Rac1-mediated mitochondrial H₂O₂ generation regulates MMP-9 gene expression in macrophages via inhibition of SP-1 and AP-1

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Running Title: Rac1 inhibits MMP-9

Summary

Aberrant matrix deposition is a hallmark of pulmonary fibrosis and is characterized by an imbalance between matrix deposition and degradation. We have previously shown that mice harboring a conditional deletion of the GTP-binding protein, Rac1, in macrophages are protected from asbestos-induced pulmonary fibrosis. To investigate the contribution of aberrant matrix degradation, we addressed the role of Rac1 in regulating expression of macrophage-specific matrix metalloproteinase-9 (MMP-9). We found that MMP-9 gene transcription was significantly greater in Rac1 null macrophages. Deletion and mutational analysis of the MMP-9 promoter revealed that the combination of SP-1 and either AP-1 binding site are essential for MMP-9 transcription. Over expression of constitutive active Rac1 (V12) revealed that H₂O₂ was derived from the mitochondria. Rac1-induced H₂O₂ generation down regulated MMP-9 gene transcription, whereas catalase over expression in WT cells enhanced MMP-9 expression. SP-1 interacted directly with both c-Jun and c-Fos, and H₂O₂ decreased this binding, suggesting that SP-1 and AP-1 function cooperatively to regulate MMP-9 transcription. Rac1-mediated H₂O₂ inhibited the ERK MAP kinase, which was essential for activation of SP-1 and AP-1. ERK activation and MMP-9 expression were recovered by over expressing catalase or transfecting siRNA for the mitochondrial iron-sulfur protein, Rieske, respectively. These observations were recapitulated in vivo. MMP-9 mRNA was higher in alveolar macrophages isolated from Rac1 null mice and WT mice given catalase. Rac1 regulates MMP-9 transcription via mitochondrial H₂O₂ generation, providing a potential mechanism by which Rac1 null mice fail to develop pulmonary fibrosis.

Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that degrade extracellular matrix (ECM) proteins and are required for ECM remodeling. They play an important role in a wide variety of physiological conditions and disease states, some of which include normal development, inflammation, wound healing and repair, vascular diseases, and cancer growth and metastasis (1-3). Under normal conditions the maintenance of the ECM is a dynamic process in which the production of ECM proteins is balanced by MMP-induced protein degradation. In contrast, in states of excessive tissue remodeling when production of ECM proteins exceeds proteolysis, tissue fibrosis can develop due to expansion of the ECM (4).

The regulation of MMP expression and activity occurs at many levels, including gene expression, post-translational modification, and proteolytic activation. MMPs are synthesized as proenzymes that require activation by proteases, such as urokinase-type plasminogen activator, plasmin, and other MMPs (5-8). MMP expression is complexly regulated by transcriptional, translational, and post-translational mechanisms. Transcriptional control of MMPs occurs through the action of transcription factors, such as SP-1 and AP-1, which are activated by signal transduction pathways, including mitogen-activated protein kinases (9-11). MMP expression is also regulated by miRNAs, which play a critical role in the post-transcriptional regulation of gene expression (12).

In this study, we investigated the role of Rac1 in regulating MMP-9 expression in macrophages. We found that MMP-9 gene transcription was significantly greater in Rac1 null macrophages. Deletion and mutational analysis of the MMP-9 promoter revealed that the combination of SP-1 and either AP-1 binding site are essential for MMP-9 transcription. Overexpression of constitutive active Rac1 (V12) revealed that H₂O₂ was derived from the mitochondria. Rac1-induced H₂O₂ generation downregulated MMP-9 gene transcription, whereas catalase overexpression in WT cells enhanced MMP-9 expression. SP-1 interacted directly with both c-Jun and c-Fos, and H₂O₂ decreased this binding, suggesting that SP-1 and AP-1 function cooperatively to regulate MMP-9 transcription. Rac1-mediated H₂O₂ inhibited the ERK MAP kinase, which was essential for activation of SP-1 and AP-1. ERK activation and MMP-9 expression were recovered by overexpressing catalase or transfecting siRNA for the mitochondrial iron-sulfur protein, Rieske, respectively. These observations were recapitulated in vivo. MMP-9 mRNA was higher in alveolar macrophages isolated from Rac1 null mice and WT mice given catalase. Rac1 regulates MMP-9 transcription via mitochondrial H₂O₂ generation, providing a potential mechanism by which Rac1 null mice fail to develop pulmonary fibrosis.
transcription, post-transcriptional processing, and pro-enzyme activation (5-6). Additional control occurs by the family of tissue inhibitors of metalloproteinases (TIMPs), which bind in a 1:1 ratio to the active site of MMPs (7). The generation of reactive oxygen species (ROS) has been shown to modulate both the expression and activity of MMPs (8-9). ROS are known to convert pro-MMPs to their active catalytic state by oxidizing the bond between a highly conserved cysteine residue and zinc in the catalytic domain (10). In addition, ROS increase the expression of MMPs via cell signaling pathways, such as MAP kinases, that are regulated by redox-sensitive phosphatases (11-13). ROS oxidize cysteine residues in the catalytic domains of these phosphatases rendering them inactive and, thereby, potentiate the signal of the respective kinases resulting in increased MMP expression. In addition to other MAP kinases, the ERK MAP kinase has been shown to modulate MMP expression (12). Inhibition of ROS by flavoenzyme inhibitors or antioxidants has been shown to decrease MMP expression (8,14-15).

The Rho family of guanosine 5’-triphosphate (GTP)-binding proteins is comprised of 20 members, one of which is Rac1 (16). These proteins play an important role in host defense. In particular, Rac1 is known to regulate assembly of the actin cytoskeleton, activation of the NADPH oxidase in non-phagocytic cells, the cellular transformation initiated by Ras oncogenes, and the migration, adhesion, and differentiation of cells (17-19). Its role in mediating ROS generation has been implicated in many disease processes, including fibrosis (17-18,20). Studies in which Rac1 has been shown to regulate MMP production via increased ROS were performed in non-phagocytic cells, such as fibroblasts, epithelial cells, and adenocarcinoma cells, and reduction of ROS decreased MMP expression and activity (14-15,21-22). However, the molecular mechanism(s) by which Rac1 modulates MMP-9 gene transcription and the basis of these actions in phagocytic cells has not been demonstrated.

We recently demonstrated that Rac1 activity in alveolar macrophages, via H2O2 production, is required for asbestos-induced pulmonary fibrosis (23). We now investigated the mechanisms for Rac1-mediated ROS signaling focusing on the transcriptional regulation of MMP-9. Our results demonstrate that MMP-9 transcription was redox-sensitive and dramatically increased in Rac1 null cells due to increased MMP-9 promoter activity and mRNA synthesis. The source of Rac1-induced H2O2 was from the mitochondria. Constitutive active Rac1 (V12) and Rac1-mediated H2O2 inhibited the ERK MAP kinase, which was essential for MMP-9 transcription. More importantly, MMP-9 expression was recovered by expressing siRNA for the mitochondrial iron-sulfur protein, Rieske. These in vitro observations were reproduced in vivo as alveolar macrophages obtained from Rac1 null mice exposed to asbestos expressed significantly more MMP-9 mRNA than cells from WT mice. The inhibitory effect of Rac1-induced H2O2 was demonstrated by finding enhanced MMP-9 expression in macrophages obtained from asbestos-exposed WT mice given catalase. Moreover, MMP-9−/− mice developed pulmonary fibrosis to the same degree as WT mice. These data indicate that Rac1-mediated mitochondrial H2O2 generation suppresses MMP-9 gene transcription and provides a mechanism by which Rac1 null mice are protected from developing pulmonary fibrosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Catalase was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Chrysotile asbestos was provided by the NAIMA Fiber Repository. Horse radish peroxidase (HRP) and p-hydroxyphenyl acetic acid (pHPA) were purchased from Sigma Chemical Company (St. Louis, MO). Anti-SP-1, anti-c-Fos, anti-phospho-c-Jun, anti-TATA binding protein (TBP), anti-collagen 1, anti-mouse IgG-HP, and anti-rabbit IgG-HP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MMP-9 polyclonal antibody and anti-phospho(T453)-SP-1 were purchased from Abcam (Boston, MA), and anti-Lamin A/C was from Cell Signaling (Boston, MA). Consensus oligonucleotides for SP-1 and AP-1 were obtained from Promega (Madison, WI).

