Platelet-Activating Factor Overturns the Transcriptional Repressor Disposition of Sp1 in the Expression of *MMP-9* in Human Corneal Epithelial Cells

Faramarz Taheri[1] *and Haydee E. P. Bazan*[1,2]

Purpose. Matrix metalloproteinase (MMP)-9 is induced in corneal epithelial cells stimulated with platelet-activating factor (PAF), and interferes with the normal reepithelialization of wounded cornea. Here the transcriptional regulation of *MMP-9* gene expression by PAF was investigated in human corneal epithelial cells (HCECs).

Methods. DNA-binding activity of NFκB, Sp1, and AP-1 was determined in quiescent and PAF-stimulated HCECs by electrophoretic mobility shift assay (EMSA). A series of 5′ deleted human *MMP-9* promoter-luciferase reporter constructs was transiently transfected into HCECs, and luciferase activity was examined after stimulation with PAF. Mutagenesis and specific deletions of some elements in the *MMP-9* promoter were also introduced and analyzed. Phosphorylation of Sp1 and MEK/ERK pathway proteins was examined by Western blot analysis. Activation of Sp1 and MMP-9 was also determined by ELISA and zymography, respectively, in the absence or presence of the MEK inhibitor PD98059.

Results. DNA-binding activity of NFκB, Sp1, and AP-1 was upregulated by PAF with a peak at 1 hour after stimulation. A region spanning −670 to −460 relative to the transcription start point was required for the induction of the *MMP-9* promoter by PAF. Mutation of the −79AP-1 or −600NFκB motif reduced the activity of *MMP-9* promoter and the induction of gene expression by PAF. In untreated HCECs, mutation of the −558Sp1 motif upregulated gene expression, but it caused a significant decrease in the promoter activity induced by PAF. Inhibition of MEK activity eliminated the PAF-induced phosphorylation and activation of Sp1 and abolished the upregulation of MMP-9 expression and activity.

Conclusions. These findings demonstrate that collaboration between several regulatory elements is required for the induction of *MMP-9* promoter activity by PAF and that PAF overturns the repressor effect of Sp1 through activation of the MEK/ERK signaling cascade. (*Invest Ophthalmol Vis Sci.* 2007;48:1931–1941) DOI:10.1167/iovs.06-1008

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Supported by United States Public Health Science Grant RO1 EY04928 from the National Eye Institute, National Institutes of Health (HEPB).

Submitted for publication August 24, 2006; revised November 17, 2006; accepted March 6, 2007.

Disclosure: **F. Taheri**, None; **H.E.P. Bazan**, None

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The corneal surface is frequently exposed to injuries, and rapid repair of corneal epithelium is essential to the recovery of clear vision. Any interference or delay in the wound-healing process may cause a number of pathologic conditions ranging from simple haze to ulceration or even perforation of the cornea.[1] Moreover, because of avascularity that reduces the number of interacting elements, the cornea may be considered an ideal model for evaluating mechanisms of tissue repair and epithelial regeneration.

Matrix metalloproteinases (MMPs) constitute a family of zinc-dependent endopeptidases that are involved in proteolytic degradation of extracellular matrix (ECM) components[2] as part of physiological or pathologic processes.[3–6] According to their structures and substrate specificities, MMPs can be classified in four categories: collagenases (MMP-1, -8, -13); gelatinases (MMP-2, -9); stromelysins (MMP-3, -7, -10, -11, -12); and membrane-type (MT) MMPs (MT1-MMP to MT6-MMP).[5,7] MMP-9 (also known as the 92-kDa type IV collagenase) catalyzes the cleavage of denatured collagens of all types and of native basement membrane components.[5,8] In the cornea, MMP-9 plays a major role in cell migration[9] because of its ability to facilitate the destruction of type IV collagen–containing basement membrane,[10] which separates the epithelial and stromal layers. MMP-9 is expressed in epithelial cells migrating to resurface the wound bed after an injury[10,11] and in the inflammatory cells infiltrating the wound.[12,13] Overexpression of MMP-9, on the other hand, is linked to imperfect reepithelialization and impaired adhesion complex integrity.[10,14] Overall, the architectural integrity of many tissues depends on a delicate balance between MMPs and their inhibitors. Hence, the regulation of gene transcription and tissue-specific expression of MMP-9 in normal and diseased states are being widely investigated in a search for new therapeutic targets.

Platelet-activating factor (PAF) is a potent lipid mediator of inflammatory reactions that is also implicated in cellular activation, intracellular signaling, and apoptosis.[15–20] Reports from our laboratory show that during sustained corneal inflammation, PAF accumulates in the cornea and contributes to tissue destruction and corneal ulcer formation.[19,20] PAF selectively upregulates gene and protein expression of MMP-1 and MMP-9 in corneal epithelial cells[21,22] and gene expression of two members of the tissue inhibitors of metalloproteinases (TIMPs), TIMP-1 and TIMP-2, with a significant imbalance toward the elevation of *MMP-9* gene expression.[23]

The molecular mechanisms responsible for the activation of *MMP-9* promoter by PAF in corneal epithelial cells are unknown. The present study was, therefore, carried out to identify the regulatory elements in the *MMP-9* promoter, the transcription factors involved, and the role of the MEK/ERK signaling pathway in PAF-induced expression and activity of MMP-9 in human corneal epithelial cells (HCECs). We identified a PAF-responding segment (PRS) spanning 670 to 460 base pairs upstream of the transcription start point of the *MMP-9* gene that plays the most important role in the regulation of gene expression by PAF in HCECs. Although the transcription factors NFκB and AP-1 are partially responsible for the basal

and the PAF-induced activation of *MMP-9* promoter, Sp1 has a divergent function with a negative regulatory effect on *MMP-9* gene expression in quiescent HCECs and an enhancing role in PAF-stimulated cells. Moreover, PAF stimulates the phosphorylation of MEK1/2 and ERK1/2 and of Sp1 in HCECs, and blocking the MEK/ERK cascade resulted in the elimination of PAF-induced MMP-9 expression and activity.

