MECHANISMS OF SIGNAL TRANSDUCTION:

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Activation of p38α MAPK Enhances Collagenase-1 (Matrix Metalloproteinase (MMP)-1) and Stromelysin-1 (MMP-3) Expression by mRNA Stabilization*

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Here, we have examined the role of distinct MAPK pathways in the regulation of collagenase-1 (matrix metalloproteinase (MMP)-1) and stromelysin-1 (MMP-3) expression by human skin fibroblasts. Tumor necrosis factor-α rapidly and transiently activated ERK1/2 and JNK in fibroblasts, whereas the activation of p38 MAPK was more persistent. Inhibition of p38 activity by SB203580 markedly (by 80–90%) inhibited induction of MMP-1 and MMP-3 expression by tumor necrosis factor-α, whereas blocking the activation of ERK1/2 by PD98059 had no effect. Activation of endogenous ERK1/2 by adenovirus-mediated transfer of constitutively active MEK1 resulted in potent induction of MMP-1 and MMP-3 expression. Activation of endogenous or adenovirally expressed p38α by adenovirally delivered constitutively active MKK3b and MKK6b also enhanced MMP-1 and MMP-3 expression and augmented the up-regulatory effect of ERK1/2 activation on the expression of these MMPs. Activation of ERK1/2 resulted in induction of c-*jun*, *junB*, and c-*fos* expression, whereas activation of p38 alone had no effect. In contrast, activation of p38α resulted in marked stabilization of MMP-1 and MMP-3 mRNAs. These results identify two distinct and complementary signaling mechanisms mediating induction of MMP-1 and MMP-3 expression in dermal fibroblasts: AP-1-dependent transcriptional activation via the ERK1/2 pathway and AP-1-independent enhancement via p38α MAPK by mRNA stabilization. It is conceivable that both modes of action play an important role in controlling the proteolytic phenotype of fibroblasts, *e.g.* in wound repair and tumor invasion.

Matrix metalloproteinases (MMPs)[1] are a family of structurally related zinc-dependent neutral endopeptidases collectively capable of degrading essentially all components of the extracellular matrix (ECM) (see Refs. 1–3). At present, 21 human members of MMP gene family have been identified, and they can be classified into subgroups of collagenases, gelatinases, stromelysins, membrane-type MMPs, and other MMPs based on their structure and substrate specificity (1–3). There is a considerable amount of evidence that MMPs play an important role in controlled tissue remodeling in physiologic situations, including developmental tissue morphogenesis, tissue repair, and angiogenesis. In addition, MMPs obviously play an important role in destruction of normal tissue architecture, *e.g.* in rheumatoid arthritis, osteoarthritis, autoimmune blistering skin disorders, cutaneous photoaging, and tumor invasion and metastasis (1–3). Recent results show that MMPs also cleave growth factors, cytokine, chemokines, and their receptors and can in this way regulate cellular growth factor response and inflammatory reaction (3).

The expression of most MMPs in unstimulated cells is low, but is induced by a variety of extracellular stimuli, including mitogenic growth factors, cytokines, tumor promoters, and contact with the ECM (4). All the above-mentioned stimuli activate the nuclear AP-1 transcription factor complex, and AP-1 dimers consisting of members of the *jun* and *fos* gene families bind to the cognate *cis*-element at around −70 in the 5′-flanking regulatory region of several genes, resulting in activation of gene transcription (see Ref. 4). The induction of c-*jun* and c-*fos* expression is mediated by mitogen-activated protein kinases (MAPKs) (see Refs. 4 and 5). Recent results show that enhancement of collagenase-1 (MMP-1) expression in normal human skin fibroblasts by the lipid second messenger ceramide (6), the tumor promoter okadaic acid (7), or by contact with collagen (8) involves coordinate activation of three distinct MAPK pathways: extracellular signal-regulated kinase (ERK)-1 and -2, c-Jun N-terminal kinase (JNK), and p38. In addition, our recent results show that the activation of ERK1/2 alone potently enhances MMP-1 promoter activity, whereas activation of p38

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[1] The abbreviations used are: MMP, matrix metalloproteinase; ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MKK, MAPK kinase; MEK, MAPK/ERK kinase; TNF-α, tumor necrosis factor-α; DRB, 5,6-dichloro-1-*b*(β)-D-ribofuranosylbenzimidazole; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; TIMP, tissue inhibitor of metalloproteinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MOPS, 4-morpholinepropanesulfonic acid; CREB, cAMP response element-binding protein; ATF, activating transcription factor; NF-κB, nuclear factor-κB; m.o.i., multiplicity of infection.

MAPK alone has a minimal effect on the transcriptional activity of the human MMP-1 gene (9).

In this study, we have examined the specific roles of distinct MAPK signaling modules in regulation of the expression of endogenous MMP-1 and stromelysin-1 (MMP-3), a potent activator of latent MMP-1 in normal human skin fibroblasts. We show that the induction of MMP-1 and MMP-3 by tumor necrosis factor-α (TNF-α) is dependent on the activity of p38 MAPK, but not on that of ERK1/2. Activation of p38α alone by constitutively active MKK3b resulted in induction of MMP-1 and MMP-3 expression by stabilization of MMP-1 and MMP-3 mRNAs, but had no effect on c-*jun*, *junB*, and c-*fos* expression. Specific activation of endogenous ERK1/2 by adenovirus-mediated expression of constitutively active MEK1 resulted in induction of MMP-1 and MMP-3 expression and activation of c-*jun*, *junB*, and c-*fos* expression. The most abundant expression of MMP-1 and MMP-3 was noted when ERK1/2 were activated in combination with JNK or p38. These results provide evidence for two distinct and complementary mechanisms mediating induction of the collagenolytic capacity of dermal fibroblasts: AP-1-dependent transcriptional activation of MMP-1 gene expression via the ERK1/2 pathway and AP-1-independent enhancement via p38 MAPK by mRNA stabilization, suggesting that both play an important role in controlling the proteolytic phenotype of fibroblasts, *e.g.* in wound repair and tumor invasion.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant TNF-α, MEK1/2 inhibitor PD98059 (2′-amino-3′-methoxyflavone), p38 inhibitor SB203580 (4-(fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole), and RNA polymerase II inhibitor 5,6-dichloro-1-*b*(β)-D-ribofuranosylbenzimidazole (DRB) were obtained from Calbiochem. Anisomycin was obtained from Sigma.

Cell Cultures—Normal human skin fibroblast cultures were established from a punch biopsy obtained from a voluntary healthy male donor (age 23). Establishment of human Ha-Ras-transformed fibroblasts (KMST-6/Ras) has been described previously (10). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin G, and 100 μg/ml streptomycin. For experiments, fibroblasts were maintained in culture medium supplemented with 0.5% FCS for 18 h; TNF-α (20 ng/ml) was added; and the incubations were continued for the indicated periods of time. In experiments involving MAPK inhibitors, these were added 1 h prior to TNF-α.

