Redox-dependent Matrix Metalloproteinase-1 Expression Is Regulated by JNK through Ets and AP-1 Promoter Motifs*

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The matrix metalloproteinase (MMP) family consists of at least 25 zinc-dependent proteases that degrade multiple components of the extracellular matrix (ECM) (1). Under normal physiological conditions MMPs are important for development and wound healing; however, their augmented expression is associated with numerous disease pathologies (2). For example, MMPs are important at many stages of metastasis and the high level expression of the MMP family member, MMP-1, has been linked to poor prognosis in many types of cancers (3–9).

Within the MMP-1 promoter, Brinckerhoff and co-workers (10) identified a single nucleotide polymorphism (SNP) guanine insertion at −1607 (1G → 2G) base pairs that enhances the basal rate of transcription by creation of an Ets binding domain. Ets transcription factors include a large family of helix-turn-helix proteins (11) that normally do not bind DNA alone, but preferentially form coactivator complexes with transcription factors, like activator protein-1 (AP-1) (12). The proto-oncoproteins Fos and Jun, which comprise the AP-1 complex, can homo- or heterodimerize and bind its cognate consensus sequence (TGACTCA) in the regulatory domains of many genes including various MMP family members (13). Both Ets and AP-1 play a critical role in regulating the expression of various MMP family members, particularly that of MMP-1 (14). The MMP-1 SNP is associated with a higher risk of metastasis in patients with a variety of distinct cancers (15–19).

More striking is the finding that both Ets-1 and MMP-1 immunoreactivity is high in stromal tissue adjacent to the leading edge of several tumor types (12).

The two-electron reduction product of oxygen, H2O2, has emerged as a potent signaling molecule and can modulate MMP expression and activity (20). H2O2-sensitive signaling molecules ERK1/2 and JNK are important in regulating MMP-1 expression (12, 21, 22). Furthermore, numerous reports indicate that transcription factors important for MMP-1 expression are also redox-sensitive (23, 24). Jun-N-terminal kinase (JNK) phosphorylates and activates AP-1 members including c-Jun and JunB (25) whereas c-Jun, c-Fos, FosB, Fra-1, and Ets family member, Ets-1, are sensitive to ERK activation (25). Because both AP-1 and Ets transcription factors are subject to regulation by JNK and/or ERK and are redox-regulated, we sought to determine the role these factors play in the H2O2-dependent regulation of MMP-1.

Using well characterized redox-engineered HT-1080 fibrosarcoma cell lines (26) this study demonstrates that the proximal Ets and AP-1 binding sites in the MMP-1 promoter are required for maximal H2O2-dependent expression. We also established that JNK confers redox sensitivity to the MMP-1 promoter whereas both ERK and/or JNK are

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* The abbreviations used are: MMP, matrix metalloproteinase; JNK, c-Jun N-terminal kinase; ERK1/2, extracellular signal-regulated kinase 1/2; SNP, single nucleotide polymorphism; NAC, N-acetyl-cysteine; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation assay; MAPK, mitogen-activated protein kinase; PTP, protein tyrosine phosphatase; ROS, reactive oxygen species; CMV, cytomegalovirus.
required for maximal basal promoter activity and the expression of AP-1 and Ets-1. Lastly, both c-Jun and Ets-1 and the histone acetyltransferase, p300 are recruited to the region of the MMP-1 SNP in response to alterations in the steady state production of H$_2$O$_2$. Thus, H$_2$O$_2$ plays an important role in regulating chromatin remodeling events that lead to optimal MMP-1 transcriptional activity. These findings indicate that diseases which are associated with augmented MMP-1 production may be amenable to targeted antioxidant based therapeutic intervention.

**MATERIALS AND METHODS**

**Cell Culture and Reagents**—All indicated cell lines were maintained in 25-cm$^2$ flasks in MEM containing 10% fetal calf serum, 1000 units/ml penicillin, 500 µg/ml streptomycin, and 1 mg/ml neomycin in a 37 °C incubator containing 5% CO$_2$. Constructions of the recombinant Sod2—An active site mutant form of human MnSOD (H26L) (abbreviated mutSod2) was generated by the Dr. Larry W. Oberley laboratory at the University of Iowa (Iowa City, IA), and shown to have 70% less activity compared with wild-type human MnSOD (30).—The full-length human MMP-1 promoter/luciferase reporter vector (Promega, Madison, WI) and were kindly provided by Dr. Constance Brinckerhoff (Dartmouth University). Transfection and analysis of MMP-1 promoter luciferase activity have been described in detail by Nelson (29). Treatments with MAPK inhibitors, PD98059 (50 µM), U0126 (50 µM), U0124 (50 µM), and SP600125 (10 µM) were performed 4-h post-transfection for 18 h following removal and addition of complete medium.

**Construction of Mutant AP-1, Sod2, and Scrambled 1G MMP-1 Promoter Constructs**—The full-length human MMP-1 promoter/luciferase reporter plasmids (1G and 2G) contained the firefly luciferase gene under the transcriptional control of the human MMP-1 promoter in a pGL3 basic reporter vector (Promega, Madison, WI) and were kindly provided by Dr. Constance Brinckerhoff (Dartmouth University). Transfection and analysis of MMP-1 promoter luciferase activity have been described in detail by Nelson et al. (29). Treatments with MAPK inhibitors, PD98059 (50 µM), SB203580 (20 µM), U0126 (50 µM), U0124 (50 µM), and SP600125 (10 µM) were performed 4-h post-transfection for 18 h following removal and addition of complete medium.