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approved by the University of Iowa Institutional Animal Care and Use Committee. Rac1 null mice were generated by selectively disrupting the Rac1 gene in cells of the myeloid lineage, as described previously (24). MMP-9 -/- mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were administered 100 µg of chrysotile asbestos in 50 µL normal saline intratracheally after being anesthetized with 3% isoflurane using a precision Fortec vaporizer (Cyprane, Keighley, UK). Twenty-one days later, mice were euthanized with an overdose of isoflurane, and bronchoalveolar (BAL) was performed. BAL cells were collected by centrifugation of the BAL for 10 min at 220g and used for the determination of MMP-9 mRNA. Over 90% of the BAL cells were macrophages.

To evaluate the effects of catalase, WT mice were exposed to asbestos as above described. The mice were then administered either catalase (2000 U/mouse) or water intratracheally every day for 20 days. BAL was performed on euthanized mice and BAL cells were collected.

**Cell Culture**—WT and Rac1 null macrophages were obtained from WT and Rac1 null mice as previously described (23). Cells were maintained in RPMI 1640 media containing 10% new born calf serum, 0.5 mM β-mercaptoethanol, and penicillin/streptomycin. THP-1 cells and HFL-1 cells were obtained from American Type Culture Collection and maintained in RPMI 1640 or DMEM media, respectively, supplemented with 10% newborn calf serum and penicillin/streptomycin. All experimental conditions were performed in phenol red-free RPMI 1640 media supplemented with 0.5% serum and penicillin/streptomycin. Where indicated chrysotile asbestos was used at a final concentration of 10µg/cm².

**H_2O_2 Generation**—Release of H_2O_2 from WT and Rac1 null macrophages was determined as described previously (25). The assay takes advantage of H_2O_2-mediated oxidation of HP to Complex I which, in turn, oxidizes pHPA to a stable fluorescent [pHPA]₂ dimer. Mitochondria were isolated by lysing the cells in a mitochondria buffer containing 10 mM Tris, pH 7.8, 320 mM sucrose, 0.2 mM EDTA, and protease inhibitors. Lysates were homogenized using a Kontes Pellet Pestle Motor and centrifuged at 1,000g for 10 min at 4 °C. The supernatant was removed and kept at 4 °C, and the pellet was lysed, homogenized, and centrifuged again. The two supernatants were pooled and centrifuged at 12,000g for 15 min at 4 °C. After the supernatant was discarded, the pellet was then resuspended in mitochondria buffer without sucrose. For membrane isolation, cells were lysed in a buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and protease inhibitors. Lysates were homogenized using a Kontes Pellet Pestle Motor and centrifuged at 3,000 rpm for 3 min at 4 °C. Supernatants were centrifuged at 100,000g for 1 h. After removal of the supernatant, the membrane pellet was resuspended in lysis buffer and incubated on ice for 30 min. Cells, mitochondrial, or membrane fractions were incubated in phenol red-, serum-free HBSS containing 1.6 mM pHPA, 95µg/mL HP, 6.5 mM glucose, 1mM HEPES, 6 mM sodium bicarbonate. The formation of the [pHPA]₂ dimer in the medium was monitored by measuring fluorescence at excitation and emission wavelengths of 317 and 400 nm, respectively.

**Adenoviral vectors**—Replication-deficient recombinant adenovirus type 5 with the E1 region replaced with DNA containing the cytomegalovirus (CMV) promoter region alone (Ad.CMV), catalase cDNA (26), V12-Rac1 cDNA, or N17-Rac1 cDNA (27) downstream of the CMV promoter were obtained from the Gene Transfer Vector Core at the University of Iowa (Iowa City, IA). Cells were infected for 48 h with the vectors at 500 moi in RPMI 1640 media containing 0.5% newborn calf serum.

**Quantitative Real-time PCR**—Total RNA from BAL cells and cells grown in culture was isolated by Trizol and, following DNase treatment, subjected to reverse transcription using the reverse transcriptase kit, Iscript (Bio-Rad Laboratories, Hercules, CA). MMP-9, HPRT, and β-actin mRNA transcripts were determined by quantitative real time PCR using Sybr Green (Bio-Rad Laboratories, Hercules, CA) and the respective primers on an IQ5 Real Time PCR machine (Bio-Rad Laboratories, Hercules, CA). The following primer sets were used: MMP-9 5'-CCA CAT CTC CCT CCA GAA A-3' and 5'-CAC TTG GTG GTT TGC TAC GA-3'; MMP-12 5'-AGA GCA GTG CCC...
CAG AGG TCA-3’ and 5’-GGG GGT TTC ACT GGC CCT CCA TA-3’; HPRT 5’-CCT CAT GGA CTG ATT ATG GAC-3’ and 5’-CAG ATT CAA CTG GGC TTC ACT GGG GCT CCA TA-3’; ß-actin 5’-AGA GGG AAA TCG TGC GTG AC-3’ and 5’-CAG ATT CAA CTT GCG CTC ATC-3’. Data were calculated by the ΔΔCT method. MMP-9 mRNA in cultured cells was normalized to HPRT mRNA, which amplified at approximately the same CT as MMP-9. MMP-9 mRNA in BAL cells was normalized to ß-Actin. Results are expressed as arbitrary units of MMP-9 mRNA relative to the respective house keeping gene.

Plasmids and luciferase assays—The 5’ flanking sequence of the human MMP-9 promoter (-1284/+21) inserted between KpnI and HindIII restriction sites in the pGL3 basic expression vector was a generous gift from Dr. Jianming Xu, Baylor College of Medicine, Houston, TX (28). Deletion constructs were generated by PCR using the full-length MMP-9 promoter (-1284/+21) as a template with forward and reverse primers containing KpnI and HindIII restriction sites, respectively. Amplified products were resolved on agarose gels, purified and directionally cloned into the KpnI and HindIII restriction sites of pGL3 basic vector.

MMP-9 promoter with mutations in one or more than one transcription factor binding sites were generated using QuickChange Multi and QuickChange Lightning Multi Site-Directed Mutagenesis Kits (Stratagene, La Jolla, CA), respectively. The following sequences were mutated as follows: N F-κB 5’-GGATT-3’ was mutated to 5’TATT-, SP-1 site 5’-CCGCCC-3’ was mutated to 5’-ATTCCC-3’, AP-1 sites 5’-TGAGCTCA-3’ was mutated to 5’-TAAGGCA-3’.

SP-1-dependent gene expression was evaluated using a luciferase reporter plasmid (pSP-1-luc) driven by the tandem copies of the SP-1 enhancer (GGGGCGGGCGGCG) linked to a herpes simplex virus thymidine kinase promoter. AP-1-dependent gene expression was evaluated by the previously described plasmid (29). Using PCR techniques, murine SP-1 cDNA (NM_013672) was directionally cloned into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen). The pCMV-HA-ERK2 (K/A) plasmid has been described and was a generous gift from Dr. Roger Davis, University of Massachusetts (30). The correct reading frame and sequence of all plasmids used in the study were verified by fluorescent automated DNA sequencing performed by the University of Iowa DNA Facility.