RNA Analysis—Total cellular RNA was isolated from cells using the single-step method (11), and Northern blot hybridizations were performed as described previously (6). The following cDNAs were used: a 2.0-kb cDNA for human MMP-1 (12), a 1.5-kb cDNA for human MMP-3 (13), a 0.7-kb human cDNA for TIMP-1 (14), a 0.4-kb human cDNA for c-*jun* (15), a 1.2-kb human cDNA for *junB* (16), a 3.1-kb human genomic fragment for c-*fos* (17), and a 1.3-kb rat cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (18). The [^{32}P]cDNA-mRNA hybrids were visualized by autoradiography and quantitated by scanning densitometry, and MMP-1 and MMP-3 mRNA levels were corrected for the levels of GAPDH mRNA or 28 S rRNA in the same samples.

Assay of MMP-1, MMP-3, and TIMP-1 Production—Equal aliquots of the conditioned medium of fibroblasts were analyzed for the amount of MMP-1, MMP-3, and TIMP-1 by Western blotting as described previously (6) using a rabbit polyclonal antiserum against MMP-1 (1:5000) (kindly provided by Dr. Henning Birkedal-Hansen, NIDCR, Bethesda, MD) or rabbit polyclonal antiserum against human MMP-3 (1:1000) or against TIMP-1 (1:1000) (both obtained from Chemicon International, Inc., Temecula, CA). For TIMP-1 Western blots, samples were reduced with 5% mercaptoethanol prior to electrophoretic fractionation. Specific binding of antibodies was detected with peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence (ECL, Amersham Biosciences). The levels of MMP-1, MMP-3, and TIMP-1 were quantitated by densitometric scanning of the x-ray films.

Assay of MAPK Activation—The levels of activated ERK1/2 and p38 were determined by Western blot analysis using antibodies specific for phosphorylated activated forms of the corresponding MAPKs (New England Biolabs Inc., Beverly, MA). Fibroblasts were maintained in DMEM with 0.5% FCS for 18 h, incubated with TNF-α for different periods of time, and lysed in 100 μl of Laemmli sample buffer. The samples were sonicated, fractionated by 10% SDS-PAGE, and transferred to Hybond ECL membrane (Amersham Biosciences). Western blotting was performed as described previously (6, 8), with phospho-specific antibodies in 1:1000 dilution. As controls, the levels of corresponding total MAPKs were determined in the same samples using specific antibodies for ERK1/2 and p38 (New England Biolabs Inc.). Binding of primary antibodies was detected with peroxidase-conjugated secondary antibodies and visualized by ECL.

To determine JNK activity, cell layers were lysed in 200 μl of lysis buffer (phosphate-buffered saline (pH 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mg/ml pepstatin) (6, 7). For immunoprecipitation of JNK1 and JNK2, cell lysates were centrifuged at 3000 × *g* for 15 min; and the supernatant was incubated with antibody generated against JNK1, which also recognizes JNK2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), coupled to Gammabind-Sepharose. Immunoprecipitates were washed once with lysis buffer, once with LiCl wash buffer (500 mM LiCl, 100 mM Tris (pH 7.6), 0.1% Triton X-100, and 1 mM dithiothreitol), and once with kinase assay buffer (20 mM MOPS (pH 7.2), 2 mM EGTA, 10 mM $MgCl_2$, 0.1% Triton X-100, and 1 mM dithiothreitol). The kinase reaction was initiated by adding to the immunoprecipitate 20 μl of kinase assay buffer with 25 μM ATP, 2.5 μCi of [α-^{32}P]ATP (Amersham Biosciences), and 3 μg of recombinant c-Jun (New England Biolabs Inc.) as substrate. The reaction was carried out for 20 min at 37 °C; the samples were resolved on 12.5% SDS-polyacrylamide gel; and c-Jun phosphorylation was detected by autoradiography.

Determination of CREB, ATF1, ATF2, and NF-κB Activation—The levels of phosphorylated CREB, ATF1, and ATF2 were determined by Western blot analysis using antibodies specific for the corresponding phosphorylated transcription factors (New England Biolabs Inc.). The activation of NF-κB was determined by detecting phosphorylation and degradation of IκBα by Western blot analysis using antibodies against phosphorylated and total IκBα (New England Biolabs Inc.).

Infection of Fibroblasts with Recombinant Adenoviruses—The recombinant replication-deficient adenovirus RAd*lacZ* (RAd35) (19), which contains the *Escherichia coli* β-galactosidase (*lacZ*) gene under the control of the cytomegalovirus immediate early promoter, and the empty adenovirus RAd66 (19) were kindly provided by Dr. Gavin W. G. Wilkinson (University of Cardiff, Cardiff, Wales). Construction and characterization of recombinant adenoviruses containing the coding regions of wild-type p38α (RAdp38α) (20) and constitutively active mutant human MEK1 (RAdMEK1ca) (21), MKK7 (RAdMKK7D) (22), MKK3b (RAdMKK3bE) (20), and MKK6b (RAdMKK6bE) (20) genes driven by the cytomegalovirus immediate early promoter have been described previously. In experiments, 5×10^5 fibroblasts in suspension were infected as previously described (23) with recombinant adenoviruses at m.o.i. = 500, which gives 100% transduction efficiency (24); plated; and incubated for 18 h. The culture medium (DMEM with 1% FCS) was changed, and the cultures were incubated for 24 h. Aliquots of the conditioned medium were analyzed for the levels of MMP-1 and MMP-3 as described above. Cell layers were harvested and used for RNA extraction or for determination of MAPK activation.

Determination of mRNA Stability—Human skin fibroblasts (5×10^5) were transduced with replication-deficient empty control adenovirus (RAd66) or with adenoviruses coding for constitutively active MKK3b (RAdMKK3bE) and wild-type p38α (RAdp38α) at m.o.i. = 500 and incubated in DMEM supplemented with 1% FCS. After 18 h, the medium was changed; the RNA polymerase II inhibitor DRB (60 μM) was added; and cultures were harvested at 3-h intervals for mRNA extraction and determination of MMP-1, MMP-3, and GAPDH mRNA abundance by Northern blot hybridizations as described previously (24).

RESULTS

Induction of Fibroblast MMP-1 and MMP-3 Expression by TNF-α Is Mediated by p38 MAPK—Our previous results indicate that activation of fibroblast MMP-1 expression by contact with collagen, by the lipid second messenger ceramide, and by the protein phosphatase inhibitor okadaic acid involves coordinate activation of ERK1/2, JNK, and p38 MAPK (6–8). In this study, we have further elucidated the specific roles of these MAPK cascades in regulation of the expression of two MMPs abundantly expressed by normal human skin fibroblasts, *i.e.* MMP-1 and MMP-3. Initially, the cells were treated with