**Construction of Mutant AP-1, Sod2, and Scrambled 1G MMP-1 Promoter Constructs**—The site mutant form of human MnSOD (H26L) (abbreviated mutSod2) was generated by the Dr. Larry W. Oberley laboratory at the University of Iowa (Iowa City, IA), and shown to have ~70% less activity compared with wild-type human MnSOD (30). The mutant 1G and AP-1 promoter constructs were prepared by changing the wild-type 1G from 5'-AAACACTGGC-3' to the form 5'-AAACCTAGCTTTATATCACA-3' (m1G) or to the mutant AP-1 and m1G form 5'-AAACCTAGCTTTATATCACA-3' (m1G mAP). An AatII and EcoRV digested fragment. The respective sense and antisense primer pairs used for mutant construction are described below: AatII: sense, 5'-GATAGTACTTTACT...3'; 5'-GATAGTACTTTACT...3'; m1G 5'-...TAGAAAGATATGGA...3'; m1G 5'-...TAGAAAGATATGGA...3'; EcoRV: antisense, 5'-CAGTGTGAGAAGACCTGGCG-3'; 1G: scrambled, 5'-AATAATTTAGAAACATTAG-CCAT-3', 5'-AATAATTTAGAAACATTAG-CCAT-3', 5'-AATAATTTAGAAACATTAG-CCAT-3', 5'-AATAATTTAGAAACATTAG-CCAT-3'. All mutants were confirmed by DNA sequencing.

**Immunoblotting**—Medica from confluent cells were analyzed for MMP-1 by Western blotting. Complete media from the cells were normalized to cell count and incubated overnight at 4 °C with 50 µl of heparin-Sepharose beads (Amersham Biosciences). The beads were centrifuged at 1,000 rpm for 5 min and boiled for 5 min in Hanks Buffer Salt Solution, followed by the addition of 5× loading dye containing 5% 2-mercaptoethanol. Eluates were then analyzed on a 10% SDS-PAGE followed by Western immunoblotting using monoclonal MMP-1 antibody (R&D Systems) at 1:400 in Tris-buffered saline containing 0.1% Tween 20 and 5% milk followed by incubation with a horseradish peroxidase-conjugated anti-mouse secondary antibody to a 1:4000 dilution for 1 h at 25 °C (Amersham Biosciences). Detection of the proteins was performed by the addition of Pierce SuperSignal Chemiluminescent Substrate for 5 min and exposure to Kodak MS radiographic film (Kodak, Rochester, NY). JNK immunoblotting was performed on cell lines grown to 90% confluence and washed three times with ice-cold 1× PBS. Nuclear preparation was performed for phosphospecific JNK, whereas whole cell lysates were used for total JNK Western blots. For total cell lysates, cells were resuspended in 1× PBS/EDTA and sonicated. Protein concentrations were determined using the BCA protein assay according to the manufacturer’s instructions (Pierce). 30 µg of protein was resolved on 10% SDS-PAGE and immunoblotted as described above. Blots were incubated with a rabbit polyclonal phosphospecific JNK antibody (BIOSOURCE, Camarillo, CA) at 1:1000 in TTBS containing 5% milk followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences) at 1:100,000 for 1 h at room temperature. Immunoreactive JNK was visualized as described above. The immobilobLOTS were stripped and reprobed using rabbit polyclonal antibody (BIOSOURCE) that recognizes JNK regardless of its phosphorylation state at a 1:1000 dilution overnight. The blot was washed and subsequently incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibody (Amersham Biosciences) at 1:100,000, and immunoreactive protein was detected as described above. Fra-1, c-Jun, c-Fos, and Ets-1 were analyzed by Western blotting with either rabbit anti-human Fra-1, Ser$^3$, c-Jun, Ser$^3$, c-Jun, c-Fos monoclonal (broad or specific), or Ets-1 polyclonal antibodies, (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 dilution. Blots were then incubated with the appropriate secondary horseradish peroxidase-conjugated antibodies (1:5000) and developed as described above. Phospho-c-Jun blots were performed using nuclear lysates.

**In-gel Phosphatase Assay**—In-gel phosphatase assays and phosphotyrosine immunoprecipitations were performed essentially as described by Meng et al. (31).

**Chromatin Immunoprecipitation (ChIP)**—Confluent cells lines were treated with 37% formaldehyde to cross-link proteins to DNA for 10 min at 37 °C. 0.33 ml of 1.25 M glycine was added to the flask to stop the reaction. The cells were washed twice with cold 1× PBS containing 1 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The cells were scraped off in this buffer and pelleted at 2000 rpm for 4 min at 4 °C. 200 µl of warm SDS lysis buffer containing protease inhibitors was added and incubated for 10 min on ice. Cells were sonicated to generate ~500-bp DNA fragments. Before adding antibody, 25 µl of lysate was saved as the input sample. Either 2 µl of Ets-1 (Santa Cruz Biotechnology) or c-Jun (Cell Signaling, Beverly, MA) antibody was added to the lysate with cold 1× PBS containing protease

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**Redox Control of MMP-1 Expression via JNK, Ets, and AP-1**

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Redox Control of MMP-1 Expression via JNK, Ets, and AP-1

