p38γ MAPK Cooperates with c-Jun in trans-Activating Matrix Metalloproteinase 9

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Mitogen-activated protein kinases (MAPKs) regulate gene expression through transcription factors. However, the precise mechanisms in this critical signal event are largely unknown. Here, we show that the transcription factor c-Jun is activated by p38γ MAPK, and the activated c-Jun then recruits p38γ as a cofactor into the matrix metalloproteinase 9 (MMP9) promoter to induce its trans-activation and cell invasion. This signaling event was initiated by hyperexpressed p38γ that led to increased c-Jun synthesis, MMP9 transcription, and MMP9-dependent invasion through p38γ interacting with c-Jun. p38γ requires phosphorylation and its C terminus to bind c-Jun, whereas both c-Jun and p38γ are required for the trans-activation of MMP9. The active p38γ/c-Jun/MMP9 pathway also exists in human colon cancer, and there is a coupling of increased p38γ and MMP9 expression in the primary tissues. These results reveal a new paradigm in which a MAPK acts both as an activator and a cofactor of a transcription factor to regulate gene expression leading to an invasive response.

MAPKs (including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38s) are critical signaling cascades that convert upstream signals into biological responses such as cell proliferation, invasion, and transformation (1). MAPKs are believed to do so by phosphorylating and activating a group of transcription factors, which through binding regulatory DNA elements lead to altered gene transcription. c-Jun is a major component of the AP-1 transcription factor downstream of MAPKs, whereas AP-1 is composed of homodimers of the Jun family or its heterodimers with another transcription factor such as c-Fos to bind the consensus DNA elements TGAg/cTCA (2). c-Jun is activated by JNK through phosphorylation at Ser-63, Ser-73, Thr-91, and Thr-93, and by ERK and p38 via increased gene expression. Activated c-Jun/AP-1 leads to a cell type-specific biological response through integrated gene expression (1). However, the exact mechanism by which c-Jun converts a MAPK activity into a target gene expression remains mostly unknown.

p38 MAPKs consist of four family members (α, β, γ, and δ) in which p38α is ubiquitously present, whereas p38γ is highly expressed in certain cancers (3). In addition to well established regulatory effects in cytokine signaling and stress response, substantial evidence suggests that the p38α pathway functions as a tumor suppressor (4–8). p38γ, on the other hand, is a 43-kDa protein with an unique C-terminal motif, KETXL, that can dock with the PDZ (PSD-95/Dlg/ZO-1 homology) domain of other proteins (9, 10). In contrast to p38α, our recent studies showed that p38γ is induced by Ras and required for Ras transformation and invasion (11, 12), indicating its oncogenic activity. The underlying mechanisms for p38γ involvement in Ras tumorigenesis, however, have not been established. In this report, we show that p38γ acts both as an activator and a cofactor for c-Jun in trans-activating MMP9, a critical matrix metalloproteinase involved in cancer invasion and metastasis (13, 14). These results reveal a novel paradigm by which p38γ increases c-Jun synthesis and activated c-Jun then recruits p38γ as a cofactor onto a target gene promoter through AP-1 recognition leading to an increased gene expression and invasion.

EXPERIMENTAL PROCEDURES

Reagents, Cell Culture, and cDNA Constructs—Cell culture materials were supplied by Invitrogen and chemicals by Sigma. p38 isoform-specific antibodies were purchased from RD Systems. Glyceraldehyde-3-phosphate dehydrogenase, c-Jun, MMP9, and MMP2 antibodies were from Santa Cruz Biotechnology. Phosphorylated p38 (p-p38) and p-c-Jun (Ser-63/73) antibodies were from Cell Signaling. Mouse monoclonal antibodies against FLAG (M2) were from Sigma. IEC-6 cells as well as the procedure for establishing the Ras-transformed subline (IEC-6/K-Ras) were described previously (11). Human colon cancer cell lines were purchased from the American Type Culture Collection. p38γ+/+ and p38γ−/− mouse embryonic fibroblasts (MEFs) have been described previously (10), and early passages of these cells were immortalized by infection.
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with retroviruses expressing H-Ras and E1A. c-Jun+/+ and c-Jun−/− cells were provided by R. Wisdom (15) and have been used previously in our laboratory (16). All cell cultures were maintained in minimum Eagle’s medium or Dulbecco’s modified Eagle’s medium containing 10% serum and antibiotics at 37 °C, 5% CO2.

