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Nonsteroidal Anti-inflammatory Drugs Inhibit Matrix Metalloproteinase-2 via Suppression of the ERK/Sp1-mediated Transcription*

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Our previous data showed that nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit matrix metalloproteinase-2 (MMP-2) expression via repression of gene transcription in lung cancer cells. In this study, we investigate the molecular mechanism by which NSAIDs inhibit MMP-2. Promoter deletion and mutation analysis indicate that NSAIDs act via the Sp1 transcription factor binding site located between −91 and −84 in the MMP-2 promoter to suppress gene expression. Electrophoretic mobility shift assays show that Sp1 and Sp3 proteins constitutively bind to this consensus sequence and overexpression of Sp1 may enhance MMP-2 expression. NSAID treatment reduces Sp1 DNA binding activity and phosphorylation and attenuates MMP-2 expression. We also investigate the signaling pathway that mediates the effect of NSAIDs. Our results suggest that ERKs are involved in this process. First, NSAIDs suppress basal and serum-stimulated ERK activity. Second, a MEK inhibitor PD98059 inhibits MMP-2 promoter activity and Sp1 phosphorylation. Third, overexpression of constitutively active MEK1 stimulates Sp1 phosphorylation and MMP-2 promoter activity and antagonizes the inhibition of NSAIDs. Collectively, our data suggest that NSAIDs inhibit MMP-2 by blocking ERK/Sp1-mediated transcription.

The matrix metalloproteinases (MMPs)[1] are a family of zinc-dependent endopeptidases that can selectively degrade components of the extra cellular matrix (1). The MMP family consists of at least 20 enzymes and may be subgrouped into different types including collagenases, stromelysins, gelatinases, matrilysin, membrane-type MMPs, and metalloelastase, based on sequence characteristics and substrate specificity (2). MMPs are synthesized as inactive precursors and are activated by proteolytic cleavage (3–5). Additionally, naturally occurring tissue inhibitor of metalloproteinases can bind and suppress MMP function (6, 7). Therefore, MMP activity can be regulated at three steps: (*a*) gene expression, (*b*) proenzyme processing, and (*c*) inhibition of enzymatic activity.

Among the human MMPs reported previously, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) show substrate specificity toward type IV collagen, the major component of basement membrane, and their expressions are strongly linked with tumor metastasis in various types of human cancer (8–10). Recent work showed a positive correlation between MMP-2 expression and tumor metastasis in human lung cancer (11). In addition, overexpression of MMP-2 is associated with unfavorable outcome and tumor recurrence in early stage lung cancer (12, 13). The mechanisms of MMP-2 gene activation in human cancer cells are not well defined. Originally, the MMP-2 gene has been thought to be refractory to modulation due to a lack of potential regulatory elements in the MMP-2 promoter (14). However, a recent study demonstrated that the human MMP-2 promoter contains a number of *cis*-acting regulatory elements and that several transcription factors including Sp1, Sp3, and Ap-2 participate in the control of constitutive MMP-2 gene expression (15). In addition, expression of MMP-2 can be modulated by different signal transduction pathways. For example, activation of the AKT kinase signaling pathway may stimulate MMP-2 activity and tumor invasion (16), whereas activation of the phosphatase and tensin homologue PTEN, a dual specificity phosphatase that effectively attenuates AKT activity, may suppress MMP-2 expression and metastasis (17).

Recent studies showed that nonsteroidal anti-inflammatory drugs (NSAIDs) exert potent anti-angiogenesis and anti-metastasis activity both *in vitro* and *in vivo* (18). Our previous study demonstrated that A549 human lung cancer cells expressed a high level of MMP-2, and two NSAIDs, NS398 and indomethacin, inhibited MMP-2 expression and enzymatic activity in these cells (19). We also showed that these two NSAIDs suppressed MMP-2 via inhibition of gene transcription. In this study, we address the molecular mechanism by which NSAIDs inhibit MMP-2 expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Agents—A549 human lung cancer cells were cultured in Dulbecco's modified Eagle's medium and F-12 nutrition mixture supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics. NS398 and indomethacin were purchased from Biomol Co. (Plymouth Meeting, PA). Antibodies against human Sp1 and Sp3 were from obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-ERK and anti-phospho-ERK antibody were obtained from New England Biolabs (Beverly, MA). Anti-phosphoserine antibody was obtained from Sigma. LipofectAMINE reagent was from Invitrogen, and luciferase assay and β-galactosidase assay systems were from Promega (Madison, WI).