FIGURE 1. Ets-1 and AP-1 sites important for Sod2-dependent MMP-1 promoter activity. A, regulatory elements of the human MMP-1 promoter region are illustrated. AP-1, activator protein-1; ETS, E 26; C/EBP, CCAAT/enhancer-binding protein-B. The full-length MMP-1 promoter contains a single guanine at −1607. A SNP of an additional guanine at −1607 creates an ETS binding site 5′-GGA-3′ near an AP-1 site at −1602. The distinct MMP-1 promoter luciferase variants are indicated, and mutations are indicated in bold lettering. B, CMV and Sod2 fibrosarcoma cells were transiently transfected with wild-type pGL3-MMP-1(1G or 2G) promoter constructs or such constructs containing a mutation in the AP-1 site mutated −1602 (mAP1), pGL3-MMP-1(1G) mutated −1607 1G (m1G), or C, with a double mutation in both the 1G and AP-1 sites (m1GmAP1) pGL3-MMP-1 promoter constructs. The cells were lysed 18 h post-transfection, and the luciferase reporter activity was determined using the Promega assay system. Protein in the lysates was quantified by Coomassie reagent (Pierce). Data were normalized to protein concentration as well as β-galactosidase activity and are reported in relative luciferase units (RLU). Error bars represent ± S.E. and significance from the HT15 2G-transfected cell lines. * p < 0.05, compared with respective CMV- or HT15 1G-transfected cells.

inhibitors and rocked overnight at 4 °C. The following day, 40 μl of protein A-agarose beads per sample (Santa Cruz Biotechnology) were washed three times with 1× PBS and twice with SDS lysis buffer, and beads were resuspended in lysis buffer containing protease inhibitors.

40 μl of beads was added to samples and incubated at 4 °C for 2 h with gentle agitation. Beads were washed twice with lysis buffer containing protease inhibitors, once with lysis buffer and 500 mM NaCl, once with 10 mM Tris-HCl, pH 8.0, 0.5 mM LiCl, 0.5% IPEGAL, 0.5% sodium deoxycholate, 1 mM EDTA without protease inhibitors, and rocked overnight at 4 °C. The following day, 40 μl of beads was added to samples and incubated at 4 °C for 2 h with gentle agitation. Beads were washed twice with lysis buffer containing protease inhibitors, once with lysis buffer and 500 mM NaCl, once with 10 mM Tris-HCl, pH 8.0, 0.5 mM LiCl, 0.5% IPEGAL, 0.5% sodium deoxycholate, 1 mM EDTA without protease inhibitors, and once with TE, pH 8.0. The precipitate was eluted from beads with 100 μl of glycogen, and 100 μl of proteinase K were added and incubated for 2 h at 37 °C. Beads were pelleted for a few seconds at 14,000 rpm, and supernatant was transferred to a new tube. The eluates and input samples were incubated at 65 °C overnight to reverse cross-links. The following day, 250 μl of TE, 5 μg of glycogen, and 100 μg of proteinase K were added and incubated for 2 h at 37 °C. 55 μl of 4 mM LiCl were added to each sample, and DNA was extracted using a standard phenol-chloroform method. The extracted DNA was kept in TE, pH 8.0 and subjected to PCR using MMP-1-specific primers to amplify the region between −1523 and −1978 of the MMP-1 promoter.

Real Time ChIP PCR—One microliter of ChIP-derived DNA was used as template in a 20-μl reaction containing 10 μl of 2× SYBR Green Master Mix (Bio-Rad). Real time thermal cycling was performed using a MyiQ Cycler thermal cycler (Bio-Rad), with continuous SYBR Green monitoring according to the manufacturer’s recommendations, using iCycler software. All PCR reactions were performed in duplicate and included negative controls (no DNA). Pre-IP and post-IP DNA samples were amplified using previously described parameters. Ct values were obtained after completion of reaction, which is the cycle at which fluorescence rises above a baseline threshold. The DNA units in the samples were calculated as 2−Ct Input × 1000.

Native Sod PAGE—PAGE was performed as described in detail by Rodriguez et al. (26).

Statistical Analysis—Analysis of variance with α = 0.05 was used for processing the data. Two sample Student’s t test was used as the post test. Values are expressed as means ± S.E. of the respective test or control group. Data are representative of at least three independent experiments.

RESULTS
Identification of Redox-responsive MMP-1 Promoter Elements—We have previously reported that H2O2 enhances the activity of MMP-1 promoter containing the 2G SNP (29, 32). Analysis of the full-length promoter containing the 2G polymorphism indicated that the region between −1702 and −1602 is required for optimal activation of the MMP-1 promoter by H2O2. Within this 100-bp region, there are many Ets and AP-1 binding sites including the Ets site created by the SNP at position −1607 and a very proximal AP-1 site at −1602 within the
MMP-1 promoter. Given the importance of the 2G SNP in MMP-1 promoter activity and that of AP-1 in MMP-1 regulation, the impact of mutations of these sites was assessed. The activities of the full-length 1G and 2G MMP-1 promoter constructs were increased in response to the mitochondrial production of H$_2$O$_2$ in cells overexpressing Sod2 (Fig. 1A). Mutating the AP-1 site in the context of the 1G (1GmAP) or 2G (2GmAP) promoter decreased MMP-1 promoter activity. Mutation of the 1G region (m1G) or both the 1G and AP-1 sites (m1GmAP1) further decreased MMP-1 promoter activity (Fig. 1B). Thus, both the 1G region and the AP-1 binding sites are required for activation of the MMP-1 promoter both basally and in response to alterations in the steady state production of H$_2$O$_2$.