An AP-1 luciferase reporter (AP-1 Luc, 3 AP-1 repeats fused to a luciferase reporter gene containing a minimal Fos promoter) was described previously (4, 17), whereas the wild-type (WT) and mutant human MMP-9 promoter (MMP9-Luc) were reported before (18). The Tet-on inducible expression system (T-Rex) was purchased from Invitrogen. A full-length human p38γ, its AGF mutant (p38γ/AGF), and the c-Jun luciferase promoter (c-Jun Luc, containing a minimal GATA promoter) were provided by J. Han (19, 20) and used previously in our laboratory (21). To generate a Tet-on system, a full-length human p38γ or p38γ/AGF cDNA was cloned into pcDNA4 vector, which was then cotransfected with pcDNA6/Tr into IEC-6 cells and selected/maintained as described previously (12). Expression of p38γ or p38γ/AGF was induced by addition of 1 μg/mL of tetracycline that alone has been shown to have no effects on cell invasion or endogenous p38γ protein expression (data not shown). The C-terminal truncated p38γ mutants (p38γΔ4 and p38γΔ13) were generated by PCR and cloned into FLAG-tagged pcDNA3 vector as described (22).

** Invasion/Migration Assays—** Invasion assays were carried out using the BioCoat Matrigel Invasion Chamber (BD Biosciences, Bedford, MA) by using 20% fetal bovine serum as a chemotactrant according to the manufacturer’s instruction, as we described previously (12). Invaded cells on the low surface of membrane were then fixed, stained, and counted. On the other hand, wound assays were used to assess the ability of the cell to migrate into a scratched area in serum-free medium.

**Immunohistochemistry Studies of Primary Human Colon Cancer Tissues—** Immunohistochemistry analyses were conducted in accordance with Institutional Review Board approval from the Medical College of Wisconsin and were performed as described previously (22, 23). A rabbit anti-p38γ (1:1200; R&D catalog no. AF1644) and a goat anti-MMP9 (1:150; Santa Cruz Biotechnology) were used as primary antibodies. Staining was carried out with 2.5% Triton X-100 buffer for 1 h at 37 °C. Primary antibodies were then incubated overnight at 4 °C with secondary antibodies (1:1000) followed by DAB (3,3′-diaminobenzidine) and counterstaining with hematoxylin. Sections were then dehydrated and coverslipped.

**Zymography—** To determine the level of MMP9 activity, Tet-on p38γ IEC-6 cells were plated in 100-mm dishes at a density such that about 70% confluence within 24 h with and without Tet addition. Thereafter, the medium was replaced with 4 mL of fresh serum-free medium. To determine the effects on MMP9 activity by its inhibitors, Tet cells were treated with 10 nm ilomastat (Chemicon International) or 1 μM SB-3CT (Calbiochem) or a solvent control. After additional 24 h, the media were collected and concentrated by centrifugation using the Amicon Ultra-4 Ultracel-50k filters (Millipore). Concentrated samples were measured for the protein concentration by BCA protein assay reagents (Bio-Rad). Gelatin zymography was used to assess MMP9 activity (25). Briefly, 20 μg of protein from each sample was mixed with SDS sample buffer in the absence of reducing reagents, which were separated on 10% SDS-polyacrylamide gels containing 0.1% gelatin. The gels were incubated with 2.5% Triton X-100 buffer for 1 h and then incubated in digestion buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 1 μM ZnCl2, and 5 mM CaCl2 overnight at 37 °C). Following staining with 5% Coomassie Brilliant Blue R-250, the gelatinolytic activities were detected as clear bands against a blue background.

** p38γ Knockdown, Transfection, Infection, and Luciferase Assays—** Endogenous p38γ was depleted by lentiviral-mediated shRNA delivery (with two separate target sequences by including a sequence from luciferase gene as a control) as described also was prepared in parallel and subjected to PCR as an input control.