Plasmids—Full-length human MMP-2 promoter-luciferase construct and a series of deletion or mutant constructs were created as described previously (15) and were kindly provided by Dr. E. N. Benveniste. CMV-Sp1, a expression vector in which Sp1 expression is under the

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[1] The abbreviations used are: MMP, matrix metalloproteinase; NSAID, nonsteroidal anti-inflammatory drug; FCS, fetal calf serum; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

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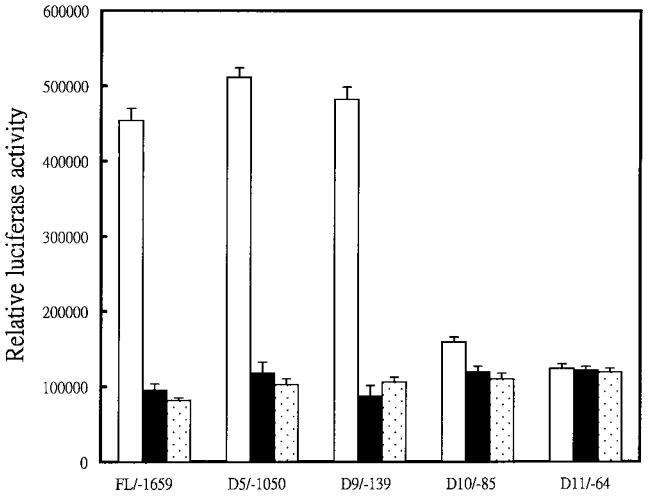

FIG. 1., **Effect of NSAIDs on human MMP-2 promoter activity.** A549 cells were co-transfected with 2 μg of different deletion MMP-2 promoter-luciferase constructs and 1 μg of pCMV-β-galactosidase plasmid. After transfection, cells were treated with vehicle (*open bar*), 100 μM NS398 (*filled bar*), or 100 μM indomethacin (*dotted bar*) for 24 h. Cells were harvested for the determination of luciferase and β-galactosidase activity. Relative luciferase activities (luciferase activity normalized with β-galactosidase activity) from three independent experiments are expressed as mean ± S.D.

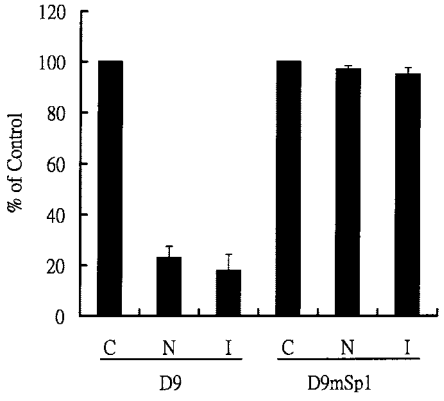

FIG. 2. **Mutation of the Sp1 site abolishes the inhibitory effect of NSAIDs on MMP-2 promoter activity.** Cells were transfected with D9 deletion construct of MMP-2 promoter or D9mSp1 deletion construct in which the Sp1 site located between −91 and −84 in the promoter was mutated. After transfection, cells were treated with vehicle (*C*) or 100 μM of NS398 (*N*) or indomethacin (*I*) for 24 h. Cells were harvested for the determination of luciferase and β-galactosidase activity. The activity of vehicle-treated cells is represented as 100%, and the activity of NS398- or indomethacin-treated cells is expressed as a percentage of the activity of vehicle-treated cells. Data are the mean ± S.D. from three independent experiments.

control of the CMV promoter, was kindly provided by Dr. S. T. Smale (20). The constitutively active MEK1 vector CMV-MEK1 (21) was a gift from Dr. M. Z. Lai.

Promoter Activity Assays—Activity of full-length or mutant MMP-2 promoter constructs was analyzed as described previously (22). In brief, cells were plated onto six-well plates at a density of 100,000 cells/well and grown overnight. Cells were co-transfected with 2 μg of MMP-2 promoter constructs and 1 μg of pCMV-β-galactosidase plasmid for 5 h by the LipofectAMINE method. After transfection, cells were cultured in 10% FCS medium with vehicle (Me_2SO) or drugs for 24 h. Luciferase and β-galactosidase activities were assayed by using the assay systems according to the procedures of the manufacturer (Promega). Luciferase activity was normalized for β-galactosidase activity in cell lysate and expressed as an average of three independent experiments. In some experiments, CMV-Sp1 or CMV-MEK1 was co-transfected with MMP-2 promoter constructs by using a similar transfection procedure.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)—Preparation of nuclear extracts from control or drug-treated cells were carried out as described previously (23). The oligonucleotide 5′-CAGAGAGGGGCGGCCCGAGTG-3′, corresponding to the human MMP-2 promoter sequence −97 to −76 was used as probe. The mutant Sp1 oligonucleotide has the sequence 5′-CAGATATCTAGATG-ATATCGTG-3′. The reaction mixture for EMSA contained 20 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 2 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1 μg of poly(dI-dC), and 8–10 μg of nuclear proteins. Unlabeled wild type or mutant oligonucleotides or preimmune, Sp1, or Sp3 antibody was added into reaction mixture and incubated for 10 min at room temperature. ^{32}P-Labeled probe DNA (300,000 cpm) was added, and the binding reaction was allowed to proceed for another 20 min. After reaction, mixtures were resolved on 6% polyacrylamide gels at 200 V for 2 h. Gels were dried and subjected to autoradiography.