H$_2$O$_2$ Regulates AP-1 and Ets-1 Expression—The levels of AP-1 factors play an important role in regulating the expression of AP-1 responsive genes. To define whether intracellular H$_2$O$_2$ affects the expression of transcription factors that control MMP-1 promoter activity we analyzed protein levels of both AP-1 and ETS-1 in the redox-engineered cell lines. Cultured media from the indicated cell lines were collected and examined for the level of secreted MMP-1 (Fig. 2A). MMP-1 levels were increased in response to enforced expression of Sod2. In addition to the Sod2-overexpressing HT-1080 cells, catalase-overexpressing cell lines were developed to test the role of H$_2$O$_2$ on gene expression. We have directed catalase expression to both the cytosolic and mitochondrial compartments that are distinct from its normal peroxisomal location. In this fashion signaling components that are sensitive to either mitochondrial or cytosolic H$_2$O$_2$ can be defined. Coexpression of catalase in the Sod2-overexpressing cells did not completely attenuate the expression of immunoreactive MMP-1 (Fig. 2A and B). Sod2 overexpression also increased the protein levels of many of the AP-1 family members including; c-Fos, phosphorylated Jun, and the Ets family member ets-1. The Sod2-dependent increase in the expression of both the AP-1 and Ets family members was reversed by coexpression of catalase in either the cytosolic or mitochondrial compartment (Fig. 2A and B). However, the AP-1 family member fra-2 was insensitive to changes in the steady state production of H$_2$O$_2$ resulting from antioxidant enzyme overexpression.

JNK historically has been studied as the primary kinase responsible for phosphorylating c-Jun (33). Analysis of phosphorylated and total forms of JNK showed that its phosphorylation state and protein expression are also redox-dependent, and these increases may account for the increase expression of the phosphorylated forms of c-Jun in response to Sod2 expression (Fig. 2A and B). These data establish that many of the factors that are involved in regulating MMP-1 expression are also under redox control. However, only a partial reduction in Sod2-driven MMP-1 expression was observed in response to catalase overexpression even when both Ets-1 and c-Fos were significantly attenuated.

The Antioxidants N-Acetyl Cysteine, Ebselen, and Antioxidant Porphyrin Treatments Restrict Sod2-mediated MMP-1 Expression—The failure of catalase overexpression to completely reverse the Sod2-mediated increase in MMP-1 expression, prompted us to evaluate the impact

**FIGURE 2.** Redox-dependent regulation of MMP-1, Ets, and AP-1 transcription factors. A, Western blot analysis of secreted MMP-1 and cytosolic Ets and AP-1 transcription factors. Confluent control (CMV) and Sod2-overexpressing cells were analyzed for secreted MMP-1 by Western blotting. B, quantitated data for secreted MMP-1 and indicated transcription factors are represented as the average band intensity from four independent blots and are plotted as fold induction over CMV (control cells). Error bars represent S.E. C, Western blot analysis of secreted MMP-1 following antioxidant treatment. Control (Con) transfected and Sod2-overexpressing cell lines were treated with N-acetyl-L-cysteine (2 mm), ebselen (0.45 µg/ml), Trolox (100 µM), butylated hydroxytoluene (25 µM), and the antioxidant porphyrin, (20 µM) for 18 h, followed by MMP-1 analyses. A representative blot is shown (n = 2).
Redox Control of MMP-1 Expression via JNK, Ets, and AP-1

A

![Graph showing ERK1/2-dependent regulation of MMP-1 and transcription factors](image)

**Figure 3.** ERK1/2-dependent regulation of MMP-1 and transcription factors. A, control CMV and Sod2-overexpressing cells were treated overnight with ERK1/2 inhibitors PD98059, U0124, or U0126, the inactive analog U0124, 30 μg of cytosolic lysate were assayed for expression of MMP-1, Ets-1, and c-Fos by Western blot analysis. Blot quantification of transcription factors is represented as the mean of three independent experiments. Error bars represent the S.E. B, blot quantification of MMP-1 expression is represented as fold from CMV (control cells). Error bars represent the S.E. * p < 0.05 comparing CMV and Sod2. # p < 0.05 comparing Sod2 cells with PD98059- and U0126-treated Sod2 cells.

B

![Graph showing MMP-1 expression](image)

of various antioxidants on the redox-dependent induction of MMP-1. Treatment of cells with the glutathione precursor, N-acetyl cysteine, or the glutathione peroxidase mimetic ebselen completely reversed the Sod2-mediated increase in MMP-1 expression, whereas the lipid hydroperoxide scavengers trolox and butylhydroxytoluene had a limited effect on MMP-1 expression. Treatment of Sod2-overexpressing cell lines with the redox-active porphyrin, an efficient H₂O₂ scavenger (34), also reversed the increase in MMP-1 expression in the Sod2-overexpressing cell lines. These findings indicate that efficient synthetic H₂O₂ scavengers can reverse the Sod2-dependent increases in MMP-1 expression.