**Real-time Quantitative Reverse Transcription PCR (qRT-PCR)—** Total RNA was prepared using a TRIzol extraction kit, and the qRT-PCR was performed using the Express One-Step Syber GreenER qPCR kit (Invitrogen). Samples were analyzed by ΔΔCT method for fold changes in expression and the ratio of MMP9 or c-Jun over β-actin (rat and human cells) or 18 S (mouse cells) was used for comparison. All experiments were repeated at least three times. Some of qRT-PCR products were also visualized on agarose gels. All primers were obtained from Integrated DNA Technologies, and their sequences are as follows: rat β-actin, 5’-ATCTGCGCAACACACTTCTAC-CATG-3’ (sense) and 5’-TTCCATGAGGATGTCAGTC- GTC-3’ (antisense); rat MMP2, 5’-CATCGTCGACCATCAGGCCCATGATC-3’ (sense) and 5’-CCCCAGGTCACACCATC- ATCATCATGACTG-3’ (antisense); rat MMP9, 5’-GAAGACTGTCGACGACTGTC-3’ (sense) and 5’- GCCAGCAGATACATCCAGGAC-3’ (antisense); human β-actin, 5’-GATATCGCCGCTGCTGTCGAC-3’ (sense) and 5’-CAGGGAGAAAGGTGAGTGCC-3’ (antisense); human MMP9, 5’-TGGGTCATCGTGACTGAC-3’ (sense) and 5’-GCCAGCAGATTCA-3’ (antisense); mouse c-Jun, 5’-GACCTTCTCAGCAGATGCG-3’ (sense) and 5’-CAAGCGCCAGCTACTGAGG-3’ (antisense).
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FIGURE 1. p38γ requires both phosphorylation and the C terminus to stimulate invasion. A–C, p38γ but not its AGF mutant, stimulates invasion and/or migration in IEC-6 cells. Cells were incubated with and without Tet for 24 h and collected for protein expression by Western blotting (A) (p-p38 antibody reacts both with p-p38α and p-p38γ and assessed for Matrigel invasion (B) and migration (C). Results of B and C are shown as relative over those without Tet from at least three separate experiments (*, p < 0.05; error bars, S.D.). D and E, p38γ increases invasion in IEC-6/K-Ras cells. Cells were transfected and selected, followed by retroviral infection and a second antibiotic selection, and then were analyzed by Western blotting (D) and Matrigel invasion (E) (*, p < 0.05 versus vector control; ** p < 0.05 versus p38γ). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Previously (22). The target sequences for individual genes are as follows: luciferase (shLuc), GTGCCGTGCTAGTACCAAC; #1shp38γ, CTCATGAAACATGAGAAGCTA; #2#1shp38γ, GAAGGAGATCATGAAGGTGAC.

To produce virus, lentiviral constructs were transfected into packaging cells, and supernatants were collected and filtered 48 h later. To deplete p38γ protein expression, human colon cancer cells were double-infected with the viruses at a 2-h interval, which were processed for invasion assays and qRT-PCR/Western blot (WB) analyses 48 and 72 h later, respectively. For reporter and promoter assays, AP-1 Luc, c-Jun-Luc, or MMP9-Luc was transiently coexpressed with various constructs, and lysates were prepared for the luciferase activity assays 48 h later using a dual luciferase kit from Promega (12). The procedures for immunoprecipitation and WB have been described previously (11).

Statistical Analyses—Results of multiple variables were analyzed by two-way analysis of variance followed by the Bonferroni post-test. Two variables were analyzed by Student’s t test. The immunohistochemistry results were analyzed by a χ² test, and the linear regression analysis was used for assessing the relationship between the normalized p38γ content with the c-Jun or MMP9. A statistically significant difference is reached when a p value is less than 0.05.

