting of MAP ki-

nase signaling pathways to TCF proteins in vivo. Targeting of MAP kinase signaling pathways by protein-protein interactions may account for this signaling specificity. Current studies in this laboratory are designed to test this hypothesis.

ACKNOWLEDGMENTS

We thank R. Zelle for preparing the p38 inhibitor. We are grateful to N. Ahn, R. A. Cerione, J. S. Gutkind, L. Kozma, J. Pouysségur, N. Tonks, and M. Weber for providing reagents. We thank P. Shore, P. Yates, and members of the Sharrocks and Davis laboratories for critical discussions.

R.J.D. is an Investigator of the Howard Hughes Medical Institute. This work was supported by grants CA 58396 and CA 65861 from the National Cancer Institute (R.J.D.), the North of England Cancer Research Campaign (A.D.S.), and the Wellcome Trust (A.D.S.).

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