Activating Transcription Factor 2 Mediates Matrix Metalloproteinase-2 Transcriptional Activation Induced by p38 in Breast Epithelial Cells

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Abstract

Mounting evidence suggests a role for matrix metalloproteinase (MMP)-2 in the malignant progression of breast cancer cells. We showed previously that H-Ras, but not N-Ras, induced invasion of MCF10A human breast epithelial cells through Rac-MKK3/6-p38 pathway resulted in MMP-2 up-regulation. Activation of p38 pathway by MKK6 caused a selective up-regulation of MMP-2. In this study, we aimed to elucidate the transcriptional regulation of MMP-2 by p38 pathway leading to the invasive phenotype of MCF10A cells. By using 5’ deletion mutant constructs of MMP-2 promoter, we showed that deletion of the region containing activator protein-1 (AP-1) site caused the greatest reduction of MMP-2 promoter activity both in MKK6- and H-Ras-activated MCF10A cells, suggesting that the AP-1 binding site is critical for the MMP-2 promoter activation. DNA binding and transcriptional activities of AP-1 were increased by MKK6 or H-Ras as evidenced by electrophoretic mobility shift assay and luciferase assay using an AP-1-driven plasmid. By doing immunoinhibition assay and chromatin immunoprecipitation assay, we revealed the activating transcription factor (ATF) 2 as a transcription factor for MMP-2 gene expression through binding to the functional AP-1 site. Activation of ATF2, which depended on p38 activity, was crucial for MMP-2 promoter activity as well as induction of invasive and migrative phenotypes in MCF10A cells. This is the first report revealing ATF2-dependent MMP-2 promoter activity as well as induction of MMP-2 promoter activity by p38 pathway leading to malignant phenotypic changes in breast epithelial cells. (Cancer Res 2006; 66(21): 10487-96)

Introduction

Tumor invasion and metastasis are often associated with enhanced synthesis of matrix metalloproteinases (MMPs), among which MMP-2 (72-kDa type IV collagenase) and MMP-9 (92-kDa type IV collagenase) are of central importance (1–4). Mounting evidence from laboratories, including ours, suggests a role for MMP-2 in the invasion of breast cancer cells and the risk for a relapse in breast cancer patients (5–7). Activity of MMP-2 can be regulated by transcriptional gene activation, proenzyme activation, and inhibition of enzyme activity. The regulatory elements in the MMP-2 promoter have not been well characterized compared with those in MMP-9. It remains to be characterized whether the putative p53, activator protein-1 (AP-1), Ets-1, CAAT/enhancer binding protein, cAMP-responsive element binding protein (CREB), PEA3, Sp1, and activator protein-2 binding elements found in the promoter region of the MMP-2 gene (8–10) are functionally active.

Efforts have been made to determine the role of MMP-2 in the induction of invasion in Ras-transformed breast cells because Ras expression has been suggested as a marker of tumor aggressiveness in breast cancer (11–13). Using MCF10A human breast epithelial cells stably expressing active mutant (Gly512 to Asp512) of H-Ras and N-Ras, we showed previously that H-Ras, but not N-Ras, induced invasion and migration through up-regulation of MMP-2 rather than MMP-9 (5, 14). We further showed that H-Ras-specific activation of Rac-MKK3/6-p38 pathway resulted in MMP-2 up-regulation, which is a key step for induction of malignant phenotypic conversion of human breast epithelial cells (15). The present study was aimed to elucidate the transcriptional regulatory mechanism of MMP-2 by p38 pathway leading to malignant phenotypic changes in breast epithelial cells. Here, we report that the activating transcription factor (ATF) 2 is a key transcription factor for induction of MMP-2 transcriptional activation by the MKK3/6-p38 signaling pathway. Our results further provide a potential implication of ATF2-dependent MMP-2 gene regulation in malignant phenotypic changes of MCF10A human breast epithelial cells.

Materials and Methods

Cell culture. Establishment and culture condition of MCF10A and H-Ras MCF10A cells were described previously (5, 14). Stable transfectants (MKK6-1 and MKK6-2) of MCF10A expressing constitutively activated mutant of MKK6 were established as described previously (15) and cultured in MCF10A medium containing 400 μg/ml G418 (Life Technologies, Grand Island, NY).

Transfection. Transfection was done using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instruction. A dominant-negative (DN) mutant construct of p38, in which the dual phosphorylated motif, Thr-Gly-Tyr, was mutated to Ala-Gly-Phe (16), was provided by Dr. Surh (Seoul National University, Seoul, Korea). A DN ATF2 (A-ATF2) construct (17, 18) was a kind gift from Dr. Vinson at National Cancer Institute, NIH (Rockville, MD).

Immunoblot analysis. Immunoblot analysis was done as described previously (14). MMP-2 and MMP-9 antibodies were purchased from R&D systems (Minneapolis, MN) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. ATF2 and phosphorylated ATF2 antibodies were from Cell Signaling Technology (Beverly, MA).