RESULTS

p38γ Requires Phosphorylation and C Terminus to Stimulate Invasion—To investigate signaling mechanisms for p38γ oncogenic activity, we focused on how forced p38γ expression leads to an increased invasion. Because normal cells express little p38γ (11), a tetracycline-inducible (Tet-on) system was used to express WT p38γ and its nonphosphorable mutant p38γ/AGF (by changing the dual phosphorylation motif TGY to AGE) in rat intestinal epithelial IEC-6 cells. Following an overnight incubation with and without Tet, cells were seeded in Matrigel-coated invasion chambers and analyzed for invasion (12). Results in Fig. 1, A and B, show that Tet-induced p38γ stimulates invasion whereas its AGF mutant is without effect, which couples with its activity to increase c-Jun but not c-Fos protein expression. The invasion-stimulatory activity of p38γ is further confirmed by an increased migration in wound assays (Fig. 1C). These results indicate that p38γ stimulates cell invasion and/or migration by phosphorylation-dependent mechanisms.

Recent studies indicate that the C-terminal PDZ motif is required for the invasive activity of c-Src (26) and TAZ (tafazzin) proteins (27), and we then explored whether the C-terminal PDZ sequence of p38γ plays a similar role. To remove the PDZ motif (-ETPL), FLAG-tagged C-terminal truncated p38γΔ4 and p38γΔ13 that lack the last four and 13 amino acids, respectively, were generated by PCR and stably expressed in IEC-6 cells by including FLAG-p38γ and FLAG-p38γ/AGF for comparison. To explore their potential roles in Ras tumorigenesis, resistant clones were pooled and infected with retrovirus expressing K-Ras and their invasive activity then compared. Consistent with the results in Tet-on cells, stable expression of p38γ significantly increases invasion over the vector control, whereas its AGF mutant has much less effect (Fig. 1, D and E). Interestingly, the invasive activity also was decreased in cells expressing both p38γΔ4 and p38γΔ13 (Fig. 1, D and E). These results together indicate that p38γ requires both phosphorylation and its C terminus to stimulate invasion.

p38γ Increases MMP9 Transcription by AP-1-dependent Mechanisms—MMPs consist of at least 23 family members and have long been associated with cancer invasion and metastases because of their role in breaking down the extracellular matrix (28). Among these family proteins, MMP9 is one of the best characterized AP-1 target genes involved in cancer invasion (13). Because p38 MAPKs were previously shown to regulate the AP-1 target gene expression (29, 30), an AP-1 reporter (4) and a 670-bp human MMP9 promoter (18) were transiently expressed in IEC-6 cells to assess whether p38γ increases their luciferase activity compared with p38α. Results in Fig. 2, A and B, show that both p38γ and p38α increase AP-1, but only p38γ...
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mRNA expression compared with MMP2. Results in Fig. 2D demonstrate that p38γ significantly increases MMP9 but not MMP2 expression; this is likely as a result of the lack of AP-1 site in the MMP2 promoter. Importantly, analyses of medium collected from cultured Tet-on p38γ cells revealed that there is an increased secreted MMP9 protein expression by WB and an elevated MMP9 gelatin activity by zymography (Fig. 2E), indicating the transcribed protein being functionally active. Consistent with these results, analysis of IEC-6/K-Ras cells stably transfected with p38γ also show that p38γ increases MMP9 RNA expression whereas all of its mutants have much less effects (Fig. 2F), which more or less correlates with their regulatory effects on AP-1 and/or MMP9 transcriptional activity (supplemental Fig. S1, A and B, respectively). These results together indicate that p38γ requires both phosphorylation and the C terminus to stimulate AP-1-dependent MMP9 transcription.

**p38γ Binds the MMP9 Promoter through a Complex Formation with c-Jun—Both human (18) and rat (32) MMP9 promoters contain two AP-1 sites, and ChIP assays were next performed to explore whether p38γ binds the endogenous MMP9 promoter around this region using primers that span the functional distal AP-1 site (see Fig. 6C). Following formaldehyde-induced DNA cross-linking with associated proteins, stably transfected p38γ in IEC-6/K-Ras cells was isolated with a FLAG antibody, and the precipitates were subjected to PCR analysis, as described previously (16). As a control, a set of plates were processed for FLAG immunoprecipitation and WB analyses to explore whether p38γ may be recruited into the MMP9 promoter through interaction with c-Jun and/or c-Fos proteins. Results in Fig. 3A (top) show that among these sublines, only precipitates from WT p38γ-expressed cells contain the MMP9 promoter. Of interest, the immunoprecipitation/WB analyses (Fig. 3A, bottom) revealed that p38γ, but not its mutants, increases c-Jun protein expression that couples with its c-Jun binding activity, whereas c-Fos remains undetectable. Because the MMP9 promoter binding couples with the p38γ activity to increase c-Jun expression and bind c-Jun protein, these results suggest a scenario in