Cells were transfected using Effectene Transfection Reagent (Qiagen, Valencia, CA) according to manufacturer’s directions. To correct for transfection efficiency, cells were co-transfected with pHl-TK vector encoding Renilla luciferase (Promega, Madison, WI). Four hours after transfection, media was replaced with fresh serum-containing media, and cells were allowed to recover for 24-48 h. Firefly and Renilla luciferase activities were determined in cell lysates using the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI), according to manufacturer’s instructions, and are expressed in real light units (RLU).

Small interfering RNA—Rac1 null cells and THP-1 cells were transfected with 100 nM control or ERK siRNA duplex (Dharmacon Research, Lafayette, Co) or human Rieske siRNA duplex (IDT, Iowa City, IA) utilizing DharmaFect 4 or 2 (Dharmacon Research, Lafayette, Co), respectively, according to the manufacturer’s instructions. Six hours after transfection, media were replaced and cells were allowed to recover for 48-72 h.

Electrophoretic Mobility Shift Assays—Nuclear proteins were extracted from WT and Rac1 null cells as previously described (31). Consensus AP-1 (5’-GGCG TTG ATG AGT CAG CCG GAA-3’) and SP-1 (5’-ATT CGA TCG GGG CGG GGC GAG C-3’) oligonucleotides were labeled with [γ^32P]ATP (NEN Life Science Products) and allowed to bind to 10 μg of nuclear proteins as previously described (31). Protein-DNA complexes were separated on a 5% non-reducing polyacrylamide gel.

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay was conducted using the SimpleChIP™ Enzymatic Chromatin IP Kit (Cell Signaling) according to the manufacturer’s instructions. Briefly, Rac1 null cells (~40 X 10^6 cells) were grown to confluence on 150-mm tissue culture plates and then treated with either vehicle or 25 μM H2O2 for 2 h. Cells were fixed using 1% formaldehyde for 15 min at room
temperature followed by termination of fixation using excess glycine. Cell were harvested, the cell nuclei were isolated, and the resulting nuclear pellets treated with Micrococcal Nuclease (150 units for 20 min at 37 °C) to digest DNA to fragments of about 0.3 kb. The resulting cross-linked chromatin preparations were used for input controls (2% of total) or for immunoprecipitation using 2 μg of anti-SP-1 or anti-c-Jun antibodies (Santa Cruz Biotechnology) or normal rabbit IgG antibody (Cell Signaling) as a negative control. Immunocomplexes were eluted, the chromatin subjected to reversal of cross-links followed by DNA purification as described in the protocol. PCR was performed using purified DNA and the following primers: 5′-GCT CCC ACA TGT GTG TGT C-3′ and 5′-CCT AGC TCC AGC AGG CTT for the murine MMP-9 SP-1 binding site (76 bp product); 5′-GTA GTG TAA ACA CAC ACA CAC A-3′ and 5′-AGT AAA ACG GAA TCA GTG ACC GAA TCA GTG ACC C-3′ for distal AP-1 site (116 bp product); and 5′-CCC CAC ACT GTA GGT TCT ATC C-3′ and 5′-ATC CTG CCT CAA AGA GCC T-3′ for proximal AP-1 site (101 bp product). RPL30 primers (Cell Signaling) were used for PCR detection of the murine ribosomal protein gene locus (159 bp product). PCR products were resolved on a 2% agarose gel.

Zymogram Analysis—WT and Rac1 null cells were incubated for 24 h in RPMI containing 0.5% new born calf serum in the absence of β-mercaptoethanol and phenol red. Conditioned media equivalent to 30 μg protein were separated on a 5% polyacrylamide gel containing 1mg/mL gelatin under denaturing and non-reducing conditions. Gels were washed twice for 10 min each in 2.5% Triton X-100 and twice for 30 min each in reaction buffer (50 mM TRIS, 5 mM calcium chloride, and 2 μM zinc chloride, pH 8) containing 2.7% Triton X-100. After removal of the Triton X-100 with washing, the gels were incubated for 72 h at 37 °C in reaction buffer. They were then stained with Coomassie Blue until clear zones representing gelatinolytic activity were observed. The bands co-migrated with purified MMP-9 protein.

Hydroxyproline Determination—BAL fluid was digested for 24 h at 112 °C with 6N hydrochloric acid. Hydroxyproline concentration was determined as previously described (23).

Immunoblot Analysis—Extraction of nuclear proteins and whole cell lysates were performed as previously described (31). Nuclear extracts, whole cell lysates, and conditioned media were separated by SDS/PAGE. Immunoblot analyses were performed with the designated antibodies followed by the appropriate secondary antibody cross-linked to HRP.

Purification of SP-1-His-tagged Protein—Rac1 null cells were transfected with Empty pcDNA3.1 vector or pcDNA3.1-SP-1-V5-His. After 24 h cells were exposed to vehicle or H2O2 (25 μM) for 2 h. After treatments, cells were harvested in Buffer B (PBS, 0.5 M NaCl, 1% Triton X-100, protease and phosphatase inhibitors), the lysates briefly sonicated on ice, and cellular debris pelleted at 12,000g for 10 min at 4 °C. Talon metal (cobalt) affinity resin (Clontech) was added to each lysate, and samples were rotated overnight at 4 °C. The SP-1-His proteins were eluted by adding protein sample buffer and heating at 95 °C for 5 min.

Statistical Analyses—Statistical comparisons were performed using either an unpaired, one-tailed t test or one-way ANOVA followed by Tukey’s T test. Values in figures are expressed as means with standard errors and p < 0.05 was considered to be significant.

RESULTS

MMP-9 expression is increased in Rac1 null cells—Our previous observations demonstrated that deletion of Rac1 from macrophages protects mice from asbestos-induced pulmonary fibrosis (23). To determine whether increased matrix degradation in Rac1 null mice accounted, in part, for this effect, we evaluated MMP-9 expression in WT and Rac1 null macrophages. WT and Rac1 null cells were cultured for 24 h. MMP-9 protein and activity in conditioned medium were determined by immunoblotting and zymogram analysis, respectively. MMP-9 protein and activity were dramatically increased in conditioned media collected from Rac1 null macrophages compared to WT cells (Figure 1A). This difference was also observed at the mRNA level (Figure 1B). To determine whether these changes were regulated at the transcriptional
level, WT and Rac1 null cells were transiently transfected with a luciferase vector driven by the MMP-9 promoter (-1284/+21 or MMP-9wat), and luciferase activity was determined 48 h after transfection. Consistent with the dramatically increased protein, activity, and mRNA levels in Rac1 null cells, MMP-9 promoter activity was 7-fold greater in Rac1 null cells compared to WT cells (Figure 1C).

Rac1-mediated inhibition was specific for MMP-9—To address the specificity of the effects of Rac1 on MMP-9, we measured relative mRNA expression of MMP-2 and MMP-12, which are other macrophage-specific MMPs. MMP-2 was not detected in WT or Rac1 null cells (data not shown), and there was no difference in mRNA expression of MMP-12 (Figure 2A).

The negative regulatory role of Rac1 was confirmed by over expressing constitutively active Rac1 (V12) in Rac1 null cells. Cells expressing V12-Rac1 had a significantly lower abundance of MMP-9 mRNA than cells infected with the empty vector (Figure 2B). Likewise, over expression of a dominant negative Rac1 (N17) in WT macrophages increased MMP-9 mRNA levels in these cells (Figure 2C). In aggregate, these results demonstrate that Rac1-mediated inhibition of macrophage-specific MMPs is exclusive for MMP-9, and Rac1 suppresses MMP-9 expression in macrophages at the level of transcription.