**ERK1/2- and JNK-dependent Redox Regulation of MMP-1 and Transcription Factors**—We have previously reported that ERK1/2 is critical for redox control of the MMP-1 promoter (32). ERK1/2 can enhance the expression or the phosphorylation state of Ets-1, c-Fos (35, 36), and c-Jun (12), and impact gene expression. Whether the redox state of the cell modulates the expression of these factors via ERK1/2 has not been established. Analysis of MMP-1 immunoreactive protein in control and Sod2-overexpressing cell lines indicates that the redox-dependent increase in its production is sensitive to inhibition by the pharmacologic inhibitors of ERK1/2, PD98059, and U0126 (Fig. 3, A and B). Furthermore, redox-dependent MMP production was not significantly altered by the inactive U0126 analog, U0124, or the P38 MAP kinase inhibitor, SB203580 (Fig. 3B). The H₂O₂-dependent induction of Ets-1 and c-Fos was also attenuated by inhibition of ERK1/2 but not the inactive analog U0124 (Fig. 3A). Overexpression of Sod2 also increased c-Jun levels and was reversed by the ERK1/2 inhibition (data not shown). These findings suggest that H₂O₂ regulates the levels of key determinants involved in regulating MMP-1 transcription through ERK1/2. Thus, the redox-dependent increase in the levels of c-Fos, c-Jun, and Ets-1 are paralleled by an increase the extracellular production of MMP-1 that is ERK1/2-sensitive.

To evaluate the influence of JNK on the redox-dependent control of Jun the various redox-engineered cell lines were treated with the pharmacological JNK inhibitor, SP600125 and phosphospecific c-Jun analyzed. Phosphorylation of Jun at Ser^63 and Ser^73 was increased in response to Sod2 overexpression relative to control cells, which was attenuated by overexpression of mitochondrial catalase but not cytosolic catalase (Fig. 4A). The JNK inhibitor decreased the phosphorylation of both serine residues suggesting that alterations in the steady state production of H₂O₂ regulate Jun phosphorylation through JNK activation. The redox-dependent phosphorylation of Jun at serine 63 and 73 may contribute to its ability to function as a transcriptional activator as phosphorylation of these sites is required for nuclear translocation.

**JNK Is Critical for Redox Control of MMP-1 Expression**—To establish a linkage between MMP-1 transcription and JNK, both control and Sod2-overexpressing cells were transiently transfected with the full-length MMP-1 promoter and then treated with the pharmacological JNK inhibitor, SP600125. The MMP-1 promoter displays significantly higher activity in the Sod2-overexpressing cells compared with control cells and the 2G promoter has substantially more MMP-1 promoter activity compared with the 1G promoter in all cases as previously reported (29). Treatment with the JNK inhibitor significantly decreased both 1G and 2G MMP-1 promoter activity (Fig. 4B). We have previously reported that ERK1/2 inhibition attenuates both basal and redox-dependent activation of the MMP-1 promoter (32). Notably, the JNK inhibitor has been the only MAPK inhibitor to completely eliminate the Sod2-dependent MMP 1G promoter activity with no effect on basal MMP-1 promoter activity (Fig. 4B). JNK inhibition significantly attenuated but did not abolish H₂O₂-dependent MMP-1 2G promoter activity. In addition, the H₂O₂-dependent regulation of endogenous MMP-1 was attenuated by JNK inhibition (Fig. 4A). These findings suggest that the redox-dependent JNK activation may elicit its effects through AP-1 in the absence of the Ets-1 site (1G promoter), whereas both the ERK and JNK pathways may coordinately regulate the MMP-1 2G promoter containing both the AP-1 and Ets-1 transcription factor binding sites.

**Redox Control of MMP-1 Chromatin Remodeling Events**—The above studies indicate that the key determinants in regulating MMP-1 transcription, AP-1 and Ets-1, are H₂O₂ responsive. Furthermore, the loss of their respective binding sites abolishes H₂O₂-dependent MMP-1 promoter activity (Fig. 1). We further investigated the effects of increases in the steady state production of H₂O₂ on recruitment of these transcription factors to the MMP-1 promoter in vivo by ChIP assay. H₂O₂-sensitive binding of Ets-1 to the MMP-1 promoter was increased in Sod2-overexpressing cells compared with control cells (Fig. 5A), whereas c-Jun displayed only slightly increased binding to the MMP-1 promoter (Fig. 6A). Thus, Sod2-mediated MMP-1 expression may be due in part to increased binding of Ets-1 and AP-1 factors to its promoter.

Histone acetylation is an important step in the process of transcriptional activation of many genes including MMP-1. Martens et al. (37) have established that the HAT p300 acetylates histones on the MMP-1 promoter. We determined whether recruitment of p300 to the MMP promoter was also sensitive to alterations in the steady state production of H₂O₂. ChIP analysis of MMP-1-bound p300 showed no significant differences in its association with the MMP-1 promoter in any of the
redox-engineered cell lines (Fig. 5B). Furthermore, the amount of input MMP-1 DNA was insensitive to alterations in the redox state of the cells. In contrast, the recruitment of acetylated histone H3 to the MMP-1 promoter was increased in response to Sod2 overexpression, and its binding was attenuated by effective H$_2$O$_2$ detoxification by both cytosolic and mitochondrial targeted catalase (Fig. 5B). These findings indicate that the redox control of events regulating MMP-1 expression extend to include key chromatin remodeling factors.

*Sod2 Activity Is Required for MMP-1-specific Signaling*—Our findings indicate that redox signaling through the JNK pathway plays a major regulatory role in MMP-1 transcription. This redox regulation can be attributed to H$_2$O$_2$, generated by the enforced expression of Sod2. To rule out possible non-enzymatic effects of Sod2 on MMP-1-specific signaling, we have generated a cell line overexpressing the active site mutant of Sod2 (mutSod2) (30). Western blot analysis and Sod zymography (Fig. 6) demonstrate that mutSod2 cells do not show increased activity though they express equivalent amounts of Sod2 immunoreactive protein as in the Sod2-overexpressing cells. Using these cells we have evaluated JNK signaling by Western blot analysis. Phosphorylation of JNK and c-Jun is not increased in cells expressing the inactive Sod2. In contrast, active Sod2 overexpression enhanced both Jnk and Jun phosphorylation with no effect on total Jnk or Jun levels. Furthermore, the expression of MMP-1 is unchanged in cells transfected with the inactive form of Sod2.