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**FIGURE 2. p38γ stimulates MMP9 via AP-1.** A–C, p38γ increases MMP9 transcription. Cells were transiently transfected with the indicated plasmids, and luciferase activities were determined 48 h later (*, \( p < 0.05 \) versus vector or no Tet control where Tet was present for total 72 h in the Tet group, 24 h before the transfection, and 48 h thereafter). D and E, p38γ activates MMP9. Cells were incubated with and without Tet for 48 h, RNAs were prepared for qRT-PCR, and levels of MMP9 and MMP2 RNAs were normalized to the ß-actin and expressed as relative over no Tet control (D, ***, \( p < 0.05 \) versus no Tet). To assess the MMP9 activity, Tet-on cells were changed to a serum-free medium for the last 24 h, and concentrated medium was assayed for MMP9 activity by zymography and MMP9 protein expression by WB (E, top) in which cell lysates were also analyzed for protein expression (E, bottom). F, stably transfected p38γ increases MMP9 RNA expression. RNA was prepared and subjected to qRT-PCR, and the ratio of MMP9/ß-actin was expressed as a fold change over the vector alone (*, \( p < 0.05 \) versus vector; **, \( p < 0.05 \) versus p38γ).
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Although c-Jun precipitates contain more c-Jun proteins in the absence of Tet, c-Jun alone, in the absence of Tet-induced p38γ expression, fails to bind the MMP9 promoter (Fig. 3B, top, lanes 6 and 7 versus bottom, lanes 1 and 2). In response to Tet, however, c-Jun binds both the p38γ protein and the MMP9 promoter, indicating a required role of p38γ in c-Jun binding to the MMP9 promoter. Moreover, the interdependent role of p38γ and c-Jun in the MMP9 promoter binding was further demonstrated by the ChIP-re-ChIP assay, and Tet-p38γ only activates MMP9 without increasing another AP-1 target gene vitamin D receptor expression (29) and without stimulating c-Jun and ATF-2 phosphorylation (Figs. 2 and 3, B–D). Our previous studies have demonstrated an increased p38γ protein expression by a p38α/p38β inhibitor SB203580 (SB) in K-Ras-transformed IEC-6 cells as a result of an antagonistic activity of p38α against p38γ (33). We explored next whether endogenous p38γ binds endogenous c-Jun and the MMP9 promoter in response to the SB treatment. Results in Fig. 3E show that there is a complex formation between p38γ and c-Jun, which is increased by SB treatment. Importantly, both endogenous c-Jun and p38γ bind to the MMP9 promoter, and the c-Jun binding activity is increased by the SB-induced p38γ/c-Jun up-regulation, further supporting the role of p38γ in c-Jun expression and its MMP9 binding activity. The SB treatment, however, failed to increase the p38γ-MMP9 binding, which may result from a general inhibitory activity of SB on MMP9 expression/activity (34, 35) and its effects on multiple AP-1 family members (36) and/or Raf pathways (37). These results together indicate a two-stage mechanism by which MMP9 is trans-activated by p38γ MAPK; p38γ first activates c-Jun by increasing its expression, and activated c-Jun then recruits p38γ into the MMP9 promoter through a complex formation.

**p38γ MAPK Both Stimulates de Novo c-Jun Synthesis and Acts as a Cofactor for c-Jun-trans-Activating MMP9**—Although p38 MAPKs are known to stimulate c-Jun promoter activity (30), so far there have been no reports about the p38-induced increase in c-Jun protein expression. c-Jun promoter contains AP-1 and myocyte-enhancing factor 2 sites that are

which p38γ first activates c-Jun by increasing its expression, and activated c-Jun then recruits p38γ onto the MMP9 promoter via a complex formation.