SP-1 and AP-1 are necessary for MMP-9 gene expression—Because MMP-9 appeared to be modulated at the level of transcription, we next addressed which regulatory elements in the MMP-9 promoter accounted for greater promoter activity in Rac1 null cells. MMP-9 gene expression is controlled by multiple transcription factors, including NF-κB and AP-1, which are known to be involved in monocyte and macrophage host defense (29,31). Two AP-1 binding sites are present in the MMP-9 promoter and are separated by 447 bp. Directly upstream of the distal AP-1 site is a GC-rich region that contains an SP-1 binding site. Therefore we investigated the role of these factors in the regulation of MMP-9 promoter activity in Rac1 null cells. Truncated constructs with the NF-κB (-584/+21), SP-1 (-544/+21), and AP-1 (-102/+21 & -69/+21) consensus sites deleted from the MMP-9 promoter were cloned into the pGL3 basic expression vector (Figure 3A). WT and Rac1 null cells were transiently transfected with each vector and luciferase activity was determined. MMP-9 promoter activity was significantly greater in Rac1 null cells compared to WT cells (Figure 3B). All the truncation mutants lacked a significant region of the promoter spanning about 616 bp upstream of the NF-κB site. We determined whether this region (-1285/-668) contributed to MMP-9 transcriptional regulatory activity. Promoter activity of Rac1 null cells transfected with the construct (-668/+21) was not significantly different than that observed in cells transfected with the full length plasmid (data not shown). Deletion of the NF-κB binding site from the full-length MMP-9 promoter did not alter the activity of the promoter in Rac1 null cells. On the other hand, successive deletions of the SP-1 and the distal and proximal AP-1 binding sites progressively attenuated the promoter activity in Rac1 null cells (Figure 3B). In contrast, none of the truncated mutants affected luciferase activity in WT cells. Deletion of all four regulatory elements, however, decreased MMP-9 promoter activity in Rac1 null cells to a similar level observed in WT cells. These results strongly suggest that the three cis-acting elements that bind SP-1 and AP-1 are required for driving MMP-9 gene expression in Rac1 null cells.

Based on the above results coupled with the fact that the truncated NF-κB mutant had no effect on MMP-9 promoter activity, we further evaluated the relative contributions of SP-1 and AP-1. MMP-9 luciferase constructs with mutations in one or all of the three binding sites were generated (Figure 3C). Results obtained with Rac1 null cells transfected with these mutants were compared to luciferase activity observed in WT cells transfected with MMP-9wt. Single mutations in SP-1 (SP-1mut), distal AP-1 (AP-1mut1), or proximal AP-1 (AP-1mut2) binding sites significantly attenuated MMP-9 promoter activity in Rac1 null cells (Figure 3D). The decreases in luciferase activities observed with SP-1mut and AP-1mut1 were similar suggesting that both these regions were of equal importance in regulating the MMP-9 promoter. Cells expressing the AP-1mut2 resulted in a relatively greater decrease in MMP-9 promoter activity.
None of these single mutations were sufficient to completely inhibit MMP-9 promoter activity in Rac1 null cells to the level observed in WT cells. In contrast, dual mutations of SP-1 and either the distal or proximal AP-1 binding sites, as well as triple mutations of all three regulatory regions, were found to decrease the activity of the MMP-9 promoter in Rac1 null cells to WT levels. Taken together, these results suggest that Rac1 inhibits MMP-9 expression by regulating SP-1 and AP-1 transcriptional activity. Furthermore, none of the three cis-acting elements alone was sufficient to regulate MMP-9 transcription in macrophages.

**SP-1 and AP-1 DNA binding and transcriptional activity are increased in Rac1 null cells**—To ascertain the role of SP-1 and AP-1 in regulating MMP-9 promoter activity, we determined whether there were differences in SP-1 and AP-1 DNA binding in WT and Rac1 null cells. Nuclear protein from WT and Rac1 null cells were incubated with labeled consensus oligonucleotides of SP-1 and AP-1 and separated on a polyacrylamide gel. There was significantly greater DNA binding of SP-1 and AP-1 in Rac1 null cells compared to WT cells (Figure 4A). In fact, WT cells had essentially no SP-1 or AP-1 DNA binding, but NF-κB DNA binding was equally present in both cells types (data not shown).

Because DNA binding does not necessarily correlate with transcriptional activity, we next determined the role of Rac1 in regulating SP-1- and AP-1-dependent transcription using a luciferase reporter plasmid. WT and Rac1 null cells were transiently transfected with either pSP-1-luc or pAP-1-luc and separated on a polyacrylamide gel. Luciferase activity in Rac1 null macrophages was at least 3-fold higher than in WT macrophages in both SP-1- (Figure 4B) and AP-1-driven luciferase expression (Figure 4C).

Total and phosphorylated SP-1 has been observed to regulate SP-1-mediated transcriptional activation (32-35). Based on the observation that the increase in MMP-9 promoter activity in Rac1 null cells was dependent, in part, on SP-1 and there was greater DNA binding and transcriptional activity of SP-1 in Rac1 null cells, we next examined SP-1 nuclear expression and its level of phosphorylation at T453, which has been associated with transcriptional activity (33-35). Rac1 null cells had significantly greater amounts of p-(T453)-SP-1 and total SP-1 in the nucleus (Figure 4D). Similar levels of SP-1 were present in the cytoplasm of both cells (data not shown).

We also determined the relative expression of c-Fos and c-Jun which form the AP-1 heterodimer in macrophages (36). We first determined levels of the phosphorylated form of c-Jun since it is necessary for transcriptional activity of AP-1. Rac1 null cells had significantly greater p-c-Jun compared to WT cells (Figure 4E). Because induction of transcriptional activity also requires the translocation of c-Fos to the nucleus, we determined the levels of c-Fos expression in these cells. Rac1 null cells had significantly more nuclear c-Fos than WT cells (Figure 4E). Taken together, these data demonstrate that Rac1 negatively regulates SP-1 and AP-1 activation and suggest that, in macrophages, optimal MMP-9 expression is modulated by SP-1 and AP-1.

**H$_2$O$_2$ inhibits MMP-9 expression**—We have previously demonstrated that asbestos-induced pulmonary fibrosis in WT mice is accompanied by increased ROS generation in alveolar macrophages and that intratracheal administration of catalase significantly attenuated pulmonary fibrosis in these mice (23). Since Rac1 has been shown to increase H$_2$O$_2$ generation (21,37), we evaluated the effect of H$_2$O$_2$ on MMP-9 expression. As expected, the generation of H$_2$O$_2$ in WT cells spontaneously increased in a time-dependent manner (Figure 5A). In contrast, H$_2$O$_2$ was barely detectable in Rac1 null cells over a prolonged period of time. This difference was associated with a higher rate of H$_2$O$_2$ production in WT cells compared to Rac1 null cells (Figure 5B).

We next determined the source of Rac1-mediated H$_2$O$_2$ generation. THP-1 cells were infected with an adenoviral vector expressing either an empty or the constitutively active Rac1 (V12). Forty-eight hours later mitochondria and membrane fractions were isolated, and H$_2$O$_2$ generation was determined. We found that over expression of Rac1 had only a slight effect on H$_2$O$_2$ generation from the membrane fraction (Figure 5C), whereas the mitochondrial H$_2$O$_2$
generation was increased greater than 6.5-fold in cells expressing constitutive active Rac1 (Figure 5D). Taken together, these data demonstrate that Rac1 increases H₂O₂ generation and that the primary source is from the mitochondria.

To determine if H₂O₂ generation contributed to MMP-9 transcription, WT cells were infected with adenoviral vectors containing either an empty or a catalase construct. After 48 h, total RNA was isolated. WT cells over expressing catalase had significantly greater MMP-9 mRNA than cells expressing the empty vector (Figure 6A). To confirm that H₂O₂ inhibited MMP-9 expression transcriptionally, WT cells were transiently co-transfected with the MMP-9wt luciferase vector and either an empty or the catalase expression vector. Luciferase activity was determined 48 h later. Similar to the mRNA levels, MMP-9 promoter activity was about 5-fold greater in WT cells over expressing catalase (Figure 6B).