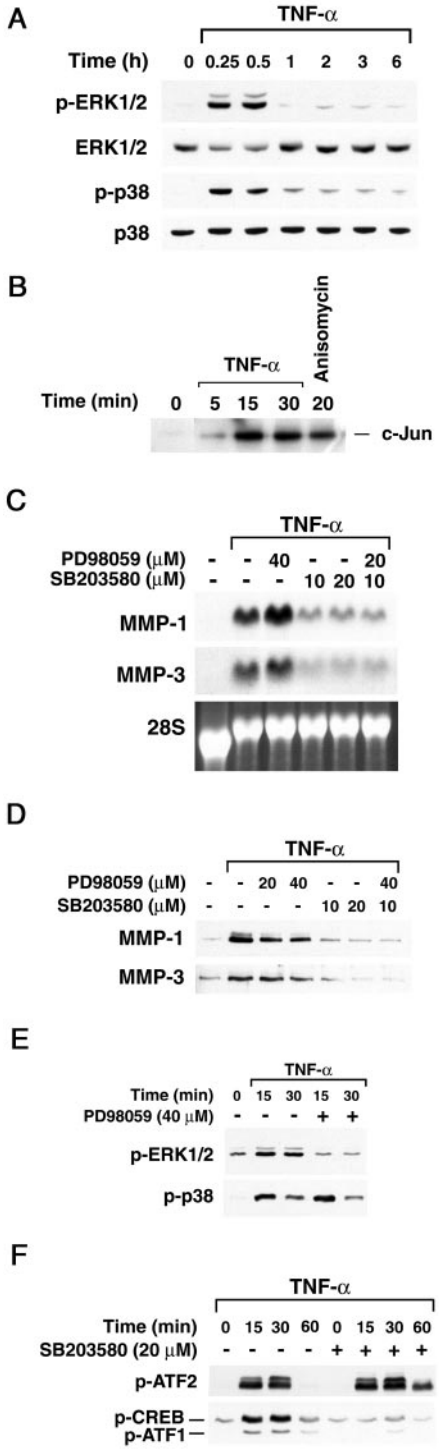

FIG. 1. **Induction of fibroblast MMP-1 and MMP-3 expression by TNF-α is mediated by p38 MAPK.** *A*, human skin fibroblasts were treated with TNF-α (20 ng/ml) for different periods of time as indicated. The levels of activated ERK1/2 (*p-ERK1/2*) and p38 (*p-p38*) were determined by Western blot analysis using phospho-specific antibodies for the corresponding MAPKs. The levels of total ERK1/2 and p38 were also determined in the same samples by Western blot analysis using specific antibodies. *B*, human skin fibroblasts were treated with TNF-α (20 ng/ml) for different periods of time as indicated. The activity of JNK immunoprecipitated from cell lysates was determined by kinase assay using c-Jun as substrate. Lysate from fibroblasts treated with anisomycin (10 μg/ml) for 20 min was used as a control. *C*, human skin fibroblasts were treated for 24 h with TNF-α (20 ng/ml) alone or in combination with PD98059, a specific inhibitor of MEK1/2, or with SB203580, a selective inhibitor of p38 MAPK, added 1 h before TNF-α at the concentrations indicated. Aliquots (15 μg) of total RNA were analyzed for levels of MMP-1 and MMP-3 mRNAs by Northern blot hybridizations. 28 S rRNA was visualized by ethidium bromide staining. *D*, human skin fibroblasts were treated as described for *C*, and the levels of MMP-1 and MMP-3 in the conditioned medium of the cells were determined by Western blot analysis. *E*, human skin fibroblasts were treated with TNF-α (20 ng/ml) for different periods of time either alone or in combination with PD98059 or SB203580, added 1 h before TNF-α at the concentrations indicated. The levels of activated ERK1/2 (*p-ERK1/2*) and p38 (*p-p38*) were determined as described for *A*. *F*, human skin fibroblasts were treated for different periods of time with TNF-α (20 ng/ml) alone or in combination with SB203580 as indicated. The levels of phosphorylated ATF2 (*p-ATF2*), CREB (*p-CREB*), and ATF1 (*p-ATF1*) were determined by Western blot analysis using phospho-specific antibodies for the corresponding transcription factors.

TNF-α (20 ng/ml) for different periods of time, and the activation of ERK1/2 and p38 was determined by Western blot analysis using antibodies against activated forms of these MAPKs. As shown in Fig. 1*A*, exposure of fibroblasts to TNF-α resulted in rapid and transient activation of ERK1/2, detected at 15 and 30 min of incubation. Interestingly, p38 MAPK was also rapidly (15 min) activated by TNF-α; but in contrast to ERK1/2, activation of p38 was more persistent and still detectable at 6 h of incubation (Fig. 1*A*). The activity of JNK, determined by kinase assay, was also potently increased by TNF-α after 15 and 30 min of incubation (Fig. 1*B*), but the activity returned to basal levels after 30 min (data not shown).

To examine the specific roles of ERK1/2 and p38 in the regulation of MMP-1 and MMP-3 expression, we treated fibroblasts with TNF-α alone or in combination with the MEK1/2 inhibitor PD98059 or with the p38 MAPK-specific inhibitor SB203580. Exposure of dermal fibroblasts to TNF-α resulted in marked up-regulation of MMP-1 and MMP-3 mRNA levels, and the enhancement of both by TNF-α was potently suppressed by 10 and 20 μM SB203580 (by 76 and 90%, respectively), whereas PD98059 (40 μM) alone or in combination with SB203580 had no effect (Fig. 1*C*). The up-regulation of pro-MMP-1 and pro-MMP-3 production by TNF-α was also potently down-regulated by SB203580, but not by PD98059 (Fig. 1*D*).

To confirm the inhibitory effect of PD98059 on ERK1/2 activation, we determined the levels of phosphorylated ERK1/2 in cells treated with TNF-α alone or in combination with PD90859. As shown in Fig. 1*E*, treatment of fibroblasts with PD98059 (40 μM) entirely inhibited the activation of ERK1/2 by TNF-α, but had no effect on the activation of p38. To corroborate the specificity of the inhibitory effect of SB203580 on p38 MAPK activity, we determined the levels of activated ATF2, a substrate for JNK (25), and the levels of phosphorylated CREB and ATF1, downstream targets of p38 (26). As shown in Fig. 1*F*, treatment of fibroblasts with TNF-α resulted in rapid and transient phosphorylation of CREB and ATF1, and the activation of both was entirely inhibited by SB203580 (20 μM). TNF-α treatment of cells also resulted in phosphorylation of ATF2 at 15 and 30 min of incubation, but the activation was not inhibited by SB203580 (Fig. 1*F*). In contrast, treatment with SB203580 resulted in prolonged activation of ATF2, still noted at 60 min (Fig. 1*F*). These results indicate that at the concentration used (20 μM), SB203580 inhibits the activity of p38 MAPK, but has no effect on endogenous JNK activity in normal dermal fibroblasts. Altogether, these results show that p38 MAPK plays an important role in induction of MMP-1 and MMP-3 expression and suggest that, in the presence of JNK and p38 activity, activation of ERK1/2 is not critical for this event.