To demonstrate the c-Jun-mediated p38γ-MMP9 promoter binding further, Tet-on IEC-6 cells were next analyzed by ChIP and WB in which endogenous c-Jun was also purified and included as a positive control. Results in Fig. 3B show that in response to Tet addition, both p38γ and c-Jun precipitates contain the MMP9 promoter by ChIP and its partner by WB, and there is an increased c-Jun protein expression, further indicating that p38γ occupies the MMP9 promoter through interacting with c-Jun. Comparative analyses of c-Jun precipitates by ChIP and WB reveal another interesting phenomenon.
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FIGURE 4. p38γ stimulates c-Jun expression through a complex formation with c-Jun and plays an important role in c-Jun/MMP9 expression. A and B, p38γ increases c-Jun promoter activity and RNA expression by phosphorylation and C terminus-dependent mechanisms. IEC-6 cells were transiently transfected with indicated constructs, and luciferase activity was analyzed 48 h later (A). To measure c-Jun RNA expression, p38γ stably expressed IEC-6/K-Ras cells were subjected to qRT-PCR analysis, and the obtained signals were normalized to β-actin (B) (*, p < 0.05 for p38γ versus vector for both A and B; error bars, S.D.). C–E, p38γ plays a role in endogenous c-Jun and MMP9 expression. Immortalized p38γ+/+ and p38γ−/− cells were analyzed for protein expression by Western blotting and for c-Jun/MMP9 RNA expression by qRT-PCR (C). To reexpress p38γ and its mutants, p38γ−/− cells were infected with retroviruses and selected with antibiotics, and resistant cells were then subjected to Western blotting (D) and qRT-PCR analyses (E) (*, p < 0.05 versus respective control for C and E).

involved in its positive autoregulation by c-Jun/AP-1 (38) and/or its stimulation by p38 MAPKs (30). Experiments were then performed to examine whether p38γ up-regulates c-Jun transcription. In this regard, a c-Jun luciferase promoter (39) was transiently cotransfected with the indicated constructs in IEC-6 cells, and the luciferase activity was assessed. Results in Fig. 4A show that p38γ, but not its mutants or p38α, increases c-Jun promoter activity. Moreover, analyses of stable transfected IEC-6/K-Ras cells further showed that only WT p38γ increases c-Jun RNA expression (Fig. 4B). These results, together with the increased c-Jun protein expression (Fig. 3A), indicate that p38γ stimulates de novo c-Jun synthesis by phosphorylation and C terminus-dependent mechanisms as observed for the AP-1/MMP9 trans-activation (supplemental Fig. S1). To examine whether p38γ may additionally stabilize c-Jun proteins, Tet-p38γ cells were treated with a protein synthesis inhibitor cycloheximide and analyzed for protein expression. As illustrated in supplemental Fig. S2A, levels of expressed c-Jun proteins are decreased similarly after cycloheximide with and without Tet and become undetectable at 9 h despite the presence of p38γ protein. These results indicate that p38γ activates c-Jun primarily by stimulating its expression and not by increasing its protein stability and/or phosphorylation (a slight increase in p-c-Jun at 3 h is likely due to the stress insult from cycloheximide). The critical role of p38γ in c-Jun and MMP9 expression was further demonstrated in p38γ+/+ and p38γ−/− MEFs (10) in which p38γ knockout decreases c-Jun and MMP9 expression that was restored by reexpressing WT p38γ but not its mutants (Fig. 4, C–E). These results indicate a required role of p38γ in c-Jun synthesis that may be critical for its MMP9 trans-activating activity.