In order to demonstrate the role of mitochondrial H₂O₂ in MMP-9 expression, THP-1 cells were transfected with siRNA for the mitochondrial iron-sulfur protein, Rieske, and MMP-9 expression was determined in conditioned media. We found that MMP-9 secretion was dramatically increased in cells expressing the Rieske siRNA (Figure 6C).

To further confirm that H₂O₂ inhibits MMP-9 expression, we examined the effects of H₂O₂ addition on MMP-9 expression. Rac1 null cells were transiently transfected with the MMP-9wt luciferase vector. After 48 h, cells were exposed to H₂O₂ over a time course up to two h. Cells stimulated with H₂O₂ had significantly less luciferase activity (Figure 6D). The decrease in MMP-9 promoter activity by H₂O₂ was similar at all times. We also examined the effect of H₂O₂ on MMP-9 in vivo utilizing a ChIP assay. Rac1 null cells were cultured with or without exposure to H₂O₂ for two h. SP-1 and c-Jun were immunoprecipitated from the DNA-protein complex, and PCR amplification was performed using primers to detect the SP-1, the distal AP-1, and the proximal AP-1 sites. We found that H₂O₂ reduced SP-1 binding and abolished AP-1 DNA binding to the MMP-9 promoter (Figure 6E). Taken together, these data demonstrate that Rac1-induced H₂O₂ generation inhibits MMP-9 expression at the level of transcription and that over expression of catalase or knockdown of the mitochondrial iron-sulfur protein, Rieske, increases MMP-9 expression.

**ERK MAP kinase is increased in Rac1 null cells and is negatively regulated by H₂O₂—**The signaling pathway linking H₂O₂ generation to inhibition of MMP-9 was evaluated by investigating the role of the ERK MAP kinase because it has been shown to be involved in regulating MMP expression (12). We found that ERK was constitutively activated in Rac1 null cells and was barely detectable in WT cells (Figure 7A). To evaluate ERK in a different manner, THP-1 cells were infected with adenoviral vectors containing an empty, a constitutive active Rac1 (V12), or a dominant negative Rac1 (N17) construct. After 48 h, cells were exposed to asbestos, and an immunoblot analysis was performed for ERK. Asbestos did not activate ERK in cells expressing the empty vector (Figure 7B). ERK was suppressed below control levels in the cells expressing V12, whereas ERK activation was significantly increased in cells expressing the dominant negative (N17) Rac1, and this activation was augmented by asbestos exposure (Figure 7B).

Because H₂O₂ generation is significantly greater in WT macrophages, we questioned whether H₂O₂ had a role in inhibiting ERK activation. THP-1 cells were infected with adenoviral vectors containing either an empty or a catalase construct, and after 48 h cells were exposed to asbestos for two h. Asbestos decreased p-ERK in cells expressing the empty vector, and this was increased significantly in cells expressing the catalase vector alone. ERK activation was further enhanced by asbestos exposure in cells over expressing catalase (Figure 7C). To address the source of H₂O₂ we transfected THP-1 cells with either scrambled or Rieske siRNA and determined ERK activation. Knockdown of the mitochondrial iron-sulfur protein, Rieske, resulted in a dramatic increase in ERK activation with no change in the expression of total ERK (Figure 7D). In aggregate, these data demonstrate that ERK is negatively regulated by Rac1 and mitochondrial H₂O₂.

**ERK modulates MMP-9 transcription by regulating SP-1 and AP-1 nuclear localization and activation—**To determine the effect of ERK
on MMP-9 expression, Rac1 null cells were transfected with a scrambled or ERK siRNA. Nuclear extracts were isolated after 48 h and separated by SDS-PAGE. Knockdown of ERK decreased SP-1 nuclear levels and SP-1 phosphorylation (Figure 8A). Likewise, c-Jun phosphorylation and c-Fos nuclear translocation were dramatically reduced in cells transfected with the ERK siRNA (Figure 8B).

To confirm that these changes in SP-1 and AP-1 proteins regulated MMP-9 transcription, Rac1 null cells were transfected with the MMP-9wt luciferase vector and either an empty or dominant negative ERK expression vector. After 48 h, cells were harvested and luciferase activity was measured. MMP-9 promoter activity in Rac1 null macrophages was significantly inhibited in cells expressing the dominant negative ERK (Figure 8C). In aggregate, these data demonstrate that ERK modulates MMP-9 transcription by regulating nuclear localization and phosphorylation of SP-1 and AP-1.

The above data coupled with our promoter mutation analysis suggest that both SP-1 and AP-1 are necessary for MMP-9 transcription in macrophages. Due to the fact that both SP-1 and AP-1 are required we determined whether there was functional interaction between these transcription factors that regulated optimal MMP-9 expression. Rac1 null macrophages were transfected with either an empty vector or SP-1-V5-His. After 24 h, cells were stimulated with H2O2 for 2 h. SP-1 expression was significantly enhanced in cells transfected with the SP-1-V5-His vector (Figure 8D). Whole cell lysates were then subjected to His pull-down. Immunoblot analysis demonstrated that both p-c-Jun and c-Fos directly interacted with SP-1-V5-His. In contrast, stimulation with H2O2 significantly inhibited this interaction (Figure 8D). Taken together, these data are the first to demonstrate that MMP-9 transcription is regulated by Rac1-mediated H2O2 generation by modulating the activation and direct interaction of SP-1 and AP-1 transcription factors.

Absence of Rac1 increases MMP-9 expression in vivo—To address whether the above in vitro observations had biological significance, we determined MMP-9 gene expression in macrophages isolated from the lungs of WT and Rac1 null mice exposed to asbestos. Asbestos induces the generation of ROS, especially H2O2, in alveolar macrophages and, thus, recapitulates the in vitro conditions (23). We first confirmed that the alveolar macrophages from Rac1 null mice did not express Rac1 by performing an immunoblot analysis of whole cell lysates of BAL cells obtained from mice exposed to chrysotile asbestos. More importantly, alveolar macrophages obtained from asbestos-exposed Rac1 null mice had an active ERK MAP kinase in contrast to WT mice (Figure 9A). To determine the relative expression of MMP-9 in mice after asbestos exposure, total RNA was isolated from alveolar macrophages, and MMP-9 mRNA was measured by quantitative real-time PCR. The in vivo data corroborated the in vitro data by demonstrating that MMP-9 mRNA was significantly greater in cells isolated from Rac1 null mice compared to WT mice (Figure 9B).

In order to confirm that these differences were due to H2O2 generation as our in vitro data suggests, we exposed WT mice to asbestos followed by daily administrations of either catalase or vehicle for 20 days. MMP-9 mRNA in macrophages obtained from WT mice administered catalase was more than 7-fold greater when compared to mice given vehicle alone, which also agrees with our in vitro results (Figure 9C).

To verify the importance of MMP-9 in regulating the development of fibrosis, WT and MMP-9-/- mice were exposed to asbestos. After 21 days, the mice were euthanized and BAL fluid was obtained. WT and MMP-9-/- mice developed a similar extent of fibrosis as determined by measuring hydroxyproline concentrations in the BAL fluid (Figure 9D). We also performed Masson’s trichome staining on lung sections obtained from WT, MMP-9-/-, and Rac1 null mice 21 days after asbestos exposure. Both WT and MMP-9-/- mice had extensive deposition of collagen in peribronchial and parenchymal lung sections, whereas Rac1 null mice has no collagen deposition after asbestos exposure (Figure 9E).