Ets-1 binding to the 2G polymorphism is an important effector of MMP-1 transcription. ChiP assays (Fig. 6, right panel) show that in comparison to the Sod2-overexpressing cell line, Ets-1 binding to the MMP-1 promoter is not increased in the mutSod2 cells. Taken together these results further confirm that the redox control of JNK signaling and MMP-1 transcription is attributed to the dismutating activity of Sod2.

H$_2$O$_2$ Modulates Tyrosine Phosphorylation—A distinguishing feature of MAPKs is that they are directly activated by phosphorylation on a tyrosine and a threonine separated by one variable residue within the kinase activation loop (38). Protein-tyrosine phosphatase (PTP) family members play an important role in regulating MAPK phosphorylation status and are sensitive to H$_2$O$_2$-dependent inactivation (31). Because of the robust increase in the steady state production of H$_2$O$_2$ by Sod2 overexpression, it is possible that phosphatase inactivation may in part contribute to the increases in MAPK activity observed in the redox engineered cell lines. To test this possibility in-gel analysis of protein-tyrosine phosphatase activity was performed. By this method, PTPs from whole cell lysates were treated with iodoacetic acid (IAA) such that any PTP having a free cysteine residue is irreversibly inactivated by IAA. However, oxidized cysteines will not be labeled with IAA and following reductive renaturation in the presence of dithiothreitol will become active upon SDS-PAGE. Phosphatase clearing of the radiolabeled substrates within the gel indicates a phosphatase, which was initially oxidized intracellularly. Analysis of phosphatase activity in the various cell lines showed a statistically significant increase in the presence of oxidatively inactivated PTPs in response to Sod2-dependent production of H$_2$O$_2$ (Fig. 7, A and B). Furthermore, the abundance of oxidized PTPs was decreased in response to the removal of H$_2$O$_2$ by catalase coexpression. Quantification of the distinct molecular weight PTPs is shown in supplemental Fig. S1. The Sod2-sensitive PTP falls within in the mass range of 80–55 kDa. Furthermore, PTPs in this range were more responsive to the overexpression of catalase than the lower molecular mass PTPs (>55 kDa). These results indicate that the PTP inactivation in the Sod2-overexpressing cells is H$_2$O$_2$-dependent.

We have previously demonstrated that the dual lipid protein phosphatase, PTEN, is sensitive to H$_2$O$_2$-dependent activation by Sod2. The PTP activity of PTEN has been shown to restrict the expression of both MMP-2 and MMP-9 by dephosphorylation of focal adhesion kinase and restricting ERK1/2 activity (39). Thus, it is possible that the redox inactivation of PTEN may contribute to the enhanced expression of MMP-1 in response to Sod2 overexpression. However, rescue of PTEN activity by enforced expression had no effect on the Sod2-mediated increase in either endogenous MMP-1 or MMP-1 promoter-driven luciferase activity (data not shown).

Inactivation of PTPs in response to alterations in the steady state production of H$_2$O$_2$ should increase overall tyrosine phosphorylation.
Coincident with the decreased PTP activity was an increase in tyrosine phosphorylation in response to H$_2$O$_2$ by Sod2 overexpression (Fig. 7, A and B). Furthermore, tyrosine phosphorylation was slightly reduced when Sod2 was coexpressed with cytosolic catalase, and was significantly reduced when Sod2 was overexpressed with the mitochondrial catalase. Thus, inactivation of PTPs and a consequent increase in protein-tyrosine phosphorylation appears to occur in response to the mitochondrial generation of H$_2$O$_2$ by Sod2. Decreased phosphatase activity may contribute to the activation of MAPK and subsequent heightened activation of target genes such as MMP-1.

**DISCUSSION**

Our findings demonstrate that increases in the steady state production of mitochondrial H$_2$O$_2$ lead to the phosphorylation and activation
of ERK1/2 at Thr202/Tyr204 (32) and JNK at Thr183 and Tyr185 (Fig. 2). This redox-sensitive signaling cascade may be activated in response to conditions that perturb mitochondrial oxidant production such as cytokine production, ionizing radiation, and bacterial lipopolysaccharides.

A growing body of evidence supports the role of MAPK in regulating MMP-1 transcription (40–43). Our studies further support MAPK involvement as treatment with ERK (32) or JNK inhibitors (Figs. 3 and 4) restrict H2O2-dependent MMP-1 promoter activity. Furthermore, H2O2 can inactivate protein-tyrosine phosphatases, which may allow for sustained signaling by alleviating dephosphorylation-dependent repression of various MAPKs (31). Activated MAPK pathways target the AP-1 and Ets transcription factor families and likely drive MMP expression (12, 40, 41). The MAPK pathways not only can increase transcription by increasing the activity via phosphorylation, but also by increasing the expression levels of the proteins themselves (44). Studies have suggested that the expression of MMP-1 in melanoma cells is dependent on the de novo synthesis of proteins (45), which may include the AP-1 and Ets families of transcription factors. In the current study, both ERK1/2 and JNK are important for enhancing phosphorylation of the AP-1 transcription factor c-Jun (Fig. 2). Additionally, ERK1/2 signals to c-Fos, c-Jun, and Ets-1 transcription factors (Fig. 3). Furthermore, Ets-1 and c-Fos expression and the phosphorylation of serines 63 and 73 on c-Jun were enhanced by Sod2-generated H2O2 (Fig. 2). Thus, many components that contribute to maximal MMP-1 expression are activated in response to the mitochondrial production of H2O2.