C-Jun has a well established AP-1 binding activity through dimerization with itself or another transcription factor, but no studies have reported thus far that a MAPK can bind the AP-1 element to regulate gene expression. Because c-Jun depends on p38γ to bind the MMP9 promoter (Fig. 3B), p38γ may act as a cofactor for c-Jun in binding the AP-1 element in the regulation of gene transcription. If this is the case, c-Jun activation alone, in the absence of p38γ, should not be active in transcription. To test this possibility, c-Jun was transiently transfected into p38γ+/+ and p38γ−/− MEFs together with the AP-1 reporter or the MMP9 promoter, and the luciferase activity was analyzed. Results in supplemental Fig. S2, B and C, show that c-Jun only stimulates AP-1/MMP9 transcription in p38γ+/+, but not in p38γ−/− cells. Conversely, p38γ transfection only increases the AP-1/MMP9 in c-Jun+/+ but not in its knock-out counterparts (15) despite similar levels of endogenous p38γ protein expression in both lines (33) (supplemental Fig. S2, D and E). These results together indicate that p38γ and c-Jun are both required for AP-1/MMP9 transcription, which thereby further
reinforces our conclusion that p38γ is an essential cofactor of c-Jun in stimulating MMP9 expression.

**p38γ Controls Endogenous c-Jun and MMP9 Expression in Human Colon Cancer and Stimulates MMP9-dependent Invasion**—To assess whether MMP9 activity is required for p38γ-induced invasion, Tet-p38γ-expressed cells were incubated with MMP9 inhibitors for 24 h and subjected to Matrigel invasion (A) and zymography (B) (*, p < 0.05 versus p38γ; **, p < 0.05 versus no Tet). Error bars, S.D. C and D, increased p38γ protein expression couples with elevated c-Jun/MMP9 expression in human colon cancer cells. Human colon cancer cells were analyzed by Western blotting for p38γ/c-Jun protein expression and by qRT-PCR for MMP9 RNA expression. Normalized MMP9 RNA levels are significantly higher in Ras-activated cells (**) than those without (*). E–H, p38γ controls endogenous c-Jun/MMP9 expression, and both p38γ and MMP9 are required for human colon cancer invasion. Human colon cancer cells were infected with lentiviral shLuc or shp38γ and subjected to Western blotting/zymography (E) and qRT-PCR (F) at 72 h and Matrigel invasion at 48 h (G). Effects of MMP9 inhibitors on invasion (H) were analyzed as in A (*, p < 0.05 versus shLuc or solvent control, F–H).

To demonstrate whether endogenous p38γ/c-Jun/MMP9 pathways intrinsically exist in human colon cancer, a group of colon cancer cell lines with known Ras status were examined for their protein expression. Results in Fig. 5C show that p38γ protein levels were increased in all three cell lines harboring Ras activations/mutations (HCT116, SW480, and LH147T) compared with those without (Caco-2, HT-29, and T48), indicating that activated Ras positively controls endogenous p38γ protein expression. Of great interest, p38γ protein expression levels were also significantly correlated with both c-Jun and MMP9 RNA expression in these cells (Fig. 5, C and D, and supplemental Fig. S3), suggesting an intrinsic p38γ/c-Jun/MMP9 pathway in human colon cancer. To demonstrate whether this pathway is functionally active, p38γ proteins were depleted by shRNA in HCT116 and SW480 cells, and its effects on c-Jun/MMP-9 expression as well as invasion were analyzed. Results in Fig. 5, E–H, show that silencing p38γ by two separate shRNAs reduces c-Jun and MMP9 expression/activity that couples with a decreased invasion in both lines in which an invasion-suppressive effect was also achieved by the pharmacolog-
ical MMP9 inhibition. These results together reveal an intrinsic p38\gamma/c-Jun/MMP9 pathway that is functionally active in stimulating colon cancer invasion.

We previously showed an increased p38\gamma expression in primary colon cancer tissues (11, 22). To demonstrate further whether hyperexpressed p38\gamma couples with an increased MMP9 expression in primary colon cancer tissues, a group of specimens were analyzed by immunohistochemistry for p38\gamma as well as MMP9 protein expression. As previously observed (42), the MMP9 immunoreactivity was detected in both normal and colon cancer tissues in contrast to the tumor-associated p38\gamma overexpression (brown color, Fig. 6A, top four panels). Interestingly, a substantial portion of strong MMP9 signals was inside lumens of the malignant but not normal tissues as previously described (42) (Fig. 6A, bottom two panels, indicated by filled and open arrows, respectively). Importantly, a high intensity of such MMP9 signals correlates with an increased p38\gamma protein expression in the malignant tissues (Fig. 6B). These results indicate that p38\gamma in the primary malignant tissues may also increase MMP9 expression, which may play a critical role in colon cancer progression.