Distinct Roles of p38 MAPK and ERK1/2 in Activation of c-jun, junB, and c-fos Expression by TNF-α—The enhancement of human MMP-1 and MMP-3 gene transcription involves activation of the AP-1 element located around −70 in the 5′-flanking regulatory region of the genes (4). The induction of the

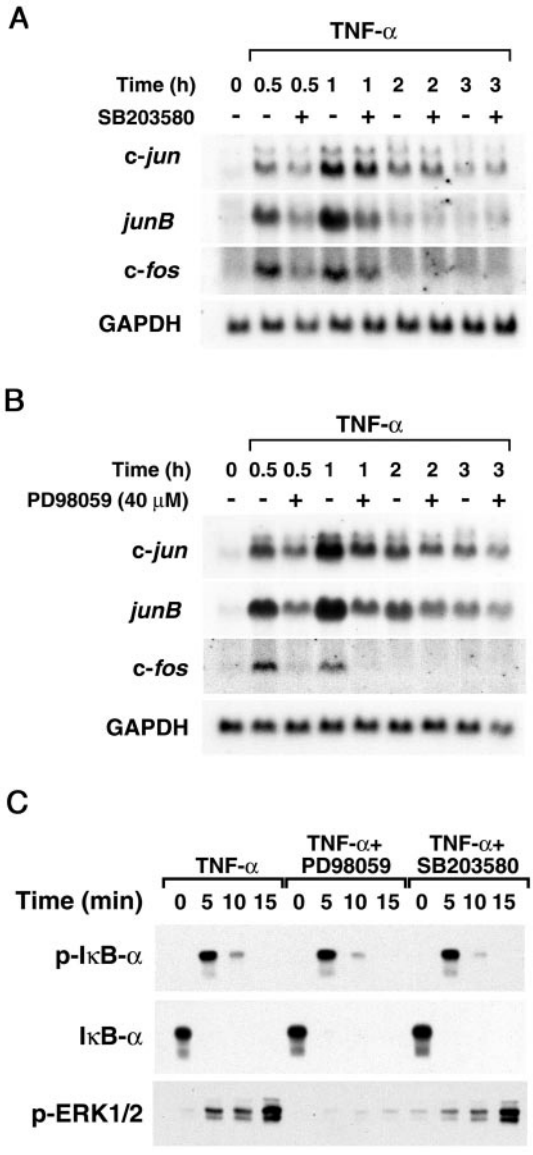

FIG. 2. **Induction of c-*jun*, *junB*, and c-*fos* expression by TNF-α is mediated by ERK1/2 and p38.** *A–C*, human skin fibroblasts were treated with TNF-α (20 ng/ml) alone or in combination with SB203580 (20 μM) or PD90859 (40 μM) for different periods of time as shown. *A* and *B*, the abundance of c-*jun*, *junB*, and c-*fos*, and GAPDH mRNAs was determined by Northern blot hybridizations. *C*, the levels of phosphorylated IκBα (*p-IκB-α*) and total IκBα were determined by Western blot analysis using specific antibodies for the corresponding forms of IκBα.

expression of the principal components of the AP-1 dimers, c-Jun and c-Fos, is mediated by distinct MAPK pathways (4, 5). We have recently shown that activation of ERK1/2 results in potent stimulation of human MMP-1 promoter activity, whereas activation of p38 by MKK3 and MKK6 has a minimal effect on MMP-1 promoter activity (9). In this context, we examined the roles of p38 and ERK1/2 MAPKs in the regulation of distinct components of the AP-1 complex. As shown in Fig. 2 (*A* and *B*), treatment of dermal fibroblasts with TNF-α resulted in rapid and transient up-regulation of c-*jun*, *junB*, and c-*fos* mRNA levels. Interestingly, pretreatment of the cells with SB203580 potently suppressed the induction of *junB* and c-*fos* mRNAs at 30-min and 1-h time points, whereas inhibition of the induction of c-*jun* mRNA abundance was less potent (Fig. 2*A*). In parallel, PD98059 entirely inhibited the induction of c-*fos* mRNA abundance by TNF-α and in part prevented the induction of c-*jun* and *junB* mRNA levels at 30-min and 1 h time points, whereas at later time points, the levels were similar both in the presence and absence of PD98059 (Fig. 2*B*). These results show that both ERK1/2 and p38 MAPK mediate the induction of c-*jun* and *junB* expression in dermal fibroblasts by TNF-α and that both pathways are essential for induction of c-*fos* expression.

Recent studies have shown that activation of NF-κB is required for interleukin-1-induced induction of MMP-1 and MMP-3 expression in rabbit dermal and synovial fibroblasts (27, 28). In this context, we also studied whether blocking ERK1/2 and p38 cascades in fibroblasts affects activation of NF-κB by TNF-α. As shown in Fig. 2*C*, treatment of fibroblasts with TNF-α resulted in rapid phosphorylation and degradation of IκBα, and this effect was not altered by cotreatment of cells with SB203580 and PD98059. These results show that inhibition of MMP-1 and MMP-3 expression by SB203580 does not involve inhibition of NF-κB activation.

Activation of ERK1/2 and p38 Results in Induction of MMP-1 and MMP-3 Expression by Fibroblasts—To directly examine the role of ERK1/2, JNK, and p38 MAPK in regulation of the expression of the endogenous MMP-1 and MMP-3 genes, we utilized adenovirus-mediated gene delivery of constitutively active MEK1, MKK7, MKK3b, and MKK6b to fibroblasts. As shown in Fig. 3 (*A* and *B*), infection of cells with the recombinant adenovirus RAdMEK1ca, harboring constitutively active MEK1, resulted in marked activation of ERK1/2, but not p38. The activation of ERK1/2 by constitutively active MEK1 was inhibited by PD98059 (40 μM). In parallel, infection of cells with adenoviruses for constitutively active MKK3b (RAdMKK3bE) and MKK6b (RAdMKK6bE) alone or in combination resulted in activation of p38, but not ERK1/2 (Fig. 3*A*). Infection of cells with RAdMEK1 and RAdMKK3bE or RAdMKK6bE resulted in coordinate activation of ERK1/2 and p38 MAPK (Fig. 3*A*).

Infection of dermal fibroblasts with the adenovirus RAdMKK7D, harboring a constitutively active mutant of MKK7, resulted in activation of JNK as determined by kinase assay using recombinant c-Jun as substrate (Fig. 3*B*), but had no effect on activation of ERK1/2 or p38 (Fig. 3*A*). In parallel, adenoviral expression of constitutively active MEK1, MKK3b, or MKK6b had no effect on JNK activity (Fig. 3*B*). Interestingly, simultaneous expression of constitutively active MKK3b or MKK6b in fibroblasts markedly potentiated the activation of JNK by MKK7D, resulting in a level of JNK activity comparable to that obtained by treatment of dermal fibroblasts with anisomycin (Fig. 3*B*). Expression of constitutively active MEK1, MKK7, MKK3b, or MKK6b either alone or in the combinations indicated had no effect on total cellular levels of ERK1/2 or p38 (Fig. 3*A*). Infection of cells with control viruses RAd66 and RAd*lacZ* had no marked effect on the activation of ERK1/2, JNK, or p38 (Fig. 3, *A* and *B*).

Activation of ERK1/2 by transduction of fibroblasts with RAdMEK1ca resulted in marked enhancement of the abundance of MMP-1 and MMP-3 mRNAs as determined by Northern blot hybridizations 24 h after adenoviral infection (Fig. 3*C*). Activation of p38 MAPK by adenovirally delivered constitutively active MKK3b and MKK6b alone or in combination also resulted in enhancement of MMP-1 and MMP-3 mRNA levels, although clearly less potently than obtained with constitutively active MEK1 (Fig. 3*C*). In addition, expression of constitutively active MKK3b and MKK6b augmented the enhancement of MMP-1 and MMP-3 mRNA levels by constitutively active MEK1 alone (Fig. 3*C*).