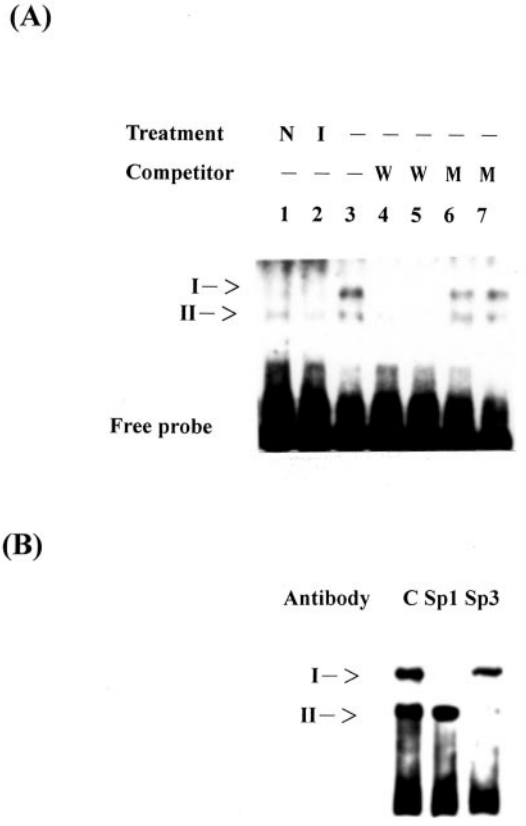

FIG. 3. **Analysis of the nuclear proteins that interact with the NSAID-responsive region in the MMP-2 promoter.** *A*, nuclear extracts from cells treated with vehicle (−) or NS398 (*N*) or indomethacin (*I*) were prepared as described under "Experimental Procedures." EMSAs were performed using a synthetic oligonucleotide corresponding to bases −76 through −97 of the wild-type human MMP-2 promoter as probe. The *arrows* indicate the specific complexes formed in EMSA experiments. Reaction mixtures were incubated with 50× (*lane 4* and *6*) or 100× (*lanes 5* and *7*) wild-type (*W*) or mutant (*M*) oligonucleotides to compete the binding between nuclear proteins and the radiolabeled probes. *B*, preimmune immunoglobulin (*C*) or human Sp1 or Sp3 antibodies were added to reaction mixtures, and EMSAs were performed to investigate the effect of antibody addition on the formation of protein-DNA complexes.

Immunoblotting and Immunoprecipitation—Cells cultured in 10% FCS medium were treated with vehicle or drugs for 24 h, washed with phosphate- buffered saline, and harvested in a lysis buffer as described previously (24). Protein concentration was measured by using a BCA protein assay kit (Pierce), and an equal amount of proteins was subjected to SDS-PAGE. Proteins were then transferred to nitrocellulose membranes, and Sp1 or Sp3 protein levels were examined by probing the membranes with different primary antibodies. Enhanced chemiluminescence reagents were used to depict the protein bands on the membranes. For immunoprecipitation, equal amounts of cell proteins prepared as described above were incubated with Sp1 or Sp3 antibody at 4 °C for 4 h, and immunocomplexes were collected by adding protein A/G-agarose beads for another 1 h. The immunoprecipitates were re-

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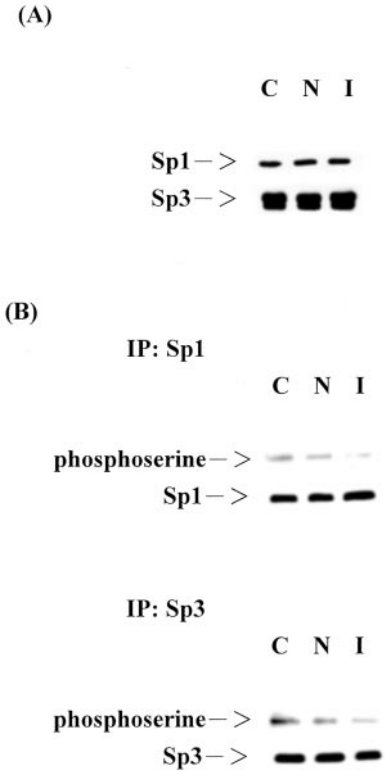

FIG. 4. **NSAIDs inhibit phosphorylation, but not protein level of Sp1 and Sp3.** *A*, cells were cultured in the vehicle (*C*) or NS398 (*N*) or indomethacin (*I*) for 24 h, and cellular proteins were harvested. Anti-Sp1 or -Sp3 antibodies were used to probe the nitrocellulose membranes and to investigate the intracellular level of these two proteins. *B*, cells were treated as described for *A*, and Sp1 or Sp3 proteins were immunoprecipitated (*IP*) by sequential incubation of specific antibody and protein A/G-agarose. The immunoprecipitates were subjected to SDS-PAGE and the membranes were probed with anti-phosphoserine antibody to investigate the phosphorylation status of Sp1 and Sp3. The membranes were also probed with anti-Sp1 or -Sp3 antibody to verify that similar amounts of Sp1 or Sp3 proteins were immunoprecipitated in the reactions.