To provide a direct link between regulation of MMP-9 expression by Rac1 and fibrosis development, human lung fibroblasts (HFL-1) were cultured for 24 h in conditioned media
obtained from WT and Rac1 null macrophages in the presence of asbestos. MMP-9 activity in conditioned media was determined by zymography. As above shown, MMP-9 activity was dramatically increased in conditioned media collected from Rac1 null macrophages compared to WT cells (Figure 9F). To determine the effect of MMP-9 on collagen deposition, we measured the amount of procollagen and collagen I secreted by HFL-1 cells incubated with WT and Rac1 null macrophage conditioned media. Fibroblasts exposed to Rac1 null conditioned media secreted significantly less procollagen and collagen I compared to cells incubated with WT media (Figure 9F). In aggregate, these results support the hypothesis that MMP-9 secreted by macrophages modulate collagen deposition by fibroblasts and provide direct evidence that lack of MMP-9 is sufficient for fibrosis development. Furthermore, these results indicate that in macrophages MMP-9 expression is regulated by Rac1-mediated mitochondrial H2O2 generation and that MMP-9 plays an important role in attenuating the development of pulmonary fibrosis.

DISCUSSION

There is limited data regarding the molecular mechanisms regulating MMP-9 expression and activity in pulmonary fibrosis. MMP-9 is a critical molecular target that regulates the fibrotic phenotype. In this study, we demonstrate for the first time that Rac1-mediated mitochondrial H2O2 generation inhibits MMP-9 transcriptional activity via inhibiting SP-1 and AP-1 DNA binding, transcriptional activity, and direct interaction in macrophages. Evidence in support of this pathway include i) Rac1 null cells exhibit constitutively high MMP-9 mRNA expression, ii) promoter deletional and mutational analysis uncovered a regulatory region (-561/-77) that confers Rac1 inhibitory response elements, iii) Rac1-induced H2O2 generation was derived from the mitochondria, iv) MMP-9 transcriptional activity was suppressed by Rac1-induced H2O2, v) Rac1 and H2O2 inhibited ERK MAP kinase which is essential for SP-1 and AP-1 transcriptional activation, vi) collagen deposition by human lung fibroblasts is abolished when cultured in conditioned media from Rac1 null cells, and vii) targeted deletion of Rac1, knockdown of the mitochondrial iron-sulfur complex III subunit, Rieske, and modulation of H2O2 in mice differentially regulates expression of MMP-9. In aggregate, these observations provide a new mechanistic model linking Rac1 and mitochondrial-generated H2O2 to the pathobiology of asbestos-induced pulmonary fibrosis.

Rac1 is a member of the family of Rho GTPases, and it regulates several cellular functions, such as actin polymerization, the assembly of NADPH oxidase in non-phagocytic cells, cell adhesion, and cell differentiation (16). Compared to the other isoforms of Rac, Rac1 is ubiquitously expressed and is abundant in macrophages (16). Rac1 activation increases the generation of H2O2 in cells (21,38). Pulmonary fibrosis is characterized by aberrant ECM remodeling, and this process can be influenced by H2O2. Previous studies have linked Rac1 to MMP activity though the activation of ROS. In fibroblasts, Rac1 increases ROS via activation of the NADPH oxidase and subsequent NF-κB-dependent genes, including collagenase-1 (21). Here, we found that WT and Rac1 null cells had similar levels of NF-κB DNA binding and that NF-κB did not regulate MMP-9 expression. Rac1 has also been shown to up-regulate TIMP-1 via ROS-dependent activation of AP-1 (39). It is not clear from this study if MMPs were inhibited by TIMP-1. We found that there was no significant difference in TIMP gene expression between WT and Rac1 null cells (data not shown), thus, we focused on the transcriptional regulation of MMP-9. Our novel results unequivocally demonstrate that Rac1 plays a crucial role in negatively regulating the expression of the macrophage-specific matrix degrading enzyme, MMP-9. This regulation is specific for MMP-9 and not other macrophage-specific MMPs.

In the current study, Rac1 regulated MMP-9 expression via H2O2 production. In non-phagocytic cells the primary supply of Rac1-mediated H2O2 is the NADPH oxidase (21,37,40). In contrast, Rac2 regulates NADPH oxidase activity in macrophages (41-43). Another important source of H2O2 is the mitochondria. Rac1 modulation of mitochondrial ROS generation has only been
described in fibroblasts via engagement of integrins (44-45). Our study is the first to demonstrate that Rac1 induces H$_2$O$_2$ generation from the mitochondria in macrophages. Moreover, our data using the Rieske siRNA suggest that complex III is important for H$_2$O$_2$ generation. These data are novel in that Rac1-mediated H$_2$O$_2$ abrogates MMP-9 transcription.

H$_2$O$_2$ is known to cause extracellular post-translational activation of MMPs via oxidative cleavage of a conserved cysteine residue (10). In addition, H$_2$O$_2$ also increases MMP-9 expression by activating cell signaling pathways such as the MAP kinase pathway (11-13). Both the ERK and p38 MAP kinase pathways have been shown to be involved in MMP expression. The mechanism by which ROS, especially H$_2$O$_2$, increase MAP kinase activation is primarily by inhibition of redox sensitive protein tyrosine and dual-specificity MAP kinase phosphatases (46-47). The novel aspect of our study is that we found that ERK is inhibited by H$_2$O$_2$ and is recovered by over expression of catalase. The relationship of Rac1 to ERK activation is uncertain. One study suggests that Rac2 and RhoA cooperate with Raf to activate ERK2 (48). In contrast, our results demonstrate that Rac1 inhibits ERK activation by increasing the generation of H$_2$O$_2$. We have previously demonstrated that oxidative inactivation of MKPs by ROS in macrophages results in p38 MAP kinase activation and inhibition of ERK (49-50). Because Rac1 is an upstream activator of the p38 MAP kinase, it is plausible that Rac1-mediated H$_2$O$_2$ negatively regulates MMP-9 expression via this differential MAP kinase activation.

ROS, including H$_2$O$_2$, regulate transcription factors, such as AP-1. The proximal AP-1 site is common to all MMPs with the exception of MMP-2 and MMP-11 and has been reported to be essential for basal promoter activity of MMP-1, MMP-3, and MMP-9 (12,51). The proximal AP-1 site has long been thought to play a dominant role in the transcriptional activation of the MMP promoters, particularly in response to stimulation with phorbol esters (51). We have found that the macrophage AP-1 is a heterodimer primarily composed of c-Fos and c-Jun (52), and both c-Fos expression and c-Jun phosphorylation have been linked to MMP gene expression (12,53). The distal AP-1 site has not been investigated in most studies, but we found that both the distal and the proximal AP-1 sites contribute to optimal promoter activity. More importantly, our data demonstrates that although both AP-1 sites contribute to MMP-9 expression, the MMP-9 gene also requires the activity of another cis-acting element, SP-1, for optimal promoter activity.

The involvement of SP-1 in regulating MMP-9 promoter activity has not been well-studied. SP-1 partially contributes to MMP expression in concert with other cis-acting elements, including NF-$\kappa$B (54-56). To our knowledge, DNA binding and transcriptional activity of SP-1 was not evaluated in any of the prior studies. In contrast, we observed significant differences in SP-1 DNA binding by electrophoretic mobility shift and ChIP assays. We also show significantly greater SP-1-driven transcription in the absence of Rac1. Depending on the site of phosphorylation, the activity of SP-1 can either increase, decrease, or not alter SP-1 activity (32). However, phosphorylation of T453 has been shown to increase SP-1 transcriptional activity in fibroblasts and smooth muscle cells (33-35). These studies corroborate our data showing that Rac1 null cells have increased SP-1 phosphorylation at T453 and SP-1-driven transcriptional activity compared to WT cells. Although SP-1 contributes to regulating MMP-9 expression in macrophages, its role is accentuated by recruiting AP-1 (c-Jun and c-Fos) to the MMP-9 promoter. SP-1 is known to interact with c-Jun in the presence of histone deacetylase 1 to enhance the expression of 12(S)-lipoxygenase (57). The novel aspect of our study is that H$_2$O$_2$ regulates MMP-9 gene transcription by modulating c-Jun/c-Fos interaction with over expressed SP-1.