Production of pro-MMP-1 and pro-MMP-3 by dermal fibroblasts was also enhanced as a result of ERK1/2 activation in cells infected with RAdMEK1ca, and this effect was inhibited by PD98059 (Fig. 3*D*). Activation of p38 MAPK by constitu-

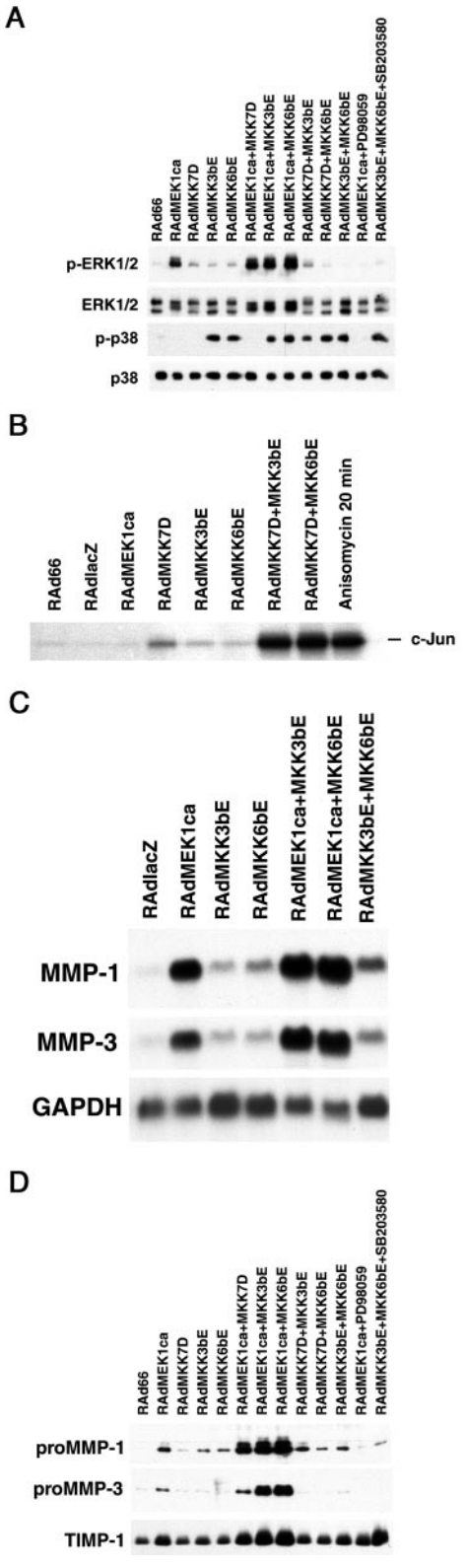

FIG. 3. **Activation of ERK1/2 and p38 results in induction of MMP-1 and MMP-3 expression by fibroblasts.** *A*, human skin fibroblasts (5×10^5) were transduced with the replication-deficient empty control adenovirus (RAd66); with the β-galactosidase-coding adenovirus (RAd*lacZ*); or with the adenovirus coding for constitutively active forms of MEK1 (RAdMEK1ca), MKK7 (RAdMKK7D), MKK3b (RAdMKK3bE), or MKK6b (RAdMKK6bE) at m.o.i. = 500 and incubated for 18 h in DMEM supplemented with 1% FCS. The MEK1/2 inhibitor PD98059 (40 μM) and the p38 inhibitor SB203580 (20 μM) were added to the cultures indicated at the time of infection. After 18 h, the medium was changed; fresh PD98059 and SB203580 were added; and incubations were continued for 24 h. The levels of activated ERK1/2 (*p-ERK1/2*) and p38 (*p-p38*) in cell lysates were determined by Western blot analysis using phospho-specific antibodies. The levels of total ERK1/2 and p38 were also determined in the same samples by Western blot analysis using specific antibodies. *B*, the activity of JNK immunoprecipitated from lysates of cells infected as described for *A* was determined by kinase assay using c-Jun as substrate. Lysate from fibroblasts treated with anisomycin (10 μg/ml) for 20 min was used as a control. *C*, human skin fibroblasts were infected and incubated as described for *A*, and aliquots (10 μg) of total RNA were analyzed for the levels of MMP-1, MMP-3, and GAPDH mRNAs by Northern blot hybridizations. *D*, human skin fibroblasts were infected and incubated as described for *A*, and the levels of pro-MMP-1, pro-MMP-3, and TIMP-1 in the conditioned medium of the cells were determined by Western blot analysis.

tively active MKK3b and MKK6b alone or in combination also enhanced production of pro-MMP-1 and pro-MMP-3, and this effect was inhibited by the p38 inhibitor SB203580. In contrast, activation of JNK by constitutively active MKK7 alone had no effect on pro-MMP-1 or pro-MMP-3 production (Fig. 3*D*). Furthermore, the potent activation of JNK noted in cells transduced with RAdMKK7D in combination with RAdMKK3bE or RAdMKK6bE did not result in induction of the production of pro-MMP-1 or pro-MMP-3 over the levels obtained by infection of cells with RAdMKK3bE and RAdMKK6bE alone (Fig. 3*D*). However, co-infection of cells with the constitutively active MKK7, MKK3b, or MKK6b adenovirus together with the MEK1 adenovirus augmented the enhancement of pro-MMP-1 and pro-MMP-3 production achieved by constitutively active MEK1 alone (Fig. 3*D*). Infection of fibroblasts with the empty control adenovirus RAd66 had no effect on production of MMP-1 or MMP-3 (Fig. 3*D*). Production of TIMP-1 was also enhanced in cells infected with RAdMEK1ca either alone or together with RAdMKK7D, RAdMKK3bE, or RAdMKK6bE, but clearly less potently than the production of pro-MMPs (Fig. 3*D*).

Activation of ERK1/2 Induces c-jun, junB, and c-fos Expression in Fibroblasts—To elucidate the role of AP-1 transcription factors in mediating the up-regulatory effect of ERK1/2 and p38 MAPK pathways on MMP-1 and MMP-3 expression, we determined the expression of distinct AP-1 components as a result of the activation of ERK1/2 and p38 MAPK. Activation of ERK1/2 by adenovirus-mediated expression of constitutively active MEK1 resulted in induction of c-*jun*, *junB*, and c-*fos* mRNA expression, and this effect was potently inhibited by PD98059 (Fig. 4). In contrast, infection of fibroblasts with RAdMKK3bE and RAdMKK6bE alone had no effect on the expression of c-*jun*, *junB*, or c-*fos* mRNA. However, co-infection of the cells with constitutively active MKK3b or MKK6b together with the MEK1 adenovirus markedly augmented the induction of c-*jun*, *junB*, and c-*fos* mRNA levels as compared with induction by constitutively active MEK1 alone (Fig. 4). These results provide evidence that the induction of fibroblast MMP-1 and MMP-3 expression as a result of ERK1/2 activation involves the AP-1 complex, whereas the up-regulatory effect of p38 activation alone on MMP-1 and MMP-3 expression is AP-1-independent.