Materials and Methods

Cell Culture

The HCEC line was kindly provided by Roger Beuerman (Department of Ophthalmology, Louisiana State University Health Sciences Center [LSUHSC], New Orleans, LA). Keratinocyte basal medium (KBM) and keratinocyte growth medium (KGM [KBM supplemented with bovine pituitary extract, recombinant human epidermal growth factor, bovine insulin, hydrocortisone, and gentamicin sulfate/amphotericin-B]) were purchased from Cambrex Bioscience Walkersville, Inc. (Walkersville, MD). HCECs were grown in KGM and subcultured twice a week. KGM was replaced with KBM for an overnight growth factor starvation of HCECs before stimulation with 100 nM methyl-carbamyl-PAF (mcPAF [1-O-alkyl-2n-methylcarbamyl-sn-glyceryl-3-phosphoryl-choline]), a nonhydrolyzable PAF analog (Cayman Chemical Co., Ann Arbor, MI), for the times specified in the experiments. LysoPAF (1-O-alkyl-sn-glycero-3-phosphocholine; Sigma, St. Louis, MO) was used as a negative control, and phorbol-12-myristate-13-acetate (PMA; Calbiochem, La Jolla, CA) was used as a positive control. In inhibition studies, HCECs were preincubated for 1 hour with KBM containing 10 μM PAF receptor antagonist LAU8080 (tetrahedral-4,7,8,10 methyl-1 (chloro-2-phenyl)6-(methoxy-4 phenyl)carbamyl-9 pyrido (4′ 3′-4,5) theine (3,2,-f) triazolo-1,2,4(4,3-a) diazopine-1,4); provided by Nicolas G. Bazan, Neuroscience Center of Excellence, LSUHSC) before stimulation with mcPAF. In the MEK inhibition experiments, cells were left untreated or were preincubated for 30 minutes with 20, 30, or 40 μM PD98059 (Calbiochem) before stimulation with PAF.

Plasmid Construction

The construct containing the regulatory sequence of the human *MMP-9* gene from position −2172 to +54 relative to the transcription start point cloned upstream of the luciferase reporter gene of pGL3-Basic vector (−2172/+54Luc) was kindly provided by Douglas D. Boyd (Department of Cancer Biology, MD Anderson Cancer Center, University of Texas, Houston, TX) and was used in this study with permission from Motoharu Seiki (Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Tokyo, Japan). The constructs containing 5′ deletions of *MMP-9* promoter sequence and an empty vector were produced by truncating the full-length plasmid using combinations of *Kpn*I and *Sma*I, *Pst*I, *Xba*I, *Eco*RV, *Bam*HI, *Pvu*II, or *Hin*dIII restriction enzymes (Promega, Madison, WI). Truncation of the −670/−460 segment from the two constructs −1511/+54Luc and −1112/+54Luc was performed using a combination of *Xba*I and *Eco*RV restriction enzymes. Herpes simplex virus thymidine kinase promoter sequence (HSV-TK) was cloned into pGL3-Basic vector (Promega) to prepare a pGL3-TK construct, which was then used to clone the two major regulatory segments from MMP-9 promoter sequence—that is, basal expression segment (BES; −1112/−670) and PRS (−670/−460)—upstream of the HSV-TK promoter to prepare the BES-TK and PRS-TK constructs. Site-specific mutated constructs with point mutations at −600/−591NFκB (GGAATTCCC to *TT*AATTCCC; mutNFκB), −558/−563Sp1 (GGGCGG to GGG*TT*G; mutSp1), or −79/−73AP-1 (TGAGTCA to T*TT*GTCA; mutAP-1) consensus sequences of a plasmid containing the −670/+53 regulatory sequence of the *MMP-9* gene cloned in pGL2-Basic vector were kindly provided by Derek A. Mann (Molecular Cell Biology, Division of Infection, Inflammation and Repair, University of Southampton School of Medicine, Southampton General Hospital, Southampton, UK). Luciferase reporter vectors containing the *cis*-acting DNA-binding element for NFκB, Sp1, and AP-1 (NFκB-Luc, Sp1-Luc, and AP-1-Luc, respectively) were purchased (Panomics Inc., Fremont, CA).

Transient Transfection Assays

HCECs were cultured in 96-well plates (ViewPlate-96; Packard, Meriden, CT) to reach 50% to 60% confluence and were cotransfected with a transfection reagent (FuGene 6; Roche, Palo Alto, CA) at a ratio of 1 μg plasmid DNA, 50 ng pRL-TK (kindly provided by Jay D. Hunt, Department of Biochemistry and Molecular Biology, LSUHSC), and 2.5 μL FuGene 6/mL KBM at 37°C overnight. Activities of the two luciferases were then measured (Dual Glo Luciferase Assay System [Promega]; TopCount Liquid Scintillation Counter [Packard]). Data were normalized to the activity of Renilla luciferase before statistical analyses were performed for comparison of various conditions.

Electrophoretic Mobility Shift Assay

HCECs at 80% confluence were left untreated or were stimulated with 100 nM mcPAF for 0.5, 1, or 2 hours. Crude nuclear extracts were prepared (NE-PER kit; Pierce Biotechnology, Rockford, IL). Consensus oligonucleotides for NFκB, Sp1, and AP-1 were obtained (Promega) and biotinylated (Biotin 3′ End DNA Labeling Kit; Pierce). Binding reactions were performed for 20 minutes at room temperature with 5 μg protein in a mixture containing 2 μL buffer (Gel Shift Binding 5× Buffer; Promega), 1 μL biotinylated consensus oligonucleotide (20 fmol/μL), and nuclease-free water (Promega) up to a total volume of 9 μL. To confirm the specificity of the shifted bands, 1 μL unlabeled consensus oligonucleotide (1.75 pmol/μL) was also included in the mixture in competition tubes. DNA–protein complexes were separated from unbound oligonucleotide by electrophoresis through polyacrylamide gels for nucleic acid analysis (6% DNA Retardation Gels; Invitrogen, Carlsbad, CA), transferred to membranes (Nytran SuperCharge; Whatman-Schleicher & Schuell, Florham Park, NJ), and ultraviolet (UV) light cross-linked (GS Gene Linker UV Chamber; Bio-Rad Laboratories, Hercules, CA) before detection of shifted bands (BrightStar BioDetect Nonisotopic Detection System; Ambion, Austin, TX) and autoradiography (Chemiluminescence BioMax films; Eastman Kodak, Rochester, NY).

DNA-Binding Activity of Sp1

HCECs at 80% confluence were left untreated or were stimulated with 100 nM mcPAF for 1 hour, and nuclear extracts were prepared (Nuclear Extract Kit; Active Motif, Carlsbad, CA), to be tested for the DNA-binding activity of Sp1 (TransAm Sp1/Sp3 Activation Assay kit; Active Motif).