The results presented in this study demonstrate a mechanism by which Rac1 null mice are protected from developing pulmonary fibrosis after asbestos exposure. Increased MMP-9 expression in Rac1 null macrophages in culture and in macrophages isolated from BAL of mice exposed to asbestos strongly suggest that increased matrix degradation protects against asbestos-induced fibrosis. Furthermore, by demonstrating that catalase administration to asbestos-exposed WT mice increased MMP-9 expression.
expression in alveolar macrophages, we emphasize the role of \textit{in vivo} \( \text{H}_2\text{O}_2 \) production on regulating MMP-9 gene expression in lung macrophages exposed to asbestos. In aggregate, these results define the molecular mechanisms by which Rac1 regulates MMP-9 expression and may provide important clues to prevent the development of pulmonary fibrosis.

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ABBREVIATIONS
AP-1, activator protein 1; BAL, bronchoalveolar lavage; ChIP assay, chromatin immunoprecipitation assay; ECM, extracellular matrix; HRP, horseradish peroxidase; MAP, mitogen-activated protein; MKP, mitogen-activated protein kinase phosphatase; MMP, matrix metalloproteinase; pHPA, p-hydroxyphenyl acetic acid; ROS, reactive oxygen species; SP-1, specificity protein 1; TBP, TATA-binding protein; TIMP, tissue inhibitors of metalloproteinases.

FIGURE LEGENDS
Figure 1. MMP-9 expression is higher in Rac1 null macrophages compared to WT cells. A, WT and Rac1 null macrophages were incubated for 24 h in RPMI 1640 medium containing 0.5% serum. MMP-9 in conditioned medium was analyzed by immunoblot analysis. MMP-9 activity was determined by zymography. B, WT and Rac1 null cells were incubated for 6 h in RPMI 1640 medium containing 0.5% serum. Total RNA was isolated and MMP-9 mRNA was determined by real-time PCR. Results show mean±SEM of arbitrary units of MMP-9 mRNA normalized to HPRT mRNA. n = 3. *p < 0.05 vs WT. C, WT and Rac1 null cells were transiently transfected with the MMP-9-luciferase vector (-1284/+21) and 48 h later luciferase activity determined. Results show mean±SEM of firefly luciferase normalized to Renilla luciferase. n = 3. *p < 0.05 vs WT.

Figure 2. Rac1 negatively regulates MMP-9 and not other MMPs. A, WT and Rac1 null cells were incubated for 6 h in RPMI 1640 medium containing 0.5% serum. Total RNA was isolated and MMP-12 mRNA was determined by real-time PCR. Rac1 null and WT cells were infected with a replication-deficient adenovirus vector expressing an empty vector (Ad.CMV) or either B, a constitutive active Rac1 (Ad5.CMV.V12Rac1) or C, dominant negative Rac1 (Ad5.CMV.N17Rac1) at 500 moi. After 48 h, MMP-9 mRNA was determined by real-time PCR. Results show mean±SEM of arbitrary units of MMP-9 mRNA normalized to HPRT mRNA. n = 3. *p < 0.05 vs Empty.

Figure 3. MMP-9 promoter activity requires SP-1 and AP-1. A, the full length (-1284/+21) and its various truncation mutants inserted into pGL3 basic expression vector are shown. Vertical lines represent the transcription factors with their 5’ end binding sites shown in parentheses. Arrows represent the transcription start site. B, WT and Rac1 null cells were transiently transfected with each of the 5 plasmid constructs shown in A. After 48 h firefly and Renilla luciferase activities were measured. Results show mean±SEM of firefly luciferase normalized to Renilla luciferase activity. n = 3. a=p < 0.05 vs respective WT control. b=p < 0.05 vs Rac1 null cells transfected with the full length MMP-9-luciferase plasmid (-1284/+21). C, the full length (-1284/+21) MMP-9 construct and constructs with mutations (X) at specific cis-acting sites that bind SP-1 and AP-1 are shown. D, WT and Rac1 null cells were transiently transfected with each of the plasmid mutants shown in C. After 48 h, firefly and Renilla luciferase activities were determined. Results show mean±SEM of firefly luciferase normalized to Renilla luciferase. n = 3. a=p < 0.05 vs WT. b=p < 0.05 vs Rac1 null cells transfected with MMP-9wt. c=p < 0.05 vs Rac1 null cells transfected with Ap-1mut1.

Figure 4. Rac1 null cells have increased SP-1 and AP-1 DNA binding. A, nuclear proteins were isolated from WT and Rac1 null cells, and binding reactions were performed with a consensus SP-1 and AP-1 oligonucleotides labeled with [γ-32P]ATP. Cells were transiently transfected with either B, pSP-1-
luc or C, pAP-1-luc. After 48 h, firefly and Renilla luciferase activities were determined. Results show mean±SEM of firefly luciferase normalized to Renilla luciferase. n = 4. *p < 0.05 vs WT. D, nuclear proteins from WT and Rac1 null cells were separated by SDS-PAGE. Immunoblot analysis was performed using p(T453)-SP-1, SP-1, E, p-c-Jun, and c-Fos antibodies. Immunoblot analysis for TBP was performed to confirm equal loading of proteins.

Figure 5. Rac1 increases H₂O₂ generation from the mitochondria. A, WT and Rac1 null cells were incubated for 4 h in phenol red–free HBSS supplemented with glucose, HEPES, sodium bicarbonate, pHPA, and HRP. The amount of H₂O₂ released into the medium was followed spectrofluorometrically by measuring the formation of the fluorescent dimer [pHPA]₂ at excitation and emission wavelengths of 323 and 400 nm, respectively. Results show H₂O₂ generated in pmol per mg cell protein. B, H₂O₂ generation from A is represented as a unit of time. THP-1 cells were infected with adenovirus containing either an empty or a constitutive active Rac1 (V12). C, membrane or D, mitochondria fractions were isolated. The amount of H₂O₂ released into the medium was followed spectrofluorometrically by measuring the formation of the fluorescent dimer [pHPA]₂ at excitation and emission wavelengths of 323 and 400 nm, respectively. H₂O₂ release is expressed as fold increase compared to control, n=4. *p < 0.05 vs Empty.

Figure 6. H₂O₂ decreases MMP-9 expression. A, WT cells were infected with a replication-deficient adenovirus vector expressing either an empty vector (Ad.CMV) or a human catalase expression vector (Ad.CMV.Catalase) at 500 moi. After 48 h, total RNA was isolated and MMP-9 mRNA was measured by real-time PCR. Results show arbitrary units of MMP-9 mRNA normalized to HPRT mRNA. n = 3. *p < 0.05 vs Empty. B, WT cells were transiently transfected with MMP-9 luciferase vector and either an empty vector or a Catalase expression vector. After 48 h, firefly and Renilla luciferase activities were determined. Results show mean±SEM of firefly luciferase normalized to Renilla luciferase. n = 3. *p < 0.05 vs Empty. C, THP-1 cells were transfected with scrambled or Rieske siRNA. After 48 h, conditioned medium was collected and cells were lysed. MMP-9 in conditioned medium and Rieske protein expression in cells were analyzed by immunoblot analysis. D, Rac1 null cells were transiently transfected with MMP-9 luciferase. After 48 h, cells were stimulated over time up to 2 h with 25 μM H₂O₂ in RPMI 1640 media containing 0.5% serum. Firefly and Renilla luciferase activities were determined. Results show mean±SEM of firefly luciferase normalized to Renilla luciferase. n = 3. *p < 0.05 vs control. E, Rac1 null cells were cultured in the presence or absence of 25 μM H₂O₂ for 2 h. ChIP assays were performed as described in experimental procedures. Cross-linked chromatin from Rac1 null cells was subjected to immunoprecipitation using anti-SP-1 or anti-c-Jun or normal rabbit IgG. Input DNA (2% of total) and immunoprecipitated DNA were then analyzed by PCR using primers to the SP-1 binding site (IP1; 76 bp product), the distal AP-1 binding site (IP2; 116 bp), the proximal AP-1 binding site (IP3; 101 bp), or ribosomal protein L30 (IP4; 159 bp) as a negative control. PCR products were separated on a 2% agarose gel.