Activation of p38α Results in Induction of MMP-1 and MMP-3 Expression by Fibroblasts—Our recent results show that, in fibroblasts, MKK3b activates p38α, whereas MKK6b activates all p38 isoforms (9). To examine in detail the role of p38α in regulation of the expression of the endogenous MMP-1 and MMP-3 genes, we utilized adenovirus-mediated gene delivery of wild-type p38α either alone or in combination with constitutively active MEK1, MKK3b, or MKK6b. Transduction of human skin fibroblasts with the p38α-coding adenovirus alone had no effect on the expression of MMP-1 and MMP-3 mRNAs (Fig. 5*A*). Activation of adenovirally delivered p38α by transduction of fibroblasts with the adenovirus for constitu-

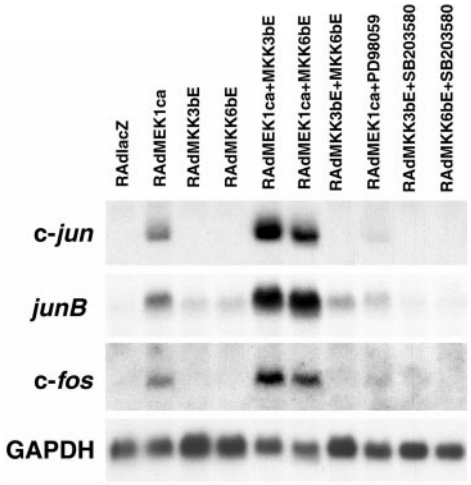

FIG. 4. **Activation of ERK1/2 induces c-*jun*, *junB*, and c-*fos* expression in fibroblasts.** Human skin fibroblasts (5×10^5) were transduced with recombinant adenoviruses (m.o.i. = 500) harboring β-galactosidase (RAd*lacZ*), constitutively active MEK1 (RAdMEK1ca), constitutively active MKK3b (RAdMKK3bE), and constitutively active MKK6b (RAdMKK6bE) and incubated for 18 h in DMEM supplemented with 1% FCS. The MEK1/2 inhibitor PD98059 (40 μM) and the p38 inhibitor SB203580 (20 μM) were added to the cultures indicated at the time of infection. After 18 h, the medium was changed; fresh PD98059 and SB203580 were added; and incubations were continued for 24 h. The cells were harvested, and aliquots (10 μg) of total RNA were analyzed for the abundance of c-*jun*, *junB*, c-*fos*, and GAPDH mRNAs by Northern blot hybridizations.

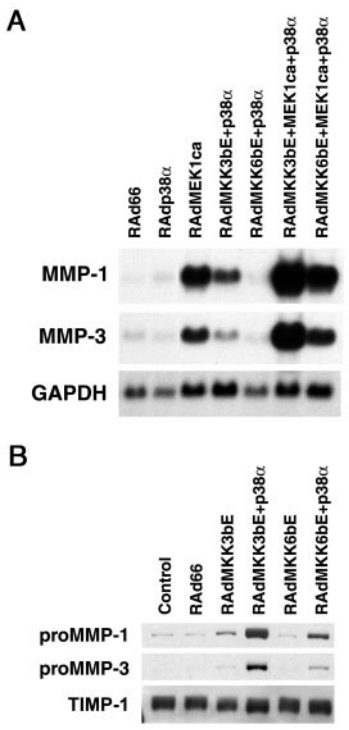

FIG. 5. **Activation of p38α increases MMP-1 and MMP-3 mRNA expression.** *A*, human skin fibroblasts (5×10^5) were transduced with the replication-deficient empty control adenovirus (RAd66) or with the adenovirus coding for wild-type p38α (RAdp38α) or constitutively active MEK1 (RAdMEK1ca), MKK3b (RAdMKK3bE), or MKK6b (RAdMKK6bE) at m.o.i. = 500 and incubated in DMEM supplemented with 1% FCS. After 18 h, the medium was changed; the incubation was continued for 24 h; and the cells were harvested for determination of MMP-1, MMP-3, and GAPDH mRNA abundance by Northern blot hybridizations. *B*, human Ha-Ras-transformed fibroblasts (KMST-6/Ras) were infected and incubated as described for *A*, and the levels of pro-MMP-1, pro-MMP-3, and TIMP-1 in the conditioned medium of the cells were determined by Western blot analysis.

tively active MKK3b potently enhanced MMP-1 and MMP-3 mRNA levels, whereas transduction of fibroblasts with p38α together with constitutively active MKK6b did not result in enhancement of MMP-1 and MMP-3 mRNA levels. Expression of constitutively active MEK1 augmented the enhancement of MMP-1 and MMP-3 mRNA abundance by p38α and MKK3b, but co-infection of MEK1, p38α, and MKK6b did not markedly enhance the expression of MMP-1 and MMP-3 over the levels obtained with MEK1 alone (Fig. 5*A*).

To gain further insight into the role of p38α in the regulation of MMP-1 and MMP-3 expression, we utilized human Ha-Ras-transformed fibroblasts (KMST-6/Ras), which display constitutive activation of ERK1/2 (9). Production of pro-MMP-1 and pro-MMP-3 in KMST-6/Ras cells was enhanced by transducing cells with the adenovirus for constitutively active MKK3b (RAdMKK3bE) alone, and this up-regulation was augmented by co-infecting the cells with the adenovirus harboring wild-type p38α (RAdp38α) (Fig. 5*B*). In contrast, infecting the cells with the constitutively active MKK6b adenovirus (RAdMKK6bE) alone did not enhance pro-MMP-1 and pro-MMP-3 production, whereas co-infection of the cells with the wild-type p38α adenovirus (RAdp38α) and RAdMKK6bE resulted in enhancement of pro-MMP-1 and pro-MMP-3 production, although clearly less potently than with MKK3b (Fig. 5*B*). The production of TIMP-1 was not altered by activation of p38α by MKK3b or MKK6b. These results show that activation of p38α specifically induces MMP-1 and MMP-3 production alone or in the presence of ERK1/2 activation.

Activation of p38α Results in Stabilization of MMP-1 and MMP-3 mRNAs—Activation of ERK1/2 results in potent stimulation of MMP-1 promoter activity and mRNA expression, whereas activation of the p38 pathway does not alone markedly enhance MMP-1 promoter activity (9). To elucidate the mechanism of p38α-mediated up-regulation of MMP-1 and MMP-3 expression, we transduced normal dermal fibroblasts either with the empty control adenovirus (RAd66) or with adenoviruses coding for constitutively active MKK3b (RAdMKK3bE) and wild-type p38α (RAdp38α) at m.o.i. = 500 (Fig. 6*A*). After an 18-h incubation in DMEM supplemented with 1% FCS, the medium was changed, and the RNA polymerase II inhibitor DRB (60 μM) was added. The control and (p38α + MKK3E)-transduced cultures were subsequently harvested at 3-h intervals for RNA extraction and determination of MMP-1, MMP-3, and GAPDH mRNA abundance by Northern blot hybridizations. At the time of addition of DRB to the cultures, the levels of MMP-1 and MMP-3 mRNAs were clearly elevated in cells in which adenovirally delivered p38α was activated by constitutively active MKK3b (Fig. 6*A*). Furthermore, activation of p38α in these cells increased the estimated half-life of MMP-1 mRNA from 2.3 to 31 h and that of MMP-3 mRNA from 2.2 to 28 h compared with control virus-infected cells (Fig. 6*B*), indicating that activation of p38α MAPK results in stabilization of MMP-1 and MMP-3 mRNAs.