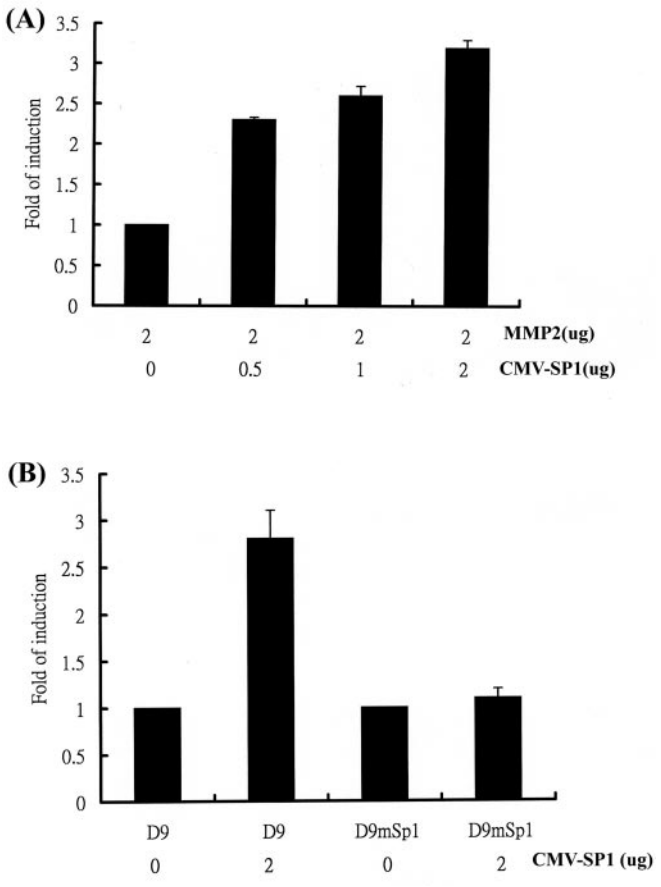

FIG. 5. **Overexpression of Sp1 stimulates MMP-2 promoter activity.** *A*, cells were co-transfected with 2 μg of full-length MMP-2 promoter construct and different amounts of CMV-Sp1 plasmid by using the LipofectAMINE method. After transfection, cells were cultured in 10% FCS medium for 24 h, and promoter activity was assayed as described in the legend to Fig. 1. Data are the mean ± S.D. from three independent experiments. *B*, D9 or D9mSp1 promoter constructs were co-transfected with different amounts of CMV-Sp1 plasmid, and luciferase activity was assayed.

solved by SDS-PAGE, and the membranes were probed with anti-phosphoserine antibody to investigate the phosphorylation status of Sp1 or Sp3 protein.

Assessment of ERK Activation—To test the effect of NSAIDs on serum- stimulated ERK activity, cells were first serum-starved for 24 h and treated with vehicle or drugs under serum-free conditions for another 24 h. Cells were then restimulated with 10% FCS for different times. After treatment, cellular proteins were extracted and were subjected to SDS-PAGE. ERK activation was investigated by probing the membranes with anti-phospho-ERK antibody. Equal loading of proteins in each lane was confirmed by probing the membranes with anti-ERK antibody. In some experiments, cells cultured under 10% FCS were incubated with vehicle or drugs for 24 h and harvested for analysis of the effect of NSAIDs on basal ERK activity in cells.