Figure 7. Rac1 and H₂O₂ modulate ERK activation. A, Whole cell lysates of WT and Rac1 null cells were isolated. B, THP-1 cells were infected with adenovirus vector containing an empty, a constitutive active Rac1 (V12), or dominant negative Rac1 (N17). After 48 h, cells were exposed to asbestos and whole cell lysates were isolated. C, THP-1 cells were infected with adenovirus containing either an empty or a catalase construct. After 48 h, the cells were exposed to asbestos, and whole cell lysates were isolated. D, THP-1 cells were transfected with 100 nM of scrambled or Rieske siRNA and 48 h later whole cell lysates were isolated. Immunoblot analyses were performed for p-ERK and ERK for activation and equal loading, respectively.

Figure 8. ERK regulates SP-1 and AP-1 activation and MMP-9 transcription. THP-1 cells were transfected with either scrambled or ERK siRNA. After 48 h, whole cells lysates and nuclear extracts were isolated. Immunoblot analyses were performed for ERK in whole cell lysates and for A, p-SP-1 and SP-1, and B, p-c-Jun and c-Fos in nuclear extracts. β-actin and Lamin A/C immunoblot analysis was
performed for equal loading. C, Rac1 null cells were transiently transfected with MMP-9 WT luciferase vector and either an empty or the dominant negative ERK (pCMV-HA-ERK2 K/A) vector. After 48 h, firefly and Renilla luciferase activities were determined. Results show mean ± SEM of firefly luciferase normalized to Renilla luciferase. n = 4. D, Rac1 null cells were transiently transfected with either an empty or pcDNA3.1-SP-1-V5-His vector. After 24 h, cells were cultured in the presence or absence of H2O2 25 μM for 2 h. Immunoblot analysis for SP-1 was performed in whole cell lysates. SP-1-V5-His proteins were purified using metal (cobalt) affinity resin overnight. Eluted proteins were separated by SDS-PAGE, and immunoblot analysis was performed for V5, p-c-Jun, and c-Fos.

Figure 9. MMP-9 expression is greater in BAL cells from Rac1 null mice and from catalase administered WT mice. A, WT and Rac1 null C57BL/6 mice were intratracheally administered 100 μg/mouse of chrysotile asbestos in 50 μL normal saline. Twenty one days later, animals were euthanized, and BAL was collected. Whole cell lysates from BAL cells were separated by SDS-PAGE, and immunoblot analysis was performed for Rac1, p-ERK, and ERK. B, WT and Rac1 null mice were exposed to asbestos as in A. Twenty one days later, animals were euthanized, and BAL cells were collected and MMP-9 mRNA was measured by real-time PCR. Results show arbitrary units of MMP-9 mRNA normalized to β-Actin mRNA. n = 3. *p < 0.05 vs WT. C, WT mice were exposed to 100 μg/mouse of chrysotile asbestos, and on each day for the next 20 days were administered either vehicle (water) or catalase (2000 U/mouse). On day 21 the animals were euthanized, and BAL cells were collected. MMP-9 mRNA was measured by real-time PCR. Results show arbitrary units of MMP-9 mRNA normalized to β-actin mRNA. n = 4. *p < 0.05 vs vehicle. D, WT and MMP-9−/− C57BL/6 mice were exposed to 100 μg of chrysotile asbestos intratracheally. Twenty one days later the animals were euthanized and BAL fluid was collected. Hydroxyproline concentration was determined in BAL fluid and is expressed as μg per mg protein. n = 6. E, WT, MMP-9−/−, and Rac1 null mice were exposed to 100 μg of chrysotile asbestos intratracheally. Twenty one days later the animals were euthanized, and lungs were removed and processed for collagen deposition using Masson’s trichome stain. Representative micrographs of one out of 5 animals are shown. Bar indicates 200 μm. F, MMP-9 activity in conditioned media from WT and Rac1 Null macrophages was determined by zymography. Human lung fibroblasts (HLF-1) were cultured for 24 h with conditioned media from WT or Rac1 null macrophages in the presence of chrysotile asbestos. Procollagen and collagen I secreted by the fibroblasts into the media were determined by immunoblot analysis. Fibroblasts cultured in media containing 0.5% serum in the absence of asbestos is shown as control.
Figure 1

(A) Secreted MMP-9

(B) MMP-9 mRNA (Arbitrary Units)

(C) MMP-9 Promoter Activity

* indicates statistically significant difference.
**Figure 2**

A. MMP-12 mRNA expression for WT and Rac1 Null.

B. MMP-9 mRNA expression for WT and Rac1 Null.

C. MMP-9 mRNA expression for N17 and V12.
Figure 3

A

NF-κB (-598)
SP-1 (-561)
AP-1 (-531)
AP-1 (-77)
-1284
-584
-544
-102
-69

B

MMP-9 Promoter Activity
Firefly/Renilla (RLU)

WT
Rac1 Null

0 250 500 750 1000

a, b

a

a, b

a, b

b
Figure 3

MMP-9 Promoter Activity
Firefly/Renilla (RLU)

0 25 50 75

WT
Null
Null
Null
Null
Null
Null
Null
Null
Null
Null
Null
Null
Null

a, b
a, b
a, b, c
b
a, b
b

MMP-9 wt
MMP-9 wt
SP-1 mut
AP-1 mut1
AP-1 mut2
SP-1 mut AP-1 mut1
SP-1 mut AP-1 mut2
SP-1 mut AP-1 mut1&2

NF-κB (-598)
SP-1 (-561)
AP-1 (-531)
AP-1 (-77)
Figure 4
Figure 5
Figure 6

A

MMP-9 mRNA
(Arbitrary Units)

WT

Empty

Catalase

B

MMP-9 Promoter Activity
Firefly/Renilla (RLU)

WT

Empty

Catalase

C

Control siRNA
Rieske siRNA

Rieske
β-Actin
MMP-9

D

MMP-9 Promoter Activity
Firefly/Renilla (RLU)

H2O2 (minutes)

Rac1 Null

0

30

60

120

* * *
Figure 7
**Figure 8**

**A** Control siRNA + - 
ERK siRNA - + 

|          | ERK | β-actin | p-SP-1 | SP-1 | Lamin A/C |
|----------|-----|---------|--------|------|-----------|
| Control  | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| ERK siRNA| ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) | ![Image](image10.png) |

**B** Control siRNA + - 
ERK siRNA - + 

|          | ERK | β-actin | p-c-Jun | c-Fos | Lamin A/C |
|----------|-----|---------|---------|-------|-----------|
| Control  | ![Image](image11.png) | ![Image](image12.png) | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) |
| ERK siRNA| ![Image](image16.png) | ![Image](image17.png) | ![Image](image18.png) | ![Image](image19.png) | ![Image](image20.png) |

**C**

MMP-9 Promoter Activity
Firefly/Renilla (RLU)

|          | Empty | DN ERK |
|----------|-------|--------|
|          | ![Image](image21.png) | ![Image](image22.png) |

**D**

Lysate
Affinity Purification

|          | SP-1 | V5 | p-c-Jun | c-Fos |
|----------|------|----|---------|-------|
| Empty Vector | ![Image](image23.png) | ![Image](image24.png) | ![Image](image25.png) | ![Image](image26.png) |
| SP-1-V5-His | ![Image](image27.png) | ![Image](image28.png) | ![Image](image29.png) | ![Image](image30.png) |

**Figure 8**
Figure 9
Figure 9
Rac1-mediated mitochondrial H$_2$O$_2$ regulates MMP-9 gene expression in macrophages via inhibition of SP-1 and AP-1
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