FIGURE 6. JNK signaling and Ets-1 binding to MMP-1 promoter is unaffected by overexpression of the inactive form of Sod2. Left panel, HT1080 fibrosarcoma cells were transfected with pCDNA (Control) or pCDNA/mutSod2 (mutSod2) constructs. The indicated cell lines were used for MMP-1 immunoblot analysis as described earlier. In addition cells were lysed and analyzed by electrophoresis in a native discontinuous polyacrylamide gel. The gel was stained with a solution containing 2.5 mM nitro blue tetrazolium, 0.008 mM riboflavin, 30 mM TEMED, and 0.005 M potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. Sod activity is identified by clearing of the gel. Western blot analysis was also performed and blots were probed with primary antibodies for phosphospecific JNK, phosphospecific c-Jun serine 73, total JNK, total c-Jun, Sod2, and GAPDH. Right panel, indicated cell lines were treated with 37% formaldehyde to cross-link proteins to DNA. Cells were lysed, and chromatin immunoprecipitation was carried out as described previously using antibody to Ets-1. Purified DNA was subjected to real time PCR using Sybr green to amplify the region between −1978 and −1523 of the MMP-1 gene. DNA units were calculated using Ct values for pre-IP and post-IP DNA obtained. Data are representative of three independent experiments.

FIGURE 7. Sod2-derived H2O2 inactivates protein-tyrosine phosphatases. A, upper panel, in-gel phosphatase analysis of indicated cell lines. A clearing in the gel represents a phosphatase which was initially inactivated by hydrogen peroxide. Lower panel, phosphotyrosine immunoreactivity. Cell lysates (500 µg) were incubated with a phosphorylated tyrosine antibody conjugated to protein G-Sepharose beads (Santa Cruz PY-20). The phosphotyrosine immunoprecipitates then were run on a 10% SDS-PAGE. Membranes were probed with distinct phosphotyrosine antibody (G104). Arrows indicate IgG immunoreactive bands, and lower panel shows a brief autoradiographic exposure indicating equivalent loading. B, densitometric analysis of representative gels from panel A. Data represent the mean relative increase ± S.E. of 5–7 independent experiments for both upper and lower panels. * represents p < 0.05 compared with CMV control, † or †† represent p < 0.05 or 0.01, respectively, compared with Sod2 control.

Redox Control of MMP-1 Expression via JNK, Ets, and AP-1

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We have also identified two motifs, the Ets site at −1607 created by the 2G SNP and the proximal AP-1 site at −1602, which are required for maximal activation of the MMP-1 promoter via enforced Sod2 expression (Fig. 1). Mutagenesis of either site led to a dramatic decrease in promoter activity that still displayed redox sensitivity, indicating the importance of other redox-sensitive DNA binding sites within the MMP-1 promoter.

The above observations are in accordance with those by Tower et al. (45), who identified the Ets site created by the 2G SNP in the MMP-1 promoter as the major target for ERK1/2-dependent signaling. However, in the absence of the 2G site, ERK1/2 inhibition required the inclusion of the region between −4372 and −3292. Potential ERK1/2-sensitive sites include an AP-1 site at −3475 base pairs and two Ets sites at −3238 and −3908 base pairs. In the absence of the 2G site at −1607 base pairs, trans-activating factors may find alternative cis-activating sequences within the MMP-1 promoter to trigger gene expression. Tower et al. (45) also suggested that under basal conditions, ERK1/2 is signaling to the AP-1 site at position −1602. This stands to reason because AP-1 factors tether Ets proteins and increase their binding affinity and are often necessary to transactivate transcription. Thus, mutation of the AP-1 site may have deleterious effects on the transcriptional activity of the 2G SNP-containing MMP-1.

To date, the Ets family member that binds to the 2G SNP within the MMP-1 promoter in any cell type remains elusive. This 2G SNP functions as a bone fide Ets site, as measured by Ets-1 gel shift competition experiments (10). Consistent with this, our chromatin immunoprecipitation data indicated that Ets-1 binding to the MMP-1 promoter between −1978 and −1523 base pairs is significantly enhanced under conditions of Sod2 overexpression (Fig. 5). Likewise, we observed that c-Jun binding to this region was slightly enhanced under the overexpression of Sod2 (Fig. 5). Within this region of the MMP-1 promoter there are at least three Ets binding sites at positions −1960, −1703, and −1633 and three AP-1 sites located at −1886, −1602, and −1557 base pairs. In the intracellular adhesion molecule (ICAM-1) promoter, Ets-1 and AP-1 factor binding sites are located within 10 base pairs of one another and cooperate to promote ICAM-1 transcription (46). The proximity of the −1607 Ets and −1602 AP-1 sites within MMP-1 marks them for similar regulatory control of MMP-1. Thus, our data suggest that Ets-1 and c-Jun are binding to at least one, if not more of their respective sites in the MMP-1 promoter in response to increases in the steady state production of H₂O₂.

Synergistic activation of gene expression plays an important role in gene regulation. DNA elements containing a single Ets binding site are often not sufficient for gene transcription but require a nearby AP-1 site to which Fos and Jun proteins bind (47). Moreover, in addition to self gene regulation. DNA elements containing a single Ets binding site are important of other redox-sensitive DNA binding sites within the MMP-1 promoter.