DISCUSSION

MAPKs play an important role in regulating cell growth, differentiation, survival, and death (4, 5). To date, three mammalian MAPK pathways have been characterized in detail: the mitogen-activated ERK1/2 pathway (Raf → MEK1/2 → ERK1/2) and the JNK (MEK kinase-1–4 → MKK4/7 → JNK1–3) and p38 (MAPK kinase kinase → MKK3/6 → p38α/β/γ/δ) pathways, activated by inflammatory cytokines and cellular stress (see Refs. 4 and 5). Phosphorylation of conserved threonine and tyrosine residues of MAPKs by specific upstream dual-specificity kinases (MAPK kinases) results in activation and nuclear translocation of MAPKs and in phosphorylation

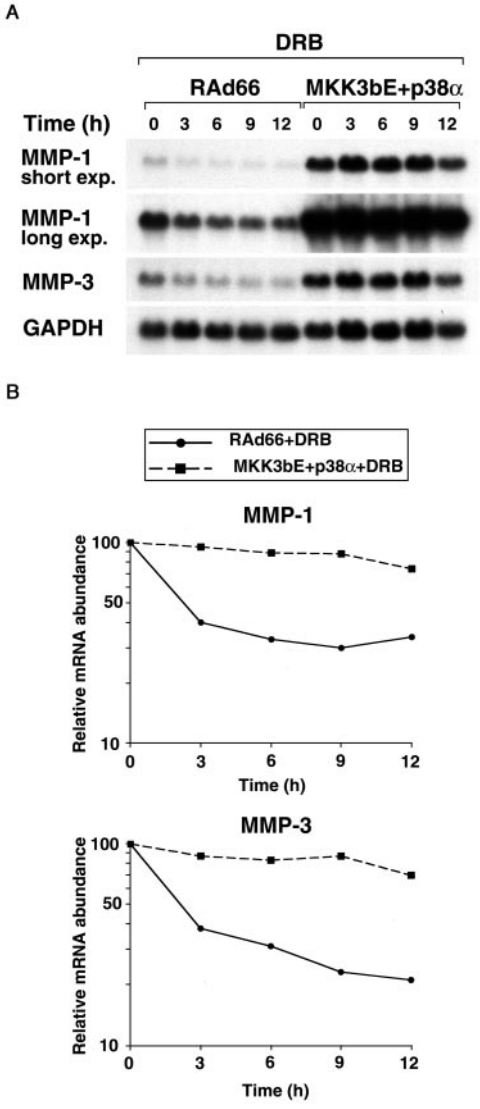

FIG. 6. **Activation of p38α results in stabilization of MMP-1 and MMP-3 mRNAs.** *A*, human skin fibroblasts (5×10^5) were transduced with the replication-deficient empty control adenovirus (RAd66) or with adenoviruses coding for constitutively active MKK3b (RAdMKK3bE) and wild-type p38α (RAdp38α) at m.o.i. = 500 and incubated for 18 h in DMEM supplemented with 1% FCS. After 18 h, the medium was changed; the RNA polymerase II inhibitor DRB (60 μM) was added; and the control (RAd66) and (p38α + MKK3bE)-transduced cultures were harvested at 3-h intervals for determination of MMP-1, MMP-3, and GAPDH mRNA abundance by Northern blot hybridizations. *B*, the relative mRNA abundance of the experiment in *A* was quantitated, and the results show the increased stability of MMP-1 and MMP-3 mRNAs. *exp.*, exposure.

and activation of their downstream effectors, nuclear protein kinases (*e.g.* MAPK-activated protein kinases-1, -2, and -3) or transcription factors (*e.g.* Elk-1, c-Jun, ATF2, and CREB), which in turn regulate, for example, expression of the components of the AP-1 complex (see Refs. 4 and 5). The specificity of MAPK pathways is controlled by cytoplasmic scaffold proteins (*e.g.* MEK partner and JNK-interacting protein-1), which can physically combine kinases of distinct MAPK cascades into effective and specific signaling modules (29, 30). Recent results have provided evidence that ERK1/2, JNK, and p38 MAPK regulate the proteolytic capacity of fibroblasts and squamous cell carcinoma cells by mediating the activation of MMP-1, MMP-3, gelatinase B (MMP-9), and collagenase-3 (MMP-13) expression (6–8, 31–34).

In this study, we have dissected the role of three distinct MAPK pathways (ERK1/2, JNK, and p38) in regulation of the proteolytic capacity of normal human skin fibroblasts. We have shown that treatment with TNF-α simultaneously activates ERK1/2, JNK, and p38 in fibroblasts and that these pathways play a distinct role in the induction of MMP-1 and MMP-3 expression. This is well demonstrated by the observation that the p38-specific inhibitor SB203580 potently inhibited the induction of fibroblast MMP-1 and MMP-3 expression by TNF-α. In contrast, blocking the ERK1/2 pathway by the MEK1/2-specific inhibitor PD98059 had no effect on the induction of MMP-1 and MMP-3 expression by TNF-α, indicating that, in the presence of JNK and p38 activation, ERK1/2 activity is not crucial for induction of MMP-1 and MMP-3 expression. Taken together, these results show that the p38 MAPK pathway plays an important role in control of the proteolytic activity of normal fibroblasts.

To examine the effect of the activation of endogenous MAPKs in regulation of the expression of MMPs in normal human skin fibroblasts, we utilized adenovirus-mediated gene delivery of constitutively active MEK1, MKK7, MKK3b, and MKK6b. Our results show that activation of ERK1/2 by constitutively active MEK1 resulted in marked induction of MMP-1 and MMP-3 expression and that the most abundant expression of both MMPs was noted when ERK1/2 was activated in combination with JNK or p38. Interestingly, activation of endogenous p38 alone by adenovirus-mediated delivery of constitutively active MKK3b or MKK6b to fibroblasts also resulted in induction of MMP-1 and MMP-3 expression, although the level of expression was lower than in cells infected with RAdMEK1ca. We have recently noted that MKK6 activates four distinct p38 isoforms (p38α, p38β, p38γ, and p38δ) in fibroblasts, whereas MKK3 activates only p38α (9). Interestingly, SB203580 has been shown to inhibit p38α and p38β, but not the p38γ and p38δ isoforms (35). The potent inhibitory effect of SB203580 on TNF-α- or MKK3-induced MMP-1 and MMP-3 expression suggests that the p38α isoform plays an important role in the regulation of MMP-1 and MMP-3 expression. This notion is also supported by our observation that specific activation of adenovirally delivered p38α results in stimulation of MMP-1 and MMP-3 expression and that this involves stabilization of the corresponding mRNAs.