DISCUSSION

MAPks function through transcription factors to convert transient regulatory signals into a sustained gene expression leading to a biological outcome. Despite extensive research, mechanisms by which a transcription factor translates a MAPK activity into gene expression remain unknown (43). Our studies show that p38\gamma activates c-Jun by increasing its expression, and the resulting c-Jun, in turn, acts as a vehicle to recruit p38\gamma into the MMP9 promoter, leading to increased MMP9 transcription and enhanced invasion. c-Jun alone, in the absence of p38\gamma, is not active in the MMP9 promoter binding, and both c-Jun and p38\gamma are required for MMP9 trans-activation, whereas p38\gamma depends on its c-Jun binding activity to bind the MMP9 promoter, to increase MMP9 transcription, and stimulate MMP9-dependent invasion. The active p38\gamma/c-Jun/MMP9 invasion pathway also was
demonstrated in Ras-activated human colon cancer, whereas hyperexpressed p38γ was shown to couple with an increased luminal MMP9 expression in primary cancer tissues. These results together reveal a novel mechanism by which p38γ MAPK acts both as a c-Jun activator and a c-Jun cofactor in the stimulation of MMP9 transcription leading to increased invasion (Fig. 6C). Because Ras-activated colon cancers are more metastatic (44) and MMP9 is a target antimetastatic therapy (14), p38γ may promote colon cancer progression through transduction of Ras signaling to MMP9 via the c-Jun-mediated promoter binding.

Previous studies showed that Hog1 in yeast (corresponding to mammalian p38α) is recruited to both promoter and coding regions of osmotic stress genes in stress response (45, 46). In mammalian cells, activated p38α similarly was shown to interact with chromatin during cell differentiation (47). In all of these studies, however, mechanisms for p38γ-DNA bindings as well as biological consequences remain unknown. Here, we show that the p38γ-MMP9 promoter binding first requires p38γ-induced c-Jun expression and then its interaction with c-Jun protein. The signaling specificity of this regulation is suggested by the fact that MMP9 is stimulated by p38γ but not by p38α, which is mediated by c-Jun (but not c-Fos or ATF2) via the AP-1 (but not NF-κB) site without significant effects on MMP2 or another AP-1 target gene vitamin D receptor expression. Although MMP9 may be one of the most documented AP-1 targets in cancer invasion and metastasis, it remains to be determined whether additional AP-1 target genes are involved in the p38γ invasive phenotype through the promoter binding.

It is important that p38γ was shown to activate c-Jun by increasing its expression, as up-regulated c-Jun protein expression has been observed in primary human colon cancer tissues (48) where it may be involved in colon cancer invasion (49). Because c-Jun is positively autoregulated by c-Jun/AP-1 (38) and p38γ increases c-Jun/AP-1/MMP9 transcription through its c-Jun binding activity, the same c-Jun-mediated promoter binding may likely operate in p38γ stimulating c-Jun as well as MMP9 transcription. With regard to the c-Jun-p38γ binding, there may be two mechanisms involved. c-Jun lacks a PDZ domain that is required for a direct interaction with a PDZ motif-containing protein such as p38γ. Therefore, the requirement of p38γ C terminus for its c-Jun binding suggests that both proteins may interact indirectly inside cells through additional PDZ proteins. On the other hand, phosphorylated p38γ is known to be localized predominantly in the nucleus (33), which may be critical for its interaction with nuclear c-Jun as a cofactor. Although these different scenarios require further study, the required role of the c-Jun binding activity in p38γ stimulating c-Jun synthesis, MMP9 transcription, and invasion highlights an essential role of this protein-complex in triggering the invasion cascade. c-Jun was previously shown to bind the MMP9 promoter in cardiac (50) but not in neuronal cells (51), and similarly we showed that c-Jun only binds the MMP9 in the presence of p38γ, indicating a determinant role of c-Jun cofactor abundances in its trans-activation of MMP9. Our results suggest that p38γ may be one of these critical cofactors to determine c-Jun transcriptional activity by increasing its expression and facilitating its target gene promoter binding.

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