Gelatin zymogram assay. Cells were cultured in serum-free DMEM/F-12 for 48 hours. Gelatinolytic activity of the conditioned medium was determined by gelatin zymogram assay as described previously (5). Areas of gelatinase activity were detected as clear bands against the blue-stained gelatin background.

Luciferase reporter assay. Luciferase and β-galactosidase activities were assayed using luciferase assay kit (Promega, Madison, WI) and Galacto-Light kit (Tropix Inc., Bedford, MA) and measured with a luminometer (Turner Research Article

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Designs, Sunnyvale, CA) as described previously (19). Briefly, cells were seeded in a six-well plate at $1 \times 10^5$ per well and transiently transfected with $4 \mu g$ promoter construct and $0.13 \mu g$ β-galactosidase expression plasmid pMDV-lacZ. The luciferase activity in $1 \mu g$ cell lysate was normalized to β-galactosidase activity. For MMP-2 and MMP-9 promoter assays, full-length human MMP-2 (9) and MMP-9 promoter-luciferase constructs (20) and a series of 5' deletion constructs of MMP-2 promoter (8), kindly provided by Dr. Benveniste (University of Alabama, Birmingham, AL), were used. For AP-1 transcriptional activity assay, the AP-1-driven plasmid pAP-1-Luc construct, which encodes luciferase under the control of heptamer AP-1 enhancer elements, seven tandem repeats of the AP-1 DNA binding sequence TGACTAA (Stratagene, La Jolla, CA), was used. A site-specific mutation (1270-TGACCTTCT-1263 → TGGATTTCT) was introduced to the AP-1 binding site in the MMP-2 promoter by site-directed mutagenesis kit (Stratagene) for the production of an AP-1 mutant construct (mAP-1).

Figure 1. MKK6 induces transcriptional activation of MMP-2, which requires AP-1 binding site. A, expressions of MMP-2 and MMP-9 were determined in vector-transfected MCF10A (vector), MKK6-1, MKK6-2, and H-Ras MCF10A (H-Ras) cell lines by immunoblot analyses of conditioned medium using antibodies against MMP-2 and MMP-9. Relative band intensities were determined by densitometry measurements. B, luciferase assays were done to detect promoter activities of MMP-2 and MMP-9 in MCF10A-1-2 and H-Ras MCF10A cells. The luciferase activity in $1 \mu g$ cell lysate was normalized to β-galactosidase activity. Data are representative of three independent experiments done in replicates. Columns, mean of triplicates; bars, SE. **, statistically different from vector-transfected cells at $P < 0.01$ by the two-tailed Student's t test. C, potential regulatory elements in the human MMP-2 promoter (8). D, effects of 5' deletion mutations on human MMP-2 promoter activity were examined. Cells were cotransfected with full-length or truncated MMP-2 promoter constructs and pMDV-lacZ galactosidase expression construct as indicated in Materials and Methods. Luciferase activity, determined in replicates, was normalized to β-galactosidase activity. Relative promoter activities of the deletion constructs compared with the full-length construct. Black columns, MMP-2 promoter activities of D3 and D4. Columns, mean of three independent experiments; bars, SE. Values indicate bp.
Role of ATF2 in p38-Induced MMP-2 Up-regulation

for control and Sp1 oligonucleotide (5'-ATTGCATCGGCGGAGGAG-3') were purchased from Santa Cruz Biotechnology. For competition experiments, nuclear extracts were incubated with molar excesses of unlabeled AP-1 or Sp1 oligonucleotide at 25°C for 30 minutes before addition of the labeled probe. For immunoinhibition analysis, the nuclear extracts were incubated with antibodies against ATF2 (Cell Signaling Technology), c-Jun, c-Fos, JunD, Fra1, or p53 (Santa Cruz Biotechnology) at 37°C for 30 minutes in binding buffer followed by an additional incubation for 30 minutes at room temperature with labeled oligonucleotide. The samples were subsequently subjected to electrophoresis on a polyacrylamide gel, dried, and visualized by autoradiography.

Chromatin immunoprecipitation assay. Chromatin Immunoprecipitation (ChIP) assay was carried out as described previously (21). One fourth of the chromatin solution was reserved for total input. The remaining solution was precleared with protein A-agarose, subsequently incubated with anti-phosphorylated ATF2 antibody for 12 hours at 4°C with shaking, and then further incubated with protein A-agarose for 2 hours. The immunoprecipitates were washed and reverse cross-linked. PCR was done on the phenol-chloroform-extracted DNA with specific primers of AP-1 site in the MMP-2 promoter [5'-ATCTCTGGGCACTTGTA-3' (sense) and 5'-TGTGACAAAGGCTCTGTA-3' (antisense)]. An amplified 275-bp PCR product was analyzed on a 1.5% agarose gel.

Inhibition of MMP-2. Small interfering RNA (siRNA) for MMP-2 was synthesized from Invitrogen. Specific oligonucleotide sequence targeting MMP-2 was 5'-AATACCATCGAGACCATGCGG-3' (sense; ref. 22). Stealth RNA interference (RNAi; Invitrogen) was used as a negative control. Cells were transfected with Stealth siRNA or siRNA directed against MMP-2 (100 pmol) using Lipofectamine 2000. Cis-9-octadeconoyl-N-hydroxylamide oeleoyl-N-hydroxylamide (OA-Hy) was purchased from Calbiochem (San Diego, CA).

In vitro invasion assay and Transwell migration assay. In vitro invasion assay and Transwell migration assay were done using a 24-well Transwell unit as described previously (14, 15).

Densitometry measurements. Relative band intensities were determined by quantification of each band with an image analyzer (Vilber Lourmat, Marne-la-Vallee Cedex 1, France).

Results

MKK6 up-regulates MMP-2 by transcriptional activation. We have shown previously that activation of MKK6 results in a marked induction of MMP-2 as evidenced by gelatin zymogram assay by using two stable transfectant MCF10A cell lines (MKK6-1 and MKK6-2), in which a constitutively active mutant of MKK6 was introduced (15). To further investigate the effect of MKK6-p38 pathway on MMP-2 expression, an immunoblot analysis was done. MMP-2 expression was prominently increased in MKK6-1 and MKK6-2 cells as well as in H-Ras MCF10A cells, whereas MMP-9 up-regulation was detected only in H-Ras MCF10A cells (Fig. 1A). The extent of MMP-2 up-regulation by MKK6 was comparable with that by H-Ras.

To investigate direct gene transcription as a potential mechanism for the MKK6-induced MMP-2 up-regulation observed, a promoter assay (luciferase reporter assay) was conducted. A significant activation of the MMP-2 promoter was detected in MKK6-1, MKK6-2, and H-Ras MCF10A cells (Fig. 1B). Increased activity of MMP-9 promoter was detected only in H-Ras MCF10A cells but not in MKK6-transfected cell lines. These results show that MKK6 induces up-regulation of MMP-2 by transcriptional activation, whereas it does not affect MMP-9 expression in MCF10A cells.

AP-1 binding site is critical for transcriptional activation of MMP-2 induced by MKK6 and H-Ras. In an attempt to identify a potential transcriptional element(s) in MMP-2 promoter responsible for transcriptional regulation by MKK6/p38 pathway, we did a promoter assay using a series 5’ deletion mutants (D2-D7 and D9-D12) based on the −1,659 bp MMP-2 promoter (Fig. 1C; ref. 8). Mutagenesis analysis of MMP-2 promoter showed that deletion of the region that contains a potential AP-1 site (D4, −1,259 bp) led to a dramatic decrease (~17% of D3) in promoter activity compared with D3 (~1,591 bp) in MKK6-1 cells (Fig. 1D). A significant reduction of MMP-2 promoter activity in −1,259-bp (D4) construct compared with −1,591 bp (D3) was also observed in MKK6-2 cells (~13% of D3) as well as in H-Ras MCF10A cells (~24% of D3). Our data showing a substantial decrease in MMP-2 promoter activity by the deletion of the AP-1 containing region located in between −1,591 and −1,259 bp show the significant role of the AP-1 site for MMP-2 gene transactivation by MKK6 and H-Ras.

MMP-2 promoter activities of D5, D6, and D7 mutants in MKK6-1 and MKK6-2 cells were increased, indicating that the region between −1,259 and −546 bp may contain potential repressor or suppressor element(s). A marked reduction was observed in −139 bp D9 compared with −546 bp D7 (45% of D7 in MKK6-1 cells and 39% of D7 in MKK6-2 cells), showing that the region of −546 to −139 bp, the potential binding sites for CREB, GCN-His, and PEA3 elements, might be also important for MMP-2 promoter activity.

Deletion mutagenesis analysis of H-Ras MCF10A cells showed that

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**Figure 2.** AP-1 binding site is critical for MMP-2 transcriptional activation induced by MKK6 and H-Ras. A, a site-specific mutagenesis (AC → CA) in the AP-1 binding site on human MMP-2 promoter (wtAP-1) was introduced, constructing a mutant AP-1 in MMP-2 promoter (mAP-1). Values indicate bp. B, cells were cotransfected with the wtAP-1 or mAP-1 construct and pMDV-lacZ galactosidase expression construct. Luciferase activity was normalized to β-galactosidase activity. Columns, mean of three independent experiments; bars, SE. *, **, statistically different from control at P < 0.05 and P < 0.01, respectively, by the two-tailed Student’s t test.
deletion of not only the AP-1 site (D4, −1,259 bp) but also the p53 site (D2, −1,611 bp) by ~24% of D3 and the first proximal Sp1 site (D10, −85 bp) by ~14% of D9 also reduced MMP-2 promoter activity. Taken together, promoter analysis using 5′ deletion mutant constructs of MMP-2 promoter showed that deletion of the AP-1 site comprising −1,591 to −1,259 bp resulted in the greatest reduction of MMP-2 promoter activity both in MKK6- and H-Ras-activated MCF10A cells, suggesting that the AP-1 binding site is important for the MMP-2 promoter activation induced by MKK6 and H-Ras in MCF10A cells.