Furthermore, Jun and Fos can physically interact with non-ZIP binding proteins including the activating transcription factors (ATFs), to which Fos and Jun proteins bind (47). Moreover, in addition to self gene regulation. DNA elements containing a single Ets binding site are important of other redox-sensitive DNA binding sites within the MMP-1 promoter.

The cascade of coactivators and transcription factors involved in the transcriptional activation of MMP-1 promoter by tetradecanoyl phorbol acetate (TPA) has been elucidated by Martens et al. (37). Upon activation by TPA, first the transcription factors c-Jun and c-Fos bind to the MMP-1 promoter followed by the basal transcription factor TATA-binding protein (TBP), RNA polymerase II, and the methyltransferase SET9. This results in the di- and trimethylation of lysine 4 on histone 3, a marker for transcriptional activation leading to the recruitment of the histone acetyl transferase p300 and a kinase RSK2, which results in acetylation and phosphorylation of histones. Finally, the chromatin remodeling complex containing Brg-1 is recruited facilitating transcriptional activation. p300 is a ubiquitous histone acetyl transferase involved in acetylating histones on specific lysine residues (37) and our findings indicate that it is recruited to the region near the 2G polymorphism. Ets-1 has been shown to interact with p300 at both its N-terminal and C-terminal regions (51) and is required for transactivation (52). Brg-1, a component of the SWI/SNF chromatin remodeling complex is also required for the transcription of MMP-2 (53) and MMP-9 (54). Mitochondrial oxidant production may play a role in modulating the recruitment of these proteins on the MMP-1 promoter. In addition ROS could affect protein-protein interactions during preinitiation complex formation leading to enhanced promoter activity. The HAT p300 contains three cysteine/histidine-rich regions (C/H1-C/H3), which fold into zinc-binding modules. These regions play a role in DNA binding as well as facilitating protein-protein interactions. SET9 also has cysteines in its SET domain, which contains the critical histidine residue needed for activity. It is probable that ROS could modulate the activity of these proteins via modification of cysteine residues. In addition Rsk2 or p90 S6 kinase has been demonstrated to be activated via the MAPK pathway leading to gene activation (55), which may be regulated by inactivation of an oxidant-sensitive phosphatase PP2Cδ, which restricts Rsk2 activity (56).

Oberley and co-workers (57) reported that the Sod2-dependent activation of MMP-2 in MCF-7 breast cancer cell lines is exacerbated by NO" inhibitors and prevented by NO" treatments (57). Our studies indicate that treatments with the NO" donor, spermine-NONOate, inhibited basal MMP-1 promoter-driven luciferase activity in control cells lines (supplemental Fig. S2). We have previously reported that Sod2 overexpression sensitizes cells to the cytostatic effects of NO donors. It has been clearly established that NO is an efficient sink for O₂" leading to the formation of peroxynitrite (ONOO⁻). Inhibition of NO" production would lead to decreased scavenging of O₂" by NO" and an increase in the Sod2-dependent production of H₂O₂. Whereas NO" donors should lead
to increased scavenging of O$_2^-$ and a decrease in the steady state production of H$_2$O$_2$ by Sod2. The net effect on Sod2-dependent H$_2$O$_2$-mediated gene expression in the presence of NO inhibitors or donors would be to increase or decrease gene expression, respectively. Oberley's studies strictly assessed the effect of these compounds on MMP-2 activity. The inhibition of MMP-1 promoter activity by NO also extends the findings of Oberley to include MMP-1 and expands upon this work to show that NO-dependent inhibition of MMP-1 expression is likely transcriptional.

In addition to their ability to detoxify H$_2$O$_2$, Ebsselen and the antioxidant porphyrin are also potent scavengers of ONOO$^-$. Efficient dismutation of O$_2^-$ by Sod2 would limit substrate availability for the generation of ONOO$^-$. So it is unlikely that the inhibitory effects of both ebsselen and the porphyrin are attributed to their ONOO$^-$ scavenging capacity. Furthermore the fact that NAC restricts MMP-1 expression also implicates H$_2$O$_2$ as the primary Sod2-derived signaling oxidant.

The enforced expression of Sod2 provides a targeted method to enhance the mitochondrial production of H$_2$O$_2$ in the absence of traditional pharmacological inhibitors. Sod2 is unique among antioxidants in that its expression and activity is increased in response to inflammatory stimuli including lipopolysaccharides, cytokines, and ionizing radiation (32). Our findings suggest that robust increases in Sod2 activity that are stimuli including lipopolysaccharides, cytokines, and ionizing radiation (32). Our findings suggest that robust increases in Sod2 activity that are.

Ptase) is inversely related to tyrosine phosphorylation and MMP-1 dependent activation of MMP-1 expression has not been established. Our findings would support this hypothesis since PTP activity (InGel Phos) is inversely related to tyrosine phosphorylation and MMP-1 expression (Fig. 7). Furthermore, we have identified that JNK plays a prominent role in the redox sensitivity of MMP-1 expression and suggest that the MAPK phosphatases, that control JNK activity, may be attractive candidates for regulating MMP-1 expression in response to alterations in the steady state production of H$_2$O$_2$ that are observed under numerous pathological conditions.

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Redox Control of MMP-1 Expression via JNK, Ets, and AP-1


Redox-dependent Matrix Metalloproteinase-1 Expression Is Regulated by JNK through Ets and AP-1 Promoter Motifs
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