RESULTS

Identification of the NSAID-responsive Elements in the MMP-2 Promoter—To determinate the critical regions in the MMP-2 promoter responsible for the transcriptional inhibition by NSAIDs, a series of 5′-deletion mutants based on the 1659-bp MMP-2 promoter were transfected into A549 cells, and the effect of NS938 and indomethacin on the promoter activity was examined. Our previous data have shown that NS398 and indomethacin inhibited MMP-2 promoter activity in a concentration-dependent manner. In this study, we routinely used 100 μM of NS398 and indomethacin to treat cells. As shown in Fig. 1, NS398 and indomethacin at this concentration inhibited about 80% of MMP-2 promoter activity when compared with that of vehicle-treated cells. MMP-2 deletion constructs consisting of 1050 bp (D5) or 139 bp (D9) proximal to the transcription start site were fully responsive to NS398 and indomethacin. Conversely, the inhibitory effect of these two NSAIDs was abolished in the D10 (−85) and D11 (−64) deletion constructs. Our data indicated that the NSAID-responsive element is located between −139 and −85 in the MMP-2 promoter. We found that a potential Sp1 consensus sequence, GGGGCGGC, is located within this region (at −91 to −84). Therefore, we tested whether mutation of this Sp1 binding site (D9mSp1) might affect the responsiveness to NSAIDs. Indeed, NS398 and indomethacin could not inhibit promoter activity of the D9mSp1 construct (Fig. 2). These results suggest that NSAIDs inhibit MMP-2 expression via the Sp1 site located at −91 to −84 in the promoter.

Sp1 Binding Activity Is Suppressed by NSAIDs—To determine whether inhibition of MMP-2 promoter activity by NSAIDs is caused by changes in the interaction between nuclear proteins with the promoter, EMSA assays were performed with oligonucleotides corresponding to the region (−98 to −76) of the wild type promoter. Our results indicated that specific nuclear proteins constitutively bound to the synthetic oligonucleotides and two DNA-protein complexes (I and II) were detected in the autoradiogram (Fig. 3*A*). NSAID treatment significantly reduced the binding of nuclear proteins to the probes. Our data also showed that interaction of nuclear proteins with the probes is sequence-specific because the bind-

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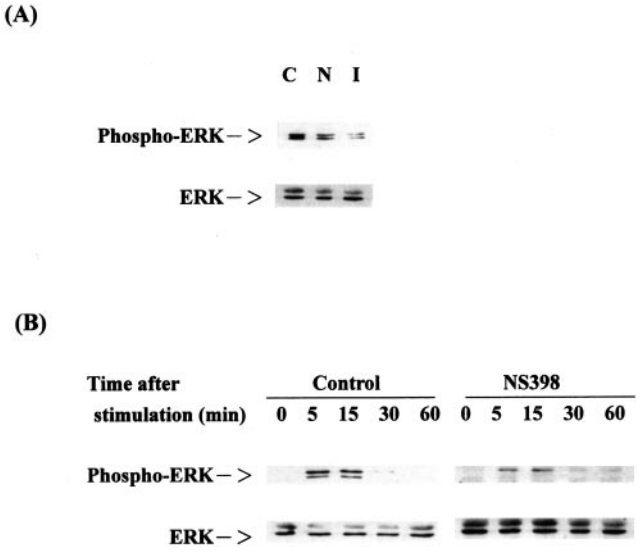

FIG. 6. **NSAIDs inhibit basal and serum-stimulated ERK activity.** *A*, cells were treated with vehicle (*C*), NS398 (*N*), or indomethacin (*I*) for 24 h, and cellular proteins were harvested. ERK activity was assayed by probing the membranes with anti-phospho-ERK antibody. Equal loading of proteins in each lane was confirmed by probing the membranes with anti-ERK antibody. *B*, cells were serum-starved for 24 h and treated with vehicle (*Control*) or NS398 for another 24 h. Cells were then restimulated with 10% FCS for different times, and cellular proteins were harvested for analysis of ERK activation as described above.

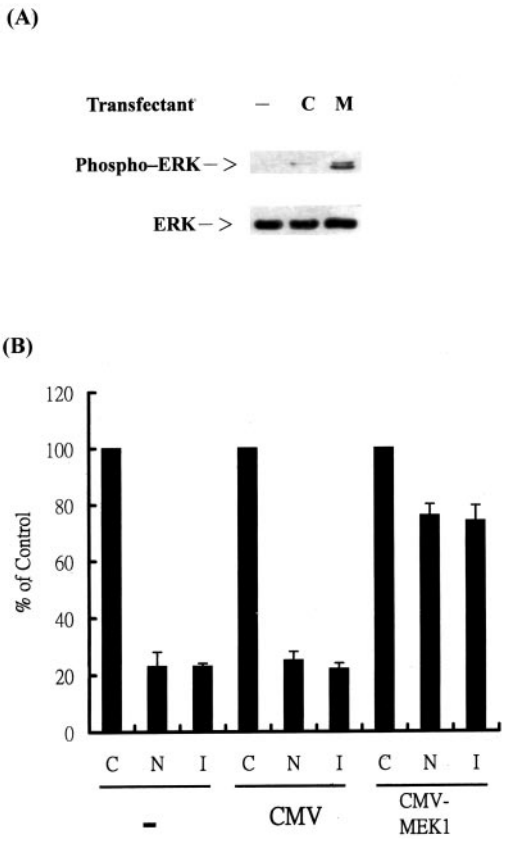

FIG. 7. **Enhancement of ERK activity counteracts NSAID-induced inhibition of MMP-2 promoter activity.** *A*, cells were co-transfected without (−) or with control vector (*C*) or MEK1 expression vector (*M*). After incubation with 10% FCS medium for 24 h, ERK activity in cell lysates was assayed by probing the membranes with anti-phospho-ERK antibody. *B*, cells were transfected as described above. After transfection, cells were cultured in 10% FCS medium for 24 h and incubated with vehicle (*C*) or NS398 (*N*) or indomethacin (*I*) for another 24 h. MMP-2 promoter activity was assayed. Data are the mean ± S.D. from three independent experiments.

ing could be competed away by an excess of unlabeled wild-type oligonucleotides but not by mutant oligonucleotides. We next tested whether the nuclear factors that bind to this region are Sp1-related proteins. As shown in Fig. 3*B*, the addition of Sp1 antibody attenuated the signal of complex I but did not affect the signal of complex II. On the contrary, Sp3 antibody inhibited the DNA-protein interaction of complex II but not complex I. The addition of preimmune antibody (Fig. 3*B*, *lane C*) had no effect on the formation of both complexes. These data suggest that Sp1 and Sp3 proteins constitutively bind to the Sp1 site located between bp −91 and −84 in the MMP-2 promoter, and this binding is suppressed by NSAIDs. We also investigated whether the attenuation of Sp1 and Sp3 binding caused by NSAID is due to reduction of Sp1 or Sp3 proteins. Control or drug-treated cells were harvested, and cellular proteins were extracted for immunoblotting. Our data showed that the protein level of Sp1 or Sp3 was not changed after treatment of NS398 and indomethacin (Fig. 4*A*). Therefore, NSAIDs modulate Sp1 binding activity via post-translational modification. Since Sp1 and Sp3 are phosphoproteins, we investigated whether NSAIDs might modulate the phosphorylation status of Sp1 and Sp3. Control or drug-treated cells were harvested, and Sp1 or Sp3 was immunoprecipitated by antibody and protein A/G-agarose beads. The immunoprecipitates were subjected to SDS-PAGE and probed with anti-phosphoserine antibody. As shown in Fig. 4*B*, phosphorylation of Sp1 and Sp3 was reduced after NSAID treatment.

Sp1 Functions as an Activator of the MMP-2 Promoter—To clarify whether Sp1 indeed activates MMP-2 promoter activity, different amounts of CMV-Sp1 expression vector were co-transfected with full-length, D9 or D9mSp1 MMP-2 promoter construct. As shown in Fig. 5*A*, CMV-Sp1 stimulated MMP-2 promoter activity in a dose-dependent manner. Similarly, CMV-Sp1 also activated the D9 promoter construct (Fig. 5*B*). Conversely, CMV-Sp1 could not significantly increase D9mSp1 promoter activity (Fig. 5*B*). Our results support the notion that Sp1 is a transactivator for the MMP-2 promoter.

NSAIDs Inhibit the ERK Signaling Pathway to Suppress MMP-2 Expression—We next studied the signaling pathway by which NSAIDs inhibit MMP-2 expression. Recent studies indicate that DNA binding activity of Sp1 can be regulated by phosphorylation. One of the protein kinases that may phosphorylate Sp1 and enhance its DNA binding activity is ERK2 (25). Previous works also demonstrated that various NSAIDs could affect the mitogen-activated protein kinase signaling pathways (26, 27). Therefore, we test whether NS398 can modulate ERK activity in A549 cells. Our results showed that NS398 and indomethacin inhibited basal ERK activity (Fig. 6*A*). Additionally, pretreatment of NS398 potently inhibited serum-stimulated ERK activation (Fig. 6*B*). Similar results were found in indomethacin-pretreated cells (data not shown).

If NSAIDs indeed suppress MMP-2 expression via inhibition of ERK activation, it is predictable that enhancement of ERK activity should be able to counteract the inhibitory effect of NSAIDs. To test this possibility, MMP-2 promoter construct was co-transfected with the CMV-MEK1 vector, which expressed the constitutively active MEK1 under control of the CMV promoter, and the effect of NSAIDs on the MMP-2 promoter activity was assayed. As shown in Fig. 7*A*, ERK activity was obviously increased in cells transfected with CMV-MEK1 vector but not control vector. We found that expression of active MEK1 indeed counteracted the inhibition

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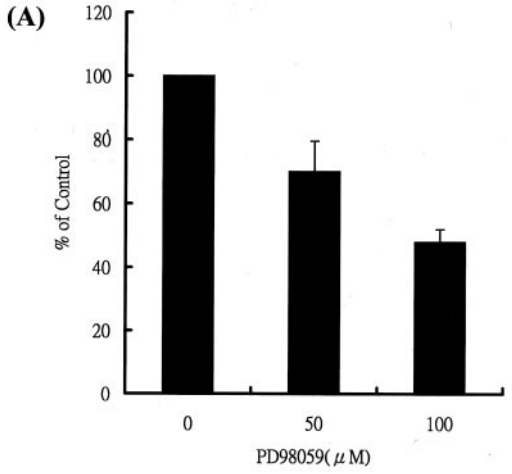


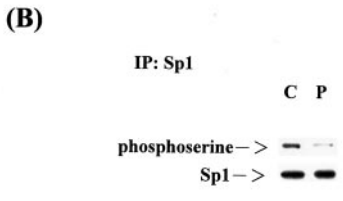
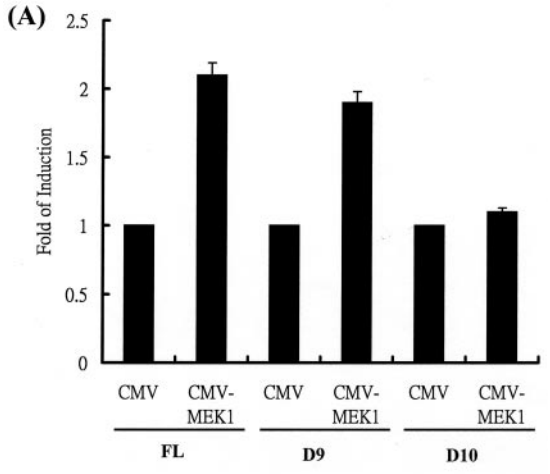


FIG. 8. **PD98059 suppresses MMP-2 promoter activity and Sp1 phosphorylation.** *A*, cells were transfected with 2 μg of MMP-2 promoter construct and were cultured in 10% FCS medium containing different concentrations of PD98059 for 24 h, and promoter activity was assayed. *B*, cells were incubated with vehicle (*C*) or 100 μM PD98059 (*P*) for 24 h and harvested for immunoprecipitation with anti-Sp1 antibody. The immunoprecipitates were subjected to SDS-PAGE and probed with anti-phosphoserine antibody to investigate the phosphorylation status of Sp1. The membranes were also probed with anti-Sp1 antibody to verify that similar amounts of Sp1 proteins were immunoprecipitated in the reactions.



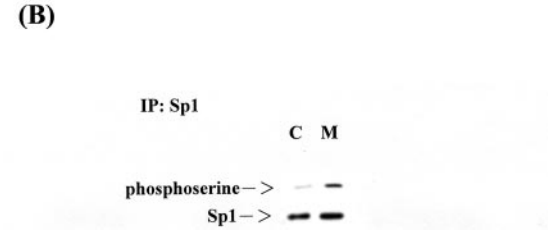


FIG. 9. **Effect of ERK activation on MMP-2 promoter activity and Sp1 phosphorylation.** *A*, cells were co-transfected with 2 μg of full-length (*FL*) MMP-2 promoter or the D9 or D10 deletion construct and with 2 μg of control or CMV-MEK1 vector. After transfection, cells were cultured in 10% FCS medium for 24 h and MMP-2 promoter activity was assayed. Data are the mean ± S.D. from three independent experiments. *B*, cells were transfected with control (*C*) or CMV-MEK1 (*M*) vector and were cultured in 10% FCS medium for 24 h. Cellular proteins were harvested for immunoprecipitation with anti-Sp1 antibody. The immunoprecipitates were subjected to SDS-PAGE and probed with anti-phosphoserine antibody to investigate Sp1 phosphorylation status. The membranes were also probed with anti-Sp1 antibody to verify that similar amounts of Sp1 proteins were immunoprecipitated in the reactions.

of MMP-2 by NSAIDs (Fig. 7*B*). These results suggest that NSAIDs inhibit MMP-2 via suppression of ERK activity.

ERK Activity Modulates MMP-2 Expression via the Sp1 Site—Our data showed that ERK and Sp1 are involved in the regulation of MMP-2. Therefore, we tested two hypotheses. First, modulation of ERK activity may affect MMP-2 expression in A549 cancer cells. We investigated the effect of a specific MEK inhibitor PD98059 on MMP-2 promoter activity, and we found that PD98059 inhibited MMP-2 promoter activity in a dose- dependent manner (Fig. 8*A*). Additionally, treatment of PD98059 reduced the phosphorylation of Sp1 in A549 cells (Fig. 8*B*). We next tested whether an increase of ERK activity by expression of CMV-MEK1 might enhance basal MMP-2 promoter activity. The full-length MMP-2 promoter construct was co-transfected with CMV-MEK1 or control vector into A549 cells. We found that a 2-fold increase of MMP-2 promoter activity was observed in cells transfected with CMV-MEK1 (Fig. 9*A*). Moreover, transfection of CMV-MEK1 increased Sp1 phosphorylation (Fig. 9*B*). Second, we investigated whether Sp1 is involved in ERK-induced MMP-2 expression. Our results indicated that CMV-MEK1 activated the D9 deletion construct but not the D10 deletion construct (Fig. 9*A*). CMV-Sp1 also could not activate the D9mSp1 promoter activity (data not shown). Collectively, our results suggest that the ERK signaling pathway regulates MMP-2 expression via the Sp1 site in the promoter region.