To determine whether the AP-1 binding site is critical for transcriptional activation of MMP-2 induced by MKK6 and H-Ras, we introduced a site-directed mutagenesis (AC → GA) in the AP-1 binding site of MMP-2 promoter (9), thereby constructing a mutant AP-1 in MMP-2 promoter (mAP-1; Fig. 2A). Promoter activity of MMP-2 was significantly inhibited when mAP-1 was used in MKK6-1 (~56% of wild-type [WT]), MKK6-2 (~53% of WT), and H-Ras MCF10A (~57% of WT) cells (Fig. 2B). The data confirm the important role of the AP-1 binding site in MMP-2 promoter for transcriptional activation of MMP-2 induced by MKK6 and H-Ras.

MKK6 and H-Ras increase DNA binding activity and transcriptional activity of AP-1. We next determined the effect of MKK6 and H-Ras activation on AP-1 DNA binding activity by EMSA using a labeled oligonucleotide containing the AP-1 element.

Figure 3. ATF2 binds to the AP-1 site in MKK6-activated cells. A, top, nuclear extracts from the cells were subjected to EMSA for the activated AP-1 using a radiolabeled oligonucleotide containing the AP-1 binding site. An AP-1 mutant oligonucleotide was used as a control. The relative band intensity was determined by densitometry measurements. Bottom, for competition assay, nuclear extracts from the cells were preincubated with unlabeled oligonucleotide containing the AP-1 binding site as a cold competitor or nonspecific Sp1 binding site with an increasing concentration (50-, 100-, and 200-fold molar excesses) and subjected to EMSA for the activated AP-1. Nuclear extract (NE) without cold oligonucleotide as a control. B, cells were transfected with a luciferase reporter plasmid with a promoter containing a heptamer repeat of the AP-1 binding sequence (pAP-1-Luc). AP-1 luciferase activity was measured. Columns, mean of triplicates; bars, SE. **, statistically different from control at P < 0.01 by the two-tailed Student’s t test. C, nuclear extracts from the cells were analyzed by EMSA for the activated AP-1 with specific antibodies against ATF2, c-Fos, Fra1, c-Jun, JunD, and p53 for the immunoinhibition study. D, ChIP assay was done on the DNA-protein complexes prepared from vector, MKK6-1, MKK6, and H-Ras MCF10A cells using anti-pATF2 antibody or pre-IgG as a negative control. The samples were PCR-amplified using specific primers of AP-1 sites in the MMP-2 promoter (~1,385 to −1,110 bp). The PCR product of 275 bp was detected. One tenth (10%) of the total input was loaded as a control.
An increased AP-1 binding activity was observed in nuclear extracts of MKK6-1, MKK6-2, and H-Ras MCF10A cells compared with vector-transfected cells, suggesting the induction of DNA binding activity of AP-1 by MKK6 and H-Ras (Fig. 3A, top). Of note, a difference in gel mobility was observed between MKK6- and H-Ras-activated MCF10A cells, suggesting that different components might bind to the AP-1 site in MKK6-1/MKK6-2 and H-Ras MCF10A cells. An AP-1 mutant oligonucleotide was used as a negative control (Control). To ensure the specific binding, a competition study with unlabeled AP-1 oligonucleotide was conducted. A gradual reduction in band intensity was observed by increasing the concentration of cold competitor AP-1 oligonucleotide, whereas the nonspecific cold competitor Sp1 oligonucleotide did not compete for the protein binding, confirming the specificity of AP-1 DNA binding (Fig. 3A, bottom). The DNA binding activity of AP-1 was not detected in the vector-transfected cells.

To examine the functional activity of AP-1, we determined the AP-1 transcriptional activity by measuring the luciferase activity of cells transfected with a luciferase reporter gene under the transcriptional control of a promoter containing a heptamer repeat of the AP-1 binding sequence (pAP-1-Luc). A significant increase in the induction of AP-1 luciferase activity was observed in MKK6-1, MKK6-2, and H-Ras MCF10A cells compared with empty vector transfectants (Fig. 3B). The results show that MKK6 and H-Ras increase AP-1 transcriptional activity in MCF10A cells.

**ATF2 binds to AP-1 site in MKK6- and H-Ras-activated cells.** To address the identity and specificity of the binding protein to AP-1 site, an immunoinhibition study was conducted with specific

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**Figure 4.** Activation of ATF2 by MKK6 is dependent on p38. A, cells were analyzed by immunoblot analyses using antibodies against phosphorylated ATF2 (pATF2) and total ATF2 (ATF2). B, cells were treated with 50 μmol/L SB203580 for 30 minutes or transiently transfected with DN p38 and then analyzed by immunoblot analyses for pATF2 and ATF2. The immunoblots shown are representative of several blots from independent experiments. Band intensities of three blots were quantitated by densitometric measurements and the ratio of pATF2 to ATF2 were plotted. Columns, mean of triplicate; bars, SE.

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**Figure 5.** Activation of ATF2 is critical for MKK6-induced MMP-2 up-regulation, invasion, and migration. A, cells transiently transfected with DN ATF2 were subjected to immunoblot analyses for pATF2 and ATF2. B, gelatin zymogram assay was done on the cells transiently transfected with DN ATF2 to analyze gelatinolytic activities of secreted MMP-2 (72 kDa) and MMP-9 (92 kDa). C, MMP-2 promoter activity was measured in the cells transiently transfected with DN ATF2. The luciferase activity in 1 μl cell lysate was normalized to β-galactosidase activity. Data are representative of three independent experiments done in triplicates. Columns, mean of triplicates; bars, SE. D, the transfected cells with DN ATF2 were subjected to the in vitro invasion assay and the Transwell migration assay. The number of invaded or migrated cells per field was counted (× 400) in 13 arbitrary visual fields. Columns, mean of triplicates; bars, SE. *,**, statistically different from control at P < 0.05 and P < 0.01, respectively, by the two-tailed Student’s t test.
antibodies directed against AP-1 family members, including ATF2, c-Fos, Fra1, c-Jun, and JunD. p53 was used as a negative control. A marked depletion of the band was detected by addition of p38 antibody to nuclear extracts from MKK6-1 and MKK6-2 cells, whereas the band intensity was not altered by other antibodies (Fig. 3C). The data suggest that, among AP-1 family members, ATF2 binds to the AP-1 site in the nucleus of MKK6-activated cells. In H-Ras MCF10A cells, the band intensity was decreased by addition of not only ATF2 antibody but also c-Fos, Fra1, and c-Jun antibodies, indicating that different components bind to the AP-1 site of MKK6-1/MKK6-2 and H-Ras MCF10A cells.

To confirm the binding of ATF2 to the AP-1 binding site of MMP-2 promoter, we did a ChIP assay using an antibody directed against phosphorylated ATF2 in combination with primers amplifying AP-1 binding site in MMP-2 promoter region. As shown in Fig. 3D, the PCR product of 275 bp (−1,385 to −1,110 bp) was detected in the MKK6-1, MKK6-2, and H-Ras MCF10A cells, indicating that phosphorylated ATF2 directly bound to AP-1 site of MMP-2 promoter. No DNA amplification from the preimmune IgG antibody verified the specificity of phosphorylated ATF2 antibody. The data reveal ATF2 as a common transcription factor, which binds to the AP-1 site of MMP-2 promoter, in both MKK6- and H-Ras-activated cells. MKK6-induced ATF2 activation depends on p38. Because the transcription factor ATF2 has been known to be phosphorylated and activated by p38 in several cell types (16, 23, 24), it is probable that ATF2 is activated by MKK6/p38 and H-Ras in MCF10A cells. To test this issue, we detected phosphorylated ATF2 in the nuclei of MKK6- and H-Ras-activated cells. A marked induction of ATF2 phosphorylation was observed in MKK6-1, MKK6-2, and H-Ras MCF10A cells (Fig. 4A), indicating that the activation of ATF2 by MKK6/p38 and H-Ras in MCF10A cells.

To determine the role of p38 pathway in the activation of ATF2 in MKK6- and H-Ras-activated MCF10A cells, we treated the cells with SB203580, a specific inhibitor of p38. Treatment with 50 μmol/L SB203580 for 30 minutes at which the p38 pathway was inhibited in MCF10A cells as shown in our previous report (14) decreased the phosphorylated ATF2 in MKK6-1, MKK6-2, and H-Ras MCF10A cells (Fig. 4B). Interestingly, whereas phosphorylation of ATF2 was markedly abolished by SB203580 in MKK6-1 and MKK6-2 cells, the inhibition of ATF2 phosphorylation by SB203580 was less intensive in H-Ras MCF10A cells. The data suggest that H-Ras activates not only p38 but also other kinase(s), such as extracellular signal-regulated kinases (ERK), which can also phosphorylate ATF2. In consistent with the results obtained by using SB203580, inactivation of ATF2 by DN p38 was observed in MKK6-1 and MKK6-2 cells (Fig. 4B). Phosphorylated ATF2 was also decreased in H-Ras MCF10A cells transfected with DN p38, to a lesser extent compared with MKK6-activated cells. The results show that p38 is required for ATF2 activation induced by MKK6 or H-Ras.

ATF2 is critical for MKK6-induced MMP-2 transcriptional activation, invasion, and migration. To test this issue, we showed that the activation of MKK6/p38 pathway phosphorylated and activated ATF2, which then bound to the AP-1 site critical for MMP-2 gene in MCF10A cells. To test this, we investigated the functional role of ATF2 in MMP-2 expression by using a DN ATF2, which acts by inhibiting the DNA binding of basic leucine zipper protein in a dimerization-dependent fashion (17). The cells were transiently transfected with DN ATF2 and the inhibitory effect on ATF2 phosphorylation was confirmed (Fig. 5A).

We examined the effect of ATF2 inhibition on MMP-2 and MMP-9 expression by gelatin zymogram assay. DN ATF2 exerted a marked down-regulation of MMP-2 in MKK6-1 and MKK6-2 cells as well as in H-Ras MCF10A cells (Fig. 5B). ATF2 inhibition had little effect on MMP-9, indicating that ATF2 activation is required for MKK6-induced up-regulation of MMP-2 but not MMP-9. A

Figure 6. p38 is required for MMP-2 up-regulation, invasion, and migration. A, cells treated with 50 μmol/L SB203580 for 30 minutes or transiently transfected with DN p38 were subjected to immunoblot analyses for MMP-2 and MMP-9. The immunoblots shown are representative of several blots from independent experiments. Band intensities of three blots were quantitated and the relative band intensities of MMP-2 were plotted. Columns, mean of triplicates; bars, SE. B, cells treated with 50 μmol/L SB203580 for 30 minutes or the transfected cells with DN p38 were subjected to the in vitro invasion assay and Transwell migration assay.
promoter assay was then done to determine whether the role of ATF2 in the MKK6-induced MMP-2 up-regulation involves transcriptional regulation of the MMP-2 gene. DN ATF2 transfection significantly inhibited the MMP-2 promoter activity in MKK6-1, MKK6-2, and H-Ras MCF10A cells (Fig. 5C), showing that ATF2 is important for transcriptional activation of MMP-2 induced by MKK6 or H-Ras.

Based on the results obtained in this work and our previous finding that MKK6-activated p38 resulted in the induction of invasive and migrative phenotypes in MCF10A cells (15), ATF2 is expected to play a pivotal role in the MKK6-induced invasive/migrative phenotypes because MMP-2 is deeply involved in tumor cell invasion. To test this possibility, we determined the invasive and migrative properties of the cells transiently transfected with DN ATF2. Invasive and migrative abilities were significantly inhibited by DN ATF2 as evidenced by in vitro invasion and migration assays (Fig. 5D). Of note, MKK6-induced invasion and migration were prominently abolished by DN ATF2, whereas the invasive and migrative phenotypes of H-Ras MCF10A cells were affected by DN ATF2 to a lesser extent. DN ATF2 resulted in approximately 56% and 46% inhibition of invasion in MKK6-1 and MKK6-2 cells, respectively, whereas only 15% inhibition was

![Diagram](https://example.com/diagram.png)

**Figure 6** Continued. C, cells were transfected with control siRNA or MMP-2 siRNA (100 pmol) and analyzed by immunoblot analysis for MMP-2. The transfected cells were subjected to the in vitro invasion assay and Transwell migration assay. The number of invaded or migrated cells per field was counted (>400) in 13 arbitrary visual fields. Columns, mean of triplicates; bars, SE. *,**, statistically different from control at \( P < 0.05 \) and \( P < 0.01 \), respectively, by the two-tailed Student’s t test. D, a proposed mechanism for the H-Ras- and MKK3/6-specific induction of malignant phenotypic conversion in MCF10A human breast epithelial cells.
observed by DN ATF2 transfection in H-Ras MCF10A cells. Similar results were obtained from Transwell migration assay. Transfection with DN ATF2 caused ~44% and ~43% inhibition of migration in MKK6-1 and MKK6-2 cells, respectively, whereas it did 20% inhibition in H-Ras MCF10A cells. The results suggest that, unlike MKK6-1 and MKK6-2 cells, in which induction of invasive/migrative phenotypes depends much on MMP-2 up-regulation by ATF2, H-Ras-induced invasion/migration involves not only MMP-2 but other protein(s), including MMP-9. MKK6-1 and MKK6-2 cells transfected with DN ATF2 showed higher invasive/migrative phenotypes than the vector control cells, possibly due to the fact that the efficiency of transient transfection was not 100%, resulting in the incomplete inactivation of ATF2.

p38 pathway is crucial for MMP-2 up-regulation, invasion, and migration. We next examined the importance of p38 in MKK6-induced MMP-2 up-regulation by using SB203580 or DN p38. Inhibition of p38 pathway resulted in a selective reduction of MMP-2 expression without affecting MMP-9 in MKK6-1, MKK6-2, and H-Ras MCF10A cells (Fig. 6A). To investigate if the enhancing effect of MKK6 on invasion and migration was mediated by p38, in vitro invasion and migration assays were done. SB203580 and DN p38 significantly inhibited invasive and migrative abilities in MKK6- and H-Ras-activated MCF10A cells (Fig. 6B). Invasion and migration were not completely abolished by SB203580 or DN p38, possibly due, at least in part, to the incomplete inhibition of p38 activity by treatment with SB203580 or transient transfection with DN p38. These results show that MKK6-induced MMP-2 up-regulation and invasive/migrative phenotypes requires p38 activity.

To address the role of MMP-2 in MKK6-induced invasion/migration of MCF10A cells, we evaluated the invasion and migration of cells treated with MMP-2 siRNA or OA-Hy, a potent inhibitor of MMP-2 (25). Inhibition of MMP-2 expression by MMP-2 siRNA or OA-Hy resulted in a significant reduction in invasion and migration of MKK6- and H-Ras-activated MCF10A cells (Fig. 6C), showing that the induction of invasive phenotype by MKK6 was indeed mediated by MMP-2 in MCF10A cells.

Discussion

A direct correlation between high level of MMP-2 expression and an increased invasive capacity of tumor cell lines has been shown in vitro and in vivo (refs. 26–28, reviewed in ref. 29). Our previous studies also support the prominent role of MMP-2 in MCF10A human breast epithelial cell invasion (5, 14). To understand the molecular basis of MMP-2 up-regulation critical for the induction of the invasive phenotype of breast epithelial cells, we identified a cis-acting element and transcription factor responsible for MMP-2 gene expression induced by p38 signaling pathway. By using a series of 5′ MMP-2 genomic deletion constructs, we showed that the region between −1,591 and −1,259 bp, containing the AP-1 binding site, is critical for MMP-2 promoter activity (Fig. 1). The contribution of the AP-1 element to MMP-2 promoter activity was confirmed by site-directed mutagenesis analysis with a mutant mAP-1 (Fig. 2). In addition to the AP-1 site, CREB, GCN-His, and PEA3 binding elements were also suggested to be important for MMP-2 promoter activity induced by MKK6-p38 signaling. Our observations on H-Ras MCF10A cells showing alterations in MMP-2 promoter activity by the constructs lacking the p53 and Sp1 sites raised the possibility that MMP-2 gene transcription may also be regulated by other transcription factor(s) in H-Ras-activated MCF10A cells. Of note, the proximal Ets-1 site (−1,255 bp) may be an important cis-acting element because it has been known to play a critical role in cancer progression due to its ability to activate transcription of metastasis- and invasion-associated genes (30–32).

AP-1 DNA binding and transcriptional activities were significantly increased in MKK6-1, MKK6-2, and H-Ras MCF10A cells, in which MMP-2 was up-regulated, suggesting the involvement of AP-1 in p38-induced MMP-2 transcriptional activation. AP-1 has been shown to play a crucial role in tumorigenesis especially in breast cancer (33–36). Although MMP-2 and MMP-9 share structural and catalytic similarities, previous studies suggest that transcription of MMP-2 and MMP-9 may be independently regulated due to distinct arrays of cis-acting elements in the promoter. Whereas the human MMP-9 gene expression is regulated through the AP-1 site (37, 38), MMP-2 had been considered to be an AP-1-unresponsive gene in many cell types (e.g., astroglia cells; refs. 8, 39, 40). Recent studies on cardiac cells and breast cancer cells, however, indicate that a functional AP-1 site mediates MMP-2 transcription (10, 41). A plausible explanation for these contradictory results would be due to the cell type–specific regulation of the MMP-2 gene.

AP-1 represents a heterogenous set of dimeric proteins consisting of members of the Jun (c-Jun, JunD, and JunB), Fos (c-Fos, FosB, Fra1, and Fra2), Maf (c-Maf, MafB, MafA, MafG/F/K, and Nrl), and ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, and JDP2) subfamily members (reviewed in ref. 42). AP-1 dimers of different composition may execute differential promoter specificity and biological activity (43). A recent report shows that the AP-1 site interacts with JunB-Fra1 and JunB-FosB heterodimers to regulate MMP-2 transcription in cardiac cells (10). This study revealed ATF2 as a transcription factor responsible for the activation of MMP-2 gene expression in MCF10A human breast epithelial cells. ATF2 binds to its target promoter as a homodimer or a heterodimer with other AP-1 family members. Further studies remain to be done to elucidate the nature of AP-1 homodimer or heterodimer complex regulating MMP-2 gene transcription in MKK6-1, MKK6-2, and H-Ras MCF10A cells.

Using a chemical inhibitor and a DN mutant of p38, we showed that p38 signaling pathway is required for the activation of ATF2 in MKK6- and H-Ras-activated MCF10A cells. Based on our previous findings that H-Ras-activated Rac-MKK3/6-p38 pathway resulted in MMP-2 up-regulation, leading to invasive and migrative phenotypes of MCF10A cells (14, 15) and the findings obtained in this study, which revealed ATF2 as an essential transcription factor linking MKK3/6-p38 signaling pathway to MMP-2 up-regulation, we propose a model for a mechanism responsible for the H-Ras-and MKK3/6-specific induction of malignant phenotypic conversion in MCF10A human breast epithelial cells (Fig. 6D). It should be noted, however, that MMP-2 may not be the sole contributor to the induction of invasive phenotype by MKK6 because the cells treated with siRNA or OA-Hy still exhibited substantial invasive/migrative activities, although MMP-2 levels were severely attenuated (Fig. 6C). Tumor cell invasion is a complex process involving multiple gene products. This study shows that transcriptional activation of MMP-2 by ATF2 is a key step for MKK6-induced invasive activity of MCF10A human breast epithelial cells.

The MKK6-2 cells showed slightly higher level of phosphorylated ATF2 (Fig. 4A) as well as invasive/migrative abilities (Figs. 5D and 6B and C) than the MKK6-1 cells. The results are consistent with our previous finding that the MKK6-2 cells exhibited higher levels of phosphorylated MKK6 and p38 than the MKK6-1 cells (15). These two cell lines, however, exerted comparable effects on the MMP-2 promoter activity (Fig. 1) and DNA binding activity of AP-1
(Fig. 3A). Although the possibility that the promoter assay and EMSA were not sensitive enough to reveal the little difference between these cell lines cannot be ruled out, it remains to be investigated whether the p38 activity, ATF2 phosphorylation, MMP-2 up-regulation, and invasiveness are quantitatively correlated in MCF10A cell system.

Recently, ATF2 has been implicated in a transcriptional response leading to cell migration and malignant tumor progression (44, 45). The mechanism for the positive correlation of ATF2 and tumor metastasis, however, has not been well defined yet. This study revealed MMP-2 as a novel target gene of ATF2, suggesting a potential role of ATF2 in invasion and migration of breast epithelial cells. DN ATF2 transfection resulted in a marked down-regulation of MMP-2, whereas it had little effect on MMP-9 expression (Fig. 5B). Given that not only MMP-2 promoter but also MMP-9 promoter contains the AP-1 site where ATF2 can bind, these data suggest that the AP-1 site may not be a sufficient element for the induction of MMP-9 gene expression. Similarly, the AP-1 site was shown to be indispensable but not sufficient for the induction of MMP-9 gene expression and the cooperation with other cis-acting element(s), such as nuclear factor-xB or the Sp1 site was required (38).

Inhibition of ATF2 caused a significant reduction in invasion and migration of MKK6-1, MKK6-2, and H-Ras MCF10A cells (Fig. 5D), supporting the notion that ATF2 functions to promote malignant conversion of MCF10A human breast epithelial cells. The inhibitory effect of DN ATF2 on MKK6-induced invasion/migration was more dramatic compared with that on H-Ras-induced invasion/migration, in which not only ATF2-induced MMP-2 but also other protein(s), including MMP-9, might be involved. This is consistent with our previous observation (15) that MKK6/p38 up-regulation of MMP-2 alone can induce invasion and migration, but less effectively than H-Ras activation, suggesting that besides MKK6/p38 pathway, H-Ras-activated ERKs involving MMP-9 up-regulation may also be necessary for the completion of H-Ras-induced invasion and migration of human breast epithelial cells.

Phosphorylation of transcription factors is a major regulatory mechanism of gene expression in many cellular responses. ATF2 is a known nuclear target of p38 and c-Jun NH2-terminal kinase; it is phosphorylated at Thr69 and Thr71 residues by the mitogen-activated protein kinases (MAPK; refs. 16, 46, 47). Whereas Ras-Raf-MAPK/ERK kinase-ERK pathway triggers ATF2 Thr71 mono-phosphorylation, the Ras-Ral-Src-p38 pathway was shown to be required for dual phosphorylation at Thr69 and Thr71, suggesting that cooperative interaction between ERK and p38 pathways is essential for growth factor–induced ATF2 activation (48). A novel phosphorylation of ATF2 has been also detected at Thr73 and Ser62 by CaM kinase IV, protein kinase A and vaccinia-related kinase 1 (49). Our data show that blocking p38 signaling pathway did not completely inhibit phosphorylation of ATF2 in H-Ras-activated cells, whereas ATF2 phosphorylation in p38-activated cells (MKK6-1 and MKK6-2 cells) was almost completely abolished by the same treatment (Fig. 4B). Given that the antibody used in this study recognizes Thr71-phosphorylated ATF2, the remaining phosphorylated ATF2 in H-Ras MCF10A cells may be due to mono-phosphorylation of ATF2 at Thr71 residue by ERK pathway, which is activated in H-Ras MCF10A cells but not in MKK6-1 and MKK6-2 cells (15). It would be of interest to dissect the differential regulation of ATF2 by ERK and p38 pathways in these cells. Identification of the phosphorylation site of ATF2 and its binding domain with coregulator(s) deserves further investigation.

Taken together, the present study elucidated the transcriptional regulation of MMP-2 by p38 pathway leading to malignant phenotypic changes in human breast epithelial cells. Here, we show that p38 signaling activates the ATF2, which then activates MMP-2 transcription through binding to the functional AP-1 site. This is the first report revealing ATF2 as an essential transcription factor linking MKK3/6/p38 signaling pathway to MMP-2 up-regulation. Our results provide an evidence for a direct role of ATF2 activation in malignant phenotypic changes of MCF10A human breast epithelial cells.

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# Activating Transcription Factor 2 Mediates Matrix Metalloproteinase-2 Transcriptional Activation Induced by p38 in Breast Epithelial Cells

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