Evidence for the role of MAPKs in malignant transformation has been provided by findings that constant activation of ERK1/2 by active mutants of Raf-1 or MEK1 results in transformation of fibroblasts (36, 37). Furthermore, it has been shown that the ERK1/2 pathway is activated in renal and breast carcinomas *in vivo* (38, 39). However, the consequences of ERK1/2 activation appear to be cell-specific, as the activation of the ERK1/2 cascade results in growth arrest in small cell lung carcinoma cells (40) and suppresses the expression of MMP-13 by squamous cell carcinoma cells (41). A recent study showed that, in Ha-Ras-transformed NIH-3T3 fibroblasts, elevated ERK1/2 (but not JNK) activity is required for growth of these cells in soft agar and that JNK activity increases the expression of the urokinase plasminogen activator (42). Our results show that MMP-1 and MMP-3 production by fibroblasts expressing constitutively active MEK1 is further enhanced when constant activation of ERK1/2 is superimposed on persistent activation of JNK or p38. This phenomenon may play an important role in invasion of malignant tumors *in vivo*, in which tumor cells and stromal fibroblasts are exposed to cytokines produced by peritumoral inflammatory cells, resulting in coordinate activation of ERK1/2, JNK, and p38 (43).

Our results show that activation of ERK1/2 results in induction of c-*jun*, *junB*, and c-*fos* mRNA expression and that simultaneous activation of p38 augments the induction of these AP-1

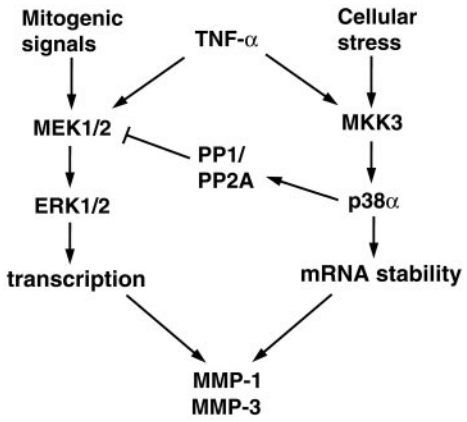

FIG. 7. **Distinct roles of ERK1/2 and p38α in the regulation of MMP-1 and MMP-3 expression.** TNF-α activates both ERK1/2 and p38α MAPK in fibroblasts. Activation of ERK1/2 first up-regulates the transcription of MMP-1 and MMP-3 genes. Activation of p38α results in inhibition of the ERK1/2 cascade at the level of MEK1/2 via protein phosphatases 1 and 2A (*PP1*/*PP2A*) and in the subsequent suppression of MMP-1 and MMP-3 gene transcription. In addition, activation of p38α results in stabilization of MMP-1 and MMP-3 mRNAs and in enhanced production of pro-MMP-1 and pro-MMP-3.

components. However, activation of p38 alone did not induce the expression of c-*jun*, *junB*, and c-*fos* mRNAs, indicating that ERK1/2 and p38 cascades regulate the expression of these genes via different mechanisms. These results provide evidence that ERK1/2-mediated enhancement of MMP-1 and MMP-3 gene expression takes place at the transcriptional level via an AP-1-dependent mechanism (9), whereas activation of the expression of these MMPs by p38 alone is AP-1-independent. Recent studies show that enhancement of fibroblast MMP-1 and MMP-3 expression by interleukin-1 and three-dimensional collagen involves activation of NF-κB (27, 28, 44). We noted that rapid phosphorylation and degradation of IκBα in fibroblasts treated with TNF-α were not affected by blocking ERK1/2 or p38 MAPK pathways, suggesting that activation of NF-κB may mediate the enhancement of MMP-1 and MMP-3 gene expression in the absence of ERK1/2 and p38 activation.

Our recent results show that activation of p38 MAPK by constitutively active MKK3b and MKK6b has no marked effect on human MMP-1 promoter activity (9). The results in the present study show that activation of p38α results in marked stabilization of both MMP-1 and MMP-3 mRNAs. These results are in accordance with previous results showing stabilization of the mRNAs of MMP-1, MMP-3, and MMP-9 in response to interleukin-1, epidermal growth factor, phorbol esters, and lipopolysaccharide (45–48). The 3′-untranslated region of human MMP-1 mRNA contains an AU-rich element, which plays a role in stabilization of the message (46). Previous studies have shown that p38 MAPK-dependent activation of cyclooxygenase-2, interleukin-6, and TNF-α gene expression involves mRNA stabilization (49–51). There is also evidence that p38 mediates lipopolysaccharide-induced expression and phosphorylation of tristetraprolin, an AU-rich element-binding zinc-finger protein that regulates the stability of TNF-α mRNA (51). Altogether, our results provide evidence that activation of p38α by MKK3b increases MMP-1 and MMP-3 expression via mRNA stabilization, whereas activation of ERK1/2 increases MMP-1 and MMP-3 expression at the transcriptional level (Fig. 7). We have recently shown that p38α mediates activation of protein phosphatases 1 and 2A and blocks the ERK1/2 cascade at the level of MEK1/2, resulting in suppression of MMP-1 promoter activity (9). Thus, activation of the ERK1/2 pathway rapidly up-regulates MMP-1 and MMP-3 mRNA transcription, whereas activation of p38α blocks the ERK1/2 pathway and enhances the expression of MMP-1 and MMP-3 via mRNA stability (Fig. 7). This model is supported by observations that mitogenic factors such as epidermal growth factor and phorbol esters, which induce the expression of MMPs, simultaneously activate both the ERK1/2 and p38 MAPK pathways (52).

Controlled degradation of the collagenous ECM is an important feature in tissue remodeling in physiologic situations such as tissue repair and angiogenesis as well as in pathologic conditions, *e.g.* rheumatoid arthritis, chronic ulcers, and tumor invasion (1–3). The ability to cleave type I collagen is essential for migration of epidermal keratinocytes and airway epithelial cells (53, 54). It is also likely that degradation of the collagenous ECM by fibroblasts alters their cell-matrix interactions and facilitates their migration capacity (55). Our results identify ERK1/2 and p38α as two distinct MAPK pathways mediating activation of the expression of collagenolytic MMP-1 and its activator MMP-3 in fibroblasts. In addition, activation of ERK1/2 in dermal fibroblasts results in marked suppression of their production of type I collagen and the collagen fibril-associated proteoglycan decorin (24, 52). Together, these results provide evidence that activation of ERK1/2 promotes a proteolytic fibroblast phenotype characterized by production of ECM-degrading MMPs and suppression of ECM deposition. Furthermore, activation of p38 is alone sufficient to induce expression of MMP-1 and MMP-3 expression in an AP-1-independent manner by stabilization of the corresponding mRNAs. However, our results show that coordinate activation of ERK1/2 in combination with JNK or p38 MAPK results in the most potent induction of MMP-1 and MMP-3 expression and apparently plays a crucial role in stimulation of the proteolytic capacity of normal fibroblasts *in vivo*, *e.g.* during wound repair and tumor invasion.

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