DISCUSSION

In the present study, we investigate the molecular mechanism by which NSAIDs inhibit MMP-2 expression, and our results show that suppression of MMP-2 by NSAIDs is mediated via the ERK/Sp1 signaling pathway. Several important findings of this work are discussed. First, recent studies have indicated that NSAIDs exhibit potent antiangiogenesis and antimetastasis activity both *in vitro* and *in vivo* (18, 28). However, the molecular mechanisms of these actions are largely unknown. Our results indicate that NSAIDs may directly suppress MMP-2 gene transcription. Additionally, inhibition of MMP-9, another MMP involved in metastasis, by NSAIDs has also been demonstrated recently (29). Based on these observations, we suggest that suppression of MMPs is one of the mechanisms by which NSAIDs inhibit angiogenesis and metastasis.

Second, we have clarified the mechanism by which NSAIDs inhibit MMP-2. Our results provide the first evidence that NSAIDs suppress MMP-2 expression via inhibition of the ERK/Sp1 signaling pathway. In accordance with our results, a recent study demonstrated that Sp1 and Sp3 are required for constitutive MMP-2 gene expression in human astroglioma cells and that the Sp1 site located between bp −91 and −84 in the promoter is critical for constitutive activity of the MMP-2 gene (15). Additionally, we verify that Sp1 is a crucial transactivator

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for MMP-2 gene expression, because overexpression of Sp1 stimulates MMP-2 promoter activity in A549 cells. Indeed, we also tested the effect of Sp1 and Sp3 on MMP-2 promoter activity in *Drosophila* SL2 cells lacking endogenous Sp factors. In accordance with the results of Qin *et al.* (15), we found that Sp1 and Sp3 are potent transactivators for MMP-2 (data not shown).

Recent studies indicate that DNA binding activity and transactivation activity of Sp1 can be regulated by post-translational modification (such as phosphorylation and glycosylation) (30, 31). Since our results showed that Sp1 DNA binding activity but not the protein level was attenuated after NSAID treatment, we speculated that NSAID may modulate a specific intracellular signaling pathway to affect Sp1 function. Our data support this speculation and suggest that the ERK signaling pathway is the target for NSAIDs. We found that block of MMP-2 expression by NSAIDs was correlated with inhibition of ERK activity and that overexpression of constitutively active MEK1 counteracted NSAID-induced inhibition of ERK activity and MMP-2 expression. However, it should be noted that enforced expression of active MEK1 cannot completely reverse the inhibitory effect of NSAIDs; it is possible that NSAIDs may affect other signaling pathways to regulate MMP-2 gene transcription.

An important issue that has been clarified in this study is how the ERK signaling pathway controls MMP-2 expression. Several works including ours have indicated that overexpression of constitutively active MEK1 can activate ERK activity and stimulate MMP-2 expression in transfected cells (32, 33). However, the mechanism by which ERK activates MMP-2 has not been elucidated. Our co-transfection experiments demonstrated that CMV-MEK1 could activate the D9 deletion construct. Conversely, the D10 deletion construct and D9mSp1 mutation construct were not stimulated by CMV-MEK1. In addition, we also demonstrated that modulation of ERK activity by PD98059 or CMV-MEK1 is correlated with the change of Sp1 phosphorylation in A549 lung cancer cells; our results strongly suggest that regulation of MMP-2 expression by the ERK signaling pathway is mediated via modulation of Sp1 DNA binding activity. A computer prediction shows that there are 7 and 10 potential ERK phosphorylation sites in Sp1 and Sp3 protein, respectively. We are now investigating the sites in which phosphorylation are increased by ERK activation and are suppressed by NSAID treatment.

ERK is a downstream effector of various signaling pathways. Genetic changes of different upstream signaling molecules such as mutation of K-*ras* or overexpression of Neu, which are frequently detected in human lung cancer cells (34, 35), may stimulate ERK activity and MMP-2 expression to increase angiogenic and metastatic potentials of cancer cells. Therefore, the ERK signaling pathway seems a rational target for prevention of tumor angiogenesis and metastasis. Collectively, results from this work indicate that the ERK/Sp1 signaling pathway controls the constitutive activity of the MMP-2 gene in lung cancer cells and NSAIDs suppress MMP-2 by inhibiting the ERK/Sp1-mediated transcription.

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