Western Blot Analysis

HCECs were cultured in six-well microplates (Corning, Corning, NY) to 80% to 90% and were stimulated as specified in each experiment. Cells from each well were extracted into 100 μL lysis buffer (20 mM Tris, pH 7.5; 150 mM NaCl; 10 mM EDTA; 200 μM Na_3VO_4; 10 mM NaF; 1 mM phenylmethylsulfonyl fluoride; 5 μg/mL leupeptin; 10 μg/mL aprotinin; 10% glycerol; 1% NP-40) for 8 minutes on ice and centrifuged at 21,000*g* for 5 minutes at 4°C. Supernatants were collected, protein concentration was determined (Bradford Protein Assay Reagent; Bio-Rad), and 10 μg protein from each sample was denatured by heating at 95°C for 7 minutes in a total volume of 25 μL, including 5 μL gel-loading buffer (62 mM Tris, pH 6.8; 6% SDS; 15% β-mercaptoethanol; 40% glycerol; 0.025% bromophenol blue). Proteins were separated on a (8% to 16% Tris-Glycine Gel; Novex, San Diego, CA) and were transferred to a PVDF membrane (Invitrolon; Invitrogen). After incubation with blocking buffer (Odyssey; LI-COR Biosciences, Lincoln, NE), membranes were probed for 1 hour at room temperature with primary antibodies. Either polyclonal rabbit anti–Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA) or monoclonal mouse anti–Sp1 (BD Biosciences PharMingen, San Diego, CA) and polyclonal rabbit anti–actin (Sigma) were used for detection of Sp1. MEK/ERK pathway

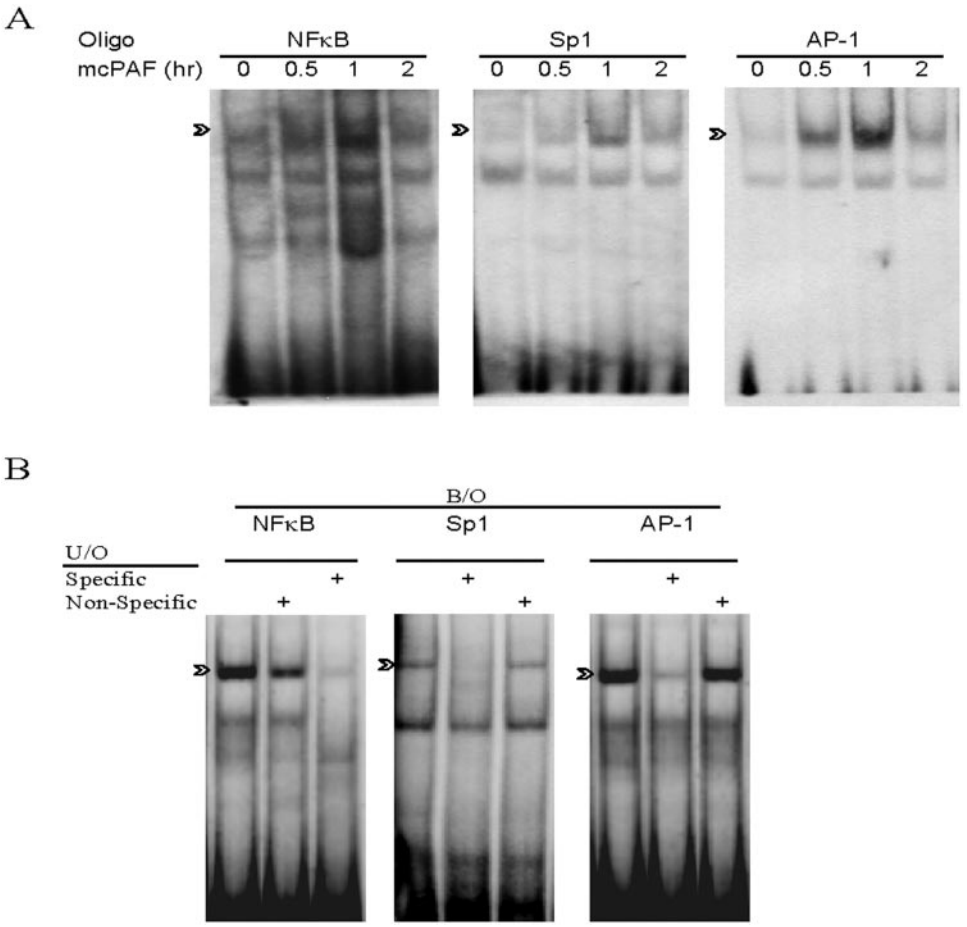

FIGURE 1. DNA-binding activity of NFκB, Sp1, and AP-1 in HCECs stimulated with PAF. (**A**) After overnight growth factor starvation, cultures of HCECs were left untreated (*lane 1*) or were stimulated with 100 nM mcPAF for 0.5, 1, or 2 hours (*lanes 2–4*, respectively), and the nuclear extracts were subjected to EMSA. (**B**) EMSA was carried out with the use of biotinylated oligonucleotide (B/O) in the absence (–) or presence (+) of unlabeled oligonucleotide (U/O) to compete out the specific shifted bands (*arrows*) in nuclear extracts of HCECs stimulated for 1 hour with 100 nM mcPAF.

proteins were detected by using anti–ERK1 (Santa Cruz), which detects ERK1 and ERK2 proteins (ERK1/2), monoclonal mouse anti–phosphorylated ERK1/2 (pERK1/2; Sigma), and rabbit anti–phosphorylated MEK1/2 (pMEK1/2; Cell Signaling, Danvers, MA). Membranes were then washed for 30 minutes with three changes of PBS–0.05% Tween 20 solution, followed by 20 minutes of incubation at room temperature with green (goat anti–rabbit; IRDye 800 [LI-COR Biosciences]) and red (Alexa Fluor 680–conjugated rabbit anti–mouse IgG; [Invitrogen]) secondary antibodies. After another washing step, as already described, specific bands were visualized by scanning (Odyssey Infrared Imaging System; LI-COR Biosciences) using both 800-nm and 700-nm channels.

Immunoprecipitation

Whole cell extract (200 μg) was mixed with anti–Sp1 antibody (4 μg; Santa Cruz Biotechnology) for 2 hours followed by overnight incubation with 25 μL protein A/G PLUS-agarose at 4°C. Beads were pelleted at 1000*g* for 5 minutes at 4°C and were washed 3 times with lysis buffer before denaturation of proteins and immunoblotting of supernatants, as described in Western Blot Analysis, using polyclonal rabbit anti–Sp1 (Santa Cruz) and either anti–phosphoserine or anti–phosphothreonine mouse monoclonal antibody (Sigma).

Zymography

HCECs were cultured to 70% to 80% confluence and were stimulated as described in each experiment. Conditioned media were collected and centrifuged to remove debris and dead cells, and 10 μL of each supernatant was mixed with an equal volume of zymogram sample buffer (Bio-Rad) and separated (10% Zymogram (Gelatin) Gel; Invitrogen) for 2 hours. The gel was then incubated for 2 hours in renaturing buffer (Zymogram; Invitrogen) and overnight in developing buffer (Zymogram; Invitrogen) before it was stained with 1% Coomassie brilliant blue (Sigma) in distilled water/methanol/acetic acid (4.5:4.5:1) for 30 minutes and washed with distilled water. Gels were examined (Odyssey Infrared Imaging System; LI-COR) using the 700-nm channel.

Statistical Analysis

Comparison between various conditions was tested by analysis of variance (ANOVA); $P < 0.05$ was considered statistically significant. All experiments were performed at least three times.

RESULTS

Activation of NFκB, Sp1, and AP-1 in HCECs Stimulated with PAF

MMP-9 is encoded by a 7.7-kb pair gene with 13 exons, the transcription of which yields a 2.5-kb mRNA[24,25] and is regulated primarily by 670 bp of regulatory sequence, which includes the binding sites for NFκB, AP-1, and Sp1.[25] We investigated the effect of PAF on the level of DNA-binding activity of these transcription factors in HCECs. After treatment of the cells with mcPAF for 0.5, 1, or 2 hours, nuclear extracts were collected, and the DNA-binding activity of the transcription factors NFκB, Sp1, and AP-1 was determined by electrophoretic mobility shift assay (EMSA). Nuclear extract from untreated cells was used as a control for the basal level of transcription factor activities. PAF induced an upregulation of NFκB, Sp1, and AP-1 DNA-binding activity, with a peak at 1 hour after stimulation (Fig. 1A). To confirm the specificity of the shifted bands, EMSA was performed in the presence of

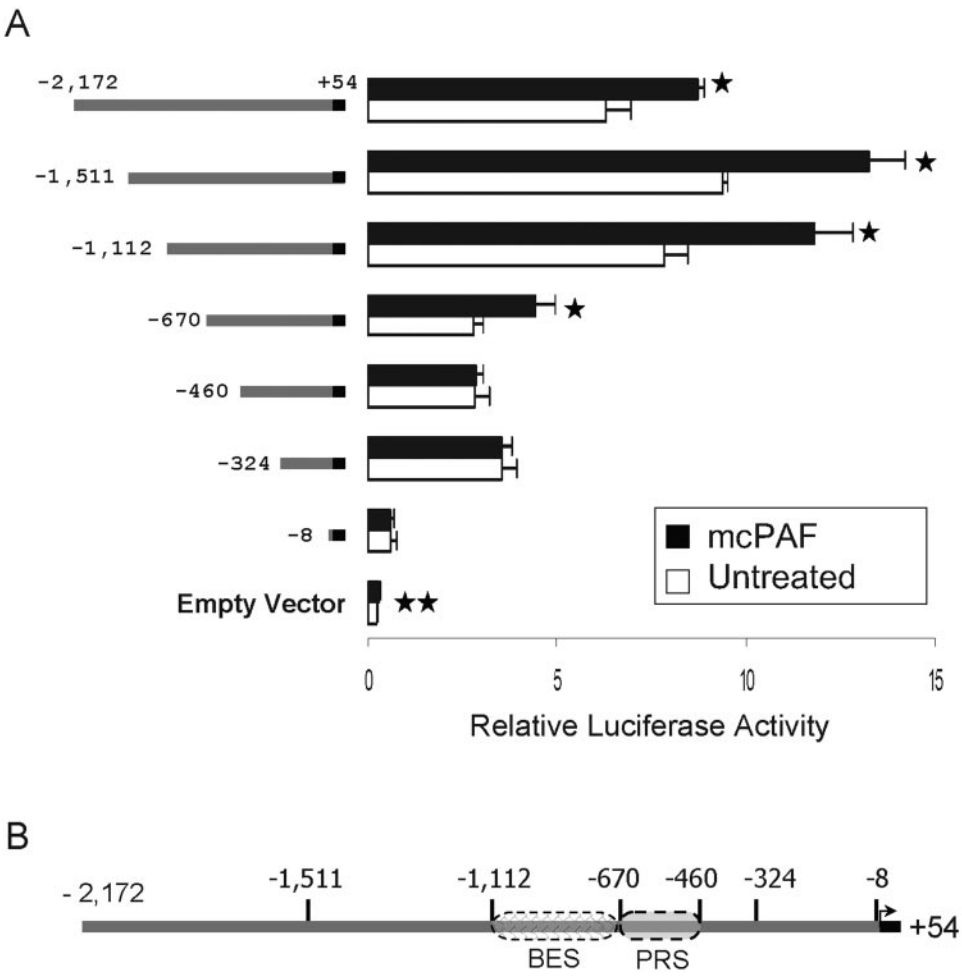

FIGURE 2. Human *MMP-9* promoter activity in HCECs stimulated by PAF. (**A**) Cultures of HCECs were cotransfected with pRL-TK and one of the various 5′-deleted *MMP-9* promoter constructs containing firefly luciferase as the reporter gene. Transiently transfected cells were left untreated or were stimulated with 100 nM mcPAF overnight, and promoter activity was tested. *Significant differences ($P < 0.05$) between PAF-stimulated and untreated conditions. **Significant differences ($P < 0.05$) between the empty vector and any of the other constructs in untreated conditions. Values are the averages (±SEM) of 5 independent experiments. (**B**) Key segments in the human *MMP-9* promoter sequence. Transcriptional regulation of the *MMP-9* gene in quiescent HCECs is mainly controlled by the BES located at −1112 to −670 relative to the transcription starting point. PRS that is largely responsible for the elevated activation of human *MMP-9* promoter sequence in response to PAF stimulation is located immediately downstream of the BES and spans −670 to −460 relative to the transcription starting point.

unlabeled oligonucleotides that competed out the shifted bands for each transcription factor (Fig. 1B).

Participation of a PAF-Responding Segment in the Induction of *MMP-9* Promoter Activity in HCECs

To investigate the contribution of various segments of *MMP-9* promoter sequence from −2172 to −8 bp relative to the transcription start point of the gene in the expression of the *MMP-9*, HCECs were transfected with the full-length promoter (−2172/+54Luc), a panel of 5′-deleted constructs, including −1511/+54Luc, −1112/+54Luc, −670/+54Luc, −460/+54Luc, −324/+54Luc, and −8/+54Luc, and the empty vector. The basal expression of luciferase reporter gene was enhanced by all 5′-deleted sequences of *MMP-9* promoter compared with the empty vector (Fig. 2A). A significant and sharp decrease in gene expression was observed when the −1112/−670 segment was deleted and was further reduced by the deletion of −324/−8 segment ($P < 0.05$). The latter segment contains the GC and TATA boxes and is indisputably essential to any regulatory activity of the promoter sequence. However, the −1112/−670 segment seems to be a major player in the basal expression of *MMP-9* gene in HCECs, and we designated it as the basal expression segment (BES; Fig. 2B). After stimulation with mcPAF, a significant increase in the expression of the reporter gene was observed ($P < 0.05$), with all the *MMP-9* promoter constructs encompassing the segment −670/−460 (−2172/+54Luc, −1511/+54Luc, −1112/+54Luc, and −670/+54Luc; Fig. 2A). The three constructs lacking this segment (−460/+54Luc, −324/+54Luc, and −8/+54Luc) failed to respond to stimulation by mcPAF, and their activity was comparable to that of their corresponding untreated conditions (Fig. 2A). This demonstrates a major regulatory role for the segment covering −670 to −460 bp of the *MMP-9* promoter sequence in the PAF-mediated expression of the gene, and we designated it as the PAF-responding segment (PRS; Fig. 2B).

We further verified our findings by exploring the effect of removing the PRS from *MMP-9* promoter on the basal and PAF-induced expression of the reporter gene. In this series of experiments, two of the constructs carrying the 5′-deleted sequences of *MMP-9* promoter, −1511/+54Luc and −1112/+54Luc, were truncated at −670/−460 to prepare the −1511+54Luc and −1112+54Luc constructs, respectively. Basal expression of the reporter gene in HCECs transfected with truncated *MMP-9* promoter sequences was significantly lower than that of their nontruncated counterparts but was significantly higher than the two 5′-deleted constructs lacking BES (−670/+54Luc and −460/+54Luc; Fig. 3), indicating that the presence of BES plays an essential role in the expression of the *MMP-9* gene in quiescent HCECs. Stimulation of HCECs transfected with truncated constructs of *MMP-9* with PAF, however, did not induce a significantly higher expression of the reporter gene compared with stimulation of untreated cells (Fig. 3). Levels of induction for the truncated constructs were only comparable to the 5′-deleted construct lacking the PRS (−460/+54Luc), whereas induction levels for nontruncated constructs were significantly higher than for their truncated counterparts and comparable to the 5′-deleted construct carrying the PRS (−670/+54Luc; Fig. 3). These findings corrobo-

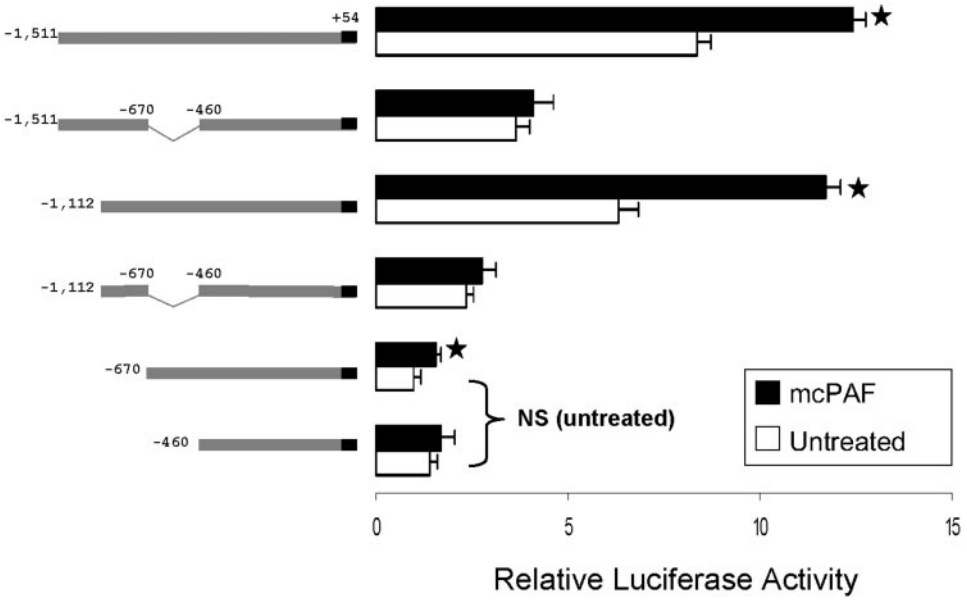

FIGURE 3. Truncation of PRS abolished the upregulation of *MMP-9* gene transcription by PAF. Cultures of HCECs were cotransfected with pRL-TK and one of the various 5′-deleted *MMP-9* promoter constructs, two of which were also truncated to remove PRS. Transiently transfected HCECs were left untreated or were stimulated with 100 nM mcPAF overnight, and promoter activity was tested. In the untreated conditions, significant differences were found between any two constructs ($P < 0.05$) but not between −670/+54Luc and −460/+54Luc constructs (NS, not significant). *Significant differences between PAF-stimulated and untreated conditions. Values are the averages (±SEM) of three independent experiments.

rate a major role for PRS in the expression of the *MMP-9* gene in HCECs stimulated with PAF.

Thymidine Kinase Promoter Response to PAF as Modified by PRS of *MMP-9* Promoter

To attest the regulatory capacity of BES and PRS in a different promoter and to determine the selectivity of PAF in the activation of *MMP-9*, we transfected HCECs with BES-TK or PRS-TK constructs carrying fusion sequences composed of BES or PRS of the *MMP-9* promoter, respectively. pGL3-Basic and pGL3-TK plasmids were used as controls. When cloned upstream of HSV-TK, both the PRS and the BES of *MMP-9* significantly upregulated basal expression of the reporter gene ($P < 0.05$) compared with that of pGL3-TK (Fig. 4). This was consistent with our findings that 5′-deleted constructs containing BES showed a significantly higher level of activity than those lacking BES (Fig. 2A) and that the gene expression of PRS-truncated constructs was significantly lower than that of their full-length counterparts (Fig. 3). PAF stimulation induced a significant increase in the expression of the reporter gene ($P < 0.05$) when PRS and not BES was present in the promoter sequence (Fig. 4). When transfected HCECs were stimulated with the physiologically inactive LysoPAF, no significant difference was observed in gene expression for any of the constructs. Pretreatment of the cells with the PAF receptor antagonist LAU8080 abolished the induction of gene transcription by PAF in PRS-TK–transfected HCECs (Fig. 4). These results indicate that only PRS exerts additional transcriptional activity to the HSV-TK promoter in PAF-treated HCECs and that PAF-induced upregulation of gene expression by PRS-TK is specific to the active form of PAF and is receptor mediated.

Mutation of Sp1 Motif and Its Divergent Effect on the *MMP-9* Promoter Activity in Quiescent and PAF-Treated HCECs

To further explore the role of transcription factors NFκB, Sp1, and AP-1 in the induction of *MMP-9* gene expression by PAF, we analyzed the contribution of their putative consensus sequences to gene expression by transfecting HCECs with wild-

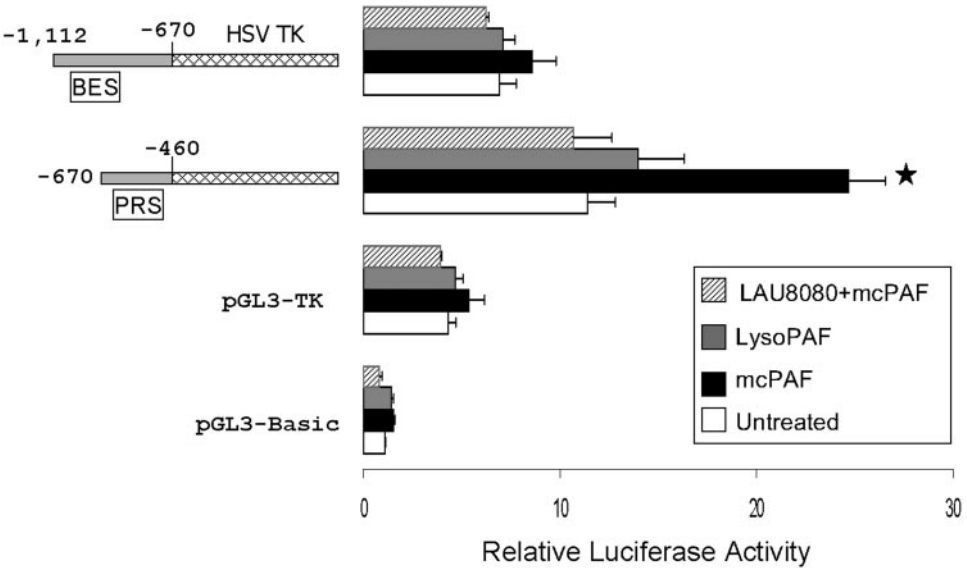

FIGURE 4. PRS conferred PAF-inducible activity to the HSV-TK promoter. Cultures of HCECs were cotransfected with pRL-TK and pGL3-Basic, pGL3-TK, or pGL3-TK with BES or PRS of the *MMP-9* promoter sequence cloned upstream of the HSV-TK promoter, as indicated in the figure. Transiently transfected HCECs were left untreated or were stimulated overnight with 100 nM lysoPAF or 100 nM mcPAF in the absence or presence of 10 μM PAF receptor antagonist LAU8080, and promoter activity was tested. In the untreated conditions, significant differences were found between any two constructs ($P < 0.05$). *Significant differences between PAF-stimulated and untreated conditions ($P < 0.05$). Values are the averages (±SEM) of three independent experiments.

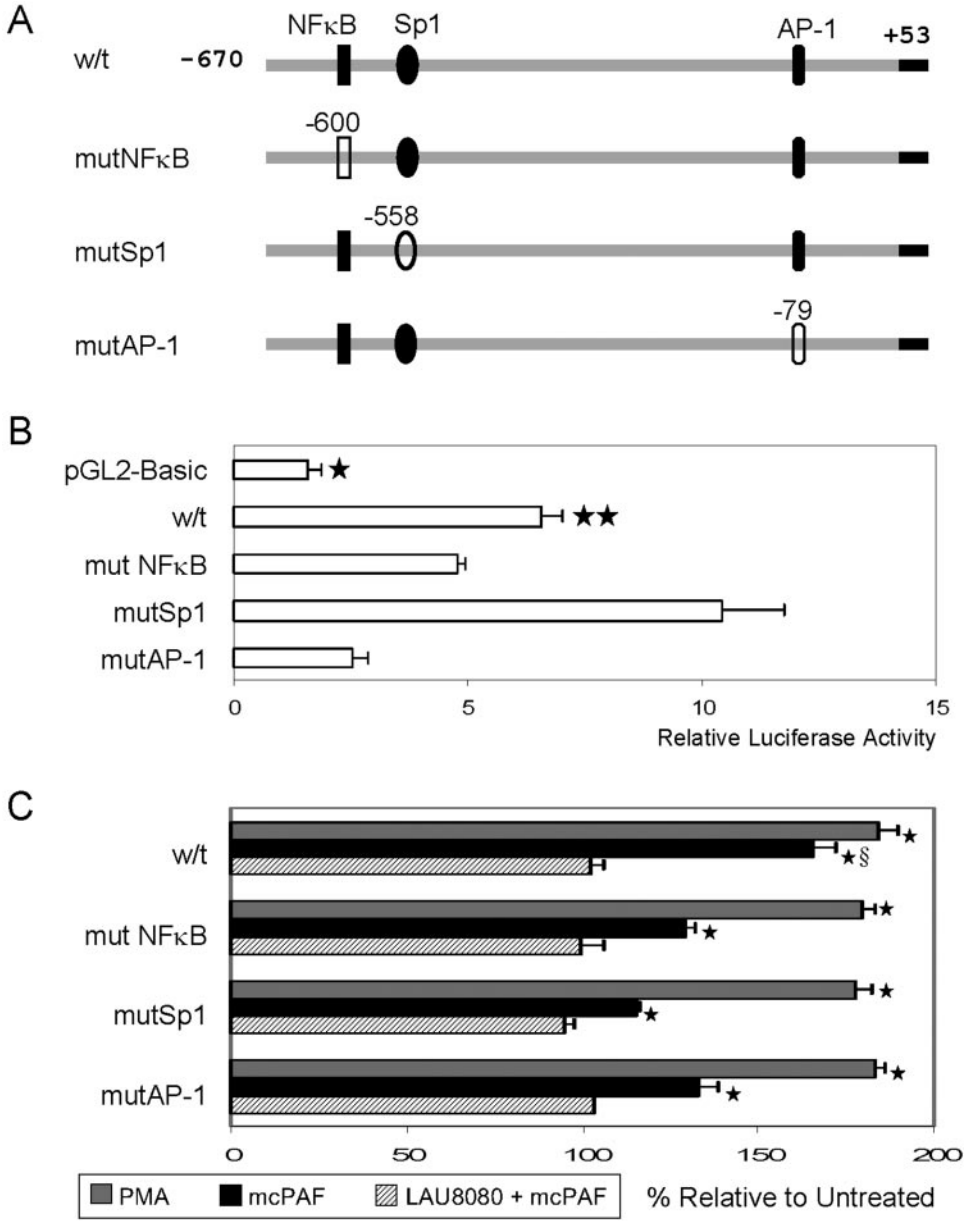

FIGURE 5. Site-directed mutations in the *MMP-9* promoter sequence. (**A**) Cultures of HCECs were cotransfected with pRL-TK and empty vector (pGL2-Basic), wild-type (w/t), or mutant *MMP-9* promoter constructs. (**B**) After overnight culture of transiently transfected cells, the promoter activity was tested. *Significant differences between pGL2-Basic and any of the *MMP-9* promoter constructs ($P < 0.05$). **Significant differences between the wild-type and any of the mutant constructs ($P < 0.05$). Values are the averages (±SEM) of four independent experiments. (**C**) Transiently transfected cells were left untreated or were stimulated overnight with 100 nM mcPAF in the absence or presence of 10 μM PAF receptor antagonist LAU8080 or with 50 nM PMA as a positive control. *Significant differences between PAF- or PMA-stimulated and untreated conditions ($P < 0.05$). §Significant differences between the wild-type and any of the mutant constructs ($P < 0.05$). Values are the averages (±SEM) of four independent experiments.

type and mutant constructs of the human *MMP-9* promoter. In this series of experiments, we used a construct consisting of 670 bp of the regulatory sequence of the human *MMP-9* gene (Fig. 5A) cloned in pGL2-basic vector (−670/+53Luc) and its mutated forms mutNFκB (−600), mutSp1 (−558), and mutAP-1 (−79). After transfection of HCECs and stimulation with mcPAF, we examined the effect of mutations on the basal and PAF-induced activities of the *MMP-9* promoter. Basal activity of the promoter for the wild-type and mutant constructs was significantly different from empty vector pGL2-Basic ($P < 0.05$), indicating that mutations at −600NFκB, −558Sp1, and −79AP-1 did not knock down the activity of *MMP-9* promoter to undetectable levels (Fig. 5B). However, there was a significant decrease of approximately 30% for mutNFκB and more than 60% for mutAP-1 constructs in the basal expression of the reporter gene ($P < 0.05$). Basal expression of the reporter gene for the mutSP1 construct, on the other hand, was increased by greater than 50% ($P < 0.05$), indicating a negative regulatory role for the −558Sp1 site in the promoter of human *MMP-9*. Stimulation with mcPAF caused a significant increase ($P < 0.05$) in the expression of the reporter gene in the HCECs transfected with any of the wild-type or mutated *MMP-9* promoter constructs (Fig. 5C). The magnitude of induction in HCECs transfected with the mutated constructs was, nevertheless, significantly lower than the wild-type *MMP-9* promoter sequence ($P < 0.05$) at 29%, 15%, and 33% induction for mutNFκB, mutSp1, and mutAP-1, respectively, compared with 65% induction for the wild type. These findings point out a cooperative participation of NFκB, Sp1, and AP-1 in the upregulation of *MMP-9* gene expression by PAF. Sp1, however, showed a unique regulatory disposition in *MMP-9* expression with a negative function in quiescent HCECs and a strong upregulating role in PAF-treated cells. Expression of the reporter gene driven by the wild-type or various mutated *MMP-9* promoter sequences in HCECs stimulated with PAF in the presence of the PAF receptor antagonist LAU8080 was comparable to those obtained in untreated conditions, indicating the specific receptor-mediated action of PAF. We also treated transfected HCECs with PMA, a well-known inducer of *MMP-9*,[26] to compare its effect with that of PAF. Stimulation of HCECs with

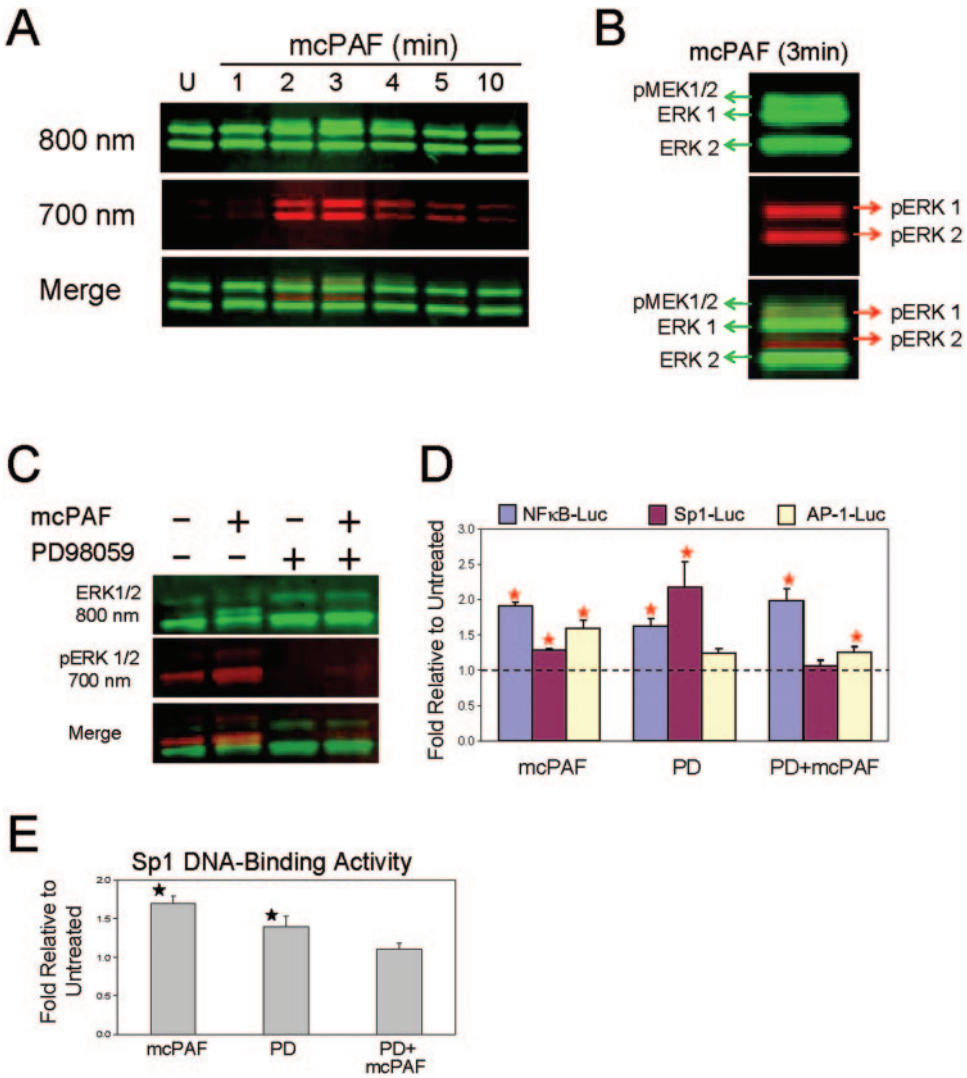

FIGURE 6. Activation of the MEK/ERK pathway in HCECs stimulated with PAF. (**A**) HCECs were left untreated (U) or were stimulated with 100 nM mcPAF for the times specified. Whole cell extracts were subjected to Western blot analysis. Images were captured using the 800-nm (*top*) or the 700-nm (*middle*) channel on an infrared imaging system and were merged together (*bottom*). (**B**) Bands for 3-minute stimulation in (**A**) are shown at a higher magnification for details. (**C**) HCECs were left untreated or were stimulated with 100 nM mcPAF for 24 hours in the absence or presence of 20 μM PD98059. Whole cell extracts were subjected to Western blot analysis, and images were captured as described in (**A**). In the merged captures, *orange* represents the binding of both antibodies to the same band, indicating that these are phosphorylated ERK1/2 proteins. (**D**) Cultures of HCECs were transiently transfected with luciferase reporter vectors containing the *cis*-acting DNA-binding element for NFκB, Sp1, or AP-1 before they were stimulated with 100 nM mcPAF in the absence or presence of 40 μM PD98059 (PD). Luciferase activity was determined and is indicated by the *dashed line* in untreated cells. (**E**) Cultures of HCECs were left untreated or were stimulated with 100 nM mcPAF in the absence or the presence of 20 μM PD98059 for 1 hour, and the nuclear extracts were collected and tested for the DNA-binding activity of Sp1. *Significant differences ($P < 0.05$) between PAF-stimulated or PD98059-treated and untreated conditions. Values are the averages (±SEM) of three independent experiments.

50 nM PMA induced a significant upregulation of reporter gene expression (approximately 80% above untreated) that was not affected by any of the mutations introduced (Fig. 5B). This demonstrates that *MMP-9* promoter activity in HCEC is regulated differently by PAF and PMA.

PAF-Stimulated MEK/ERK Pathway in HCECs

Previous studies in our laboratory showed that ERK1/2 is activated in rabbit corneal epithelial cells stimulated with PAF.[27] To investigate the activation profile of the MEK/ERK signaling pathway in HCECs stimulated with PAF, we examined the phosphorylation status of MEK1/2 and ERK1/2 by Western blot analysis. The infrared imaging system allowed us to examine the phosphorylation of MEK and ERK proteins on the same membrane. MEK1/2 and ERK1/2 proteins were rapidly phosphorylated after stimulation (Fig. 6A). A thin band in the 800-nm channel image that was heavier than 44-kDa ERK1 (Fig. 6A, top) and represented the phosphorylated form of 45-kDa MEK1/2 proteins (pMEK1/2) appeared 2 minutes after stimulation and peaked at 3 minutes, followed by a gradual decrease thereafter. Phosphorylated ERK1 and ERK2 proteins (pERK1/2) are shown as two bands in the 700-nm channel image (Fig. 6A, middle) that also peaked at 3 minutes after stimulation but lasted longer and were detectable at 10 minutes as well. The phosphorylated ERK1 and ERK2 bands are clearly indicated in the merged image (Fig. 6A, bottom), and details are illustrated in Figure 6B. After 24 hours of stimulation with PAF, ERK2 phosphorylation was more prominent than ERK1 (Fig. 6C, top and middle) and is clearly indicated in orange in the merged capture representing the dual immunoblotting of the pERK1 and pERK2 by the total (green) and phosphospecific (red) antibodies (Fig. 6C, bottom). Inhibition of MEK1/2 activity resulted in a minimal phosphorylation of ERK1/2 after stimulation with mcPAF (Fig. 6C). Pretreatment with PD98059 did not affect the total expression of ERK1/2. These results revealed a rapid activation of the MEK/ERK pathway by PAF in HCEC that after 24 hours is mainly observed as ERK2 phosphorylation. This could have important effects on transcriptional regulatory mechanisms targeted by this pathway.

Involvement of the MEK/ERK Pathway in the Activation of Sp1 DNA Binding by PAF

Sp1 showed a unique and bipolar role in the activation of the *MMP-9* promoter in HCECs that was different from the functions of NFκB and AP-1 (see Fig. 5). To determine whether the activation of the MEK/ERK pathway by PAF was involved in the alterations in Sp1 disposition, we studied the expression profile of luciferase reporter gene driven by the *cis*-acting DNA-binding element for NFκB, Sp1, or AP-1 in transiently trans-

fected HCECs stimulated with mcPAF in the absence or presence of the MEK inhibitor PD98059. Stimulation with PAF significantly upregulated reporter gene expression driven by any of the three *cis*-acting DNA-binding elements (Fig. 6D). Although treatment of the HCECs with PD98059 increased the transcriptional activity of NFκB and, more prominently, Sp1 in quiescent cells, stimulation of the PD98059 pretreated HCECs with PAF caused an increase in the expression of the reporter gene driven by NFκB or AP-1 motifs but not Sp1 (Fig. 6D). This indicates that interference with the activity of MEK/ERK signaling cascade only affected the induction of Sp1 transcriptional activity by PAF. To further verify this finding, we examined whether the inhibition of MEK activity interfered with the PAF-induced DNA-binding activity of Sp1. HCECs were stimulated with PAF in the absence or presence of PD98059, and nuclear extracts were collected. Sp1 DNA-binding activity showed a significant increase in PAF-stimulated HCECs compared with the activity of quiescent cells (Fig. 6E). Furthermore, treatment with PD98059 caused a smaller but significant increase in the Sp1 DNA-binding activity in quiescent HCECs and abolished any upregulation by PAF (Fig. 6E), indicating that the binding activity of Sp1 is regulated by the MEK/ERK pathway.

Role of MEK/ERK Pathway in the Phosphorylation of Sp1 by PAF

The involvement of MEK/ERK signaling pathway in the phosphorylation of Sp1 in PAF-stimulated HCECs was examined by Western blotting. Sp1 phosphorylation was upregulated in HCEC stimulated with PAF for 24 hours (Fig. 7A). This was more prominent when the intensity of the bands was normalized to the actin band. To confirm the phosphorylation status of Sp1, extracts of HCECs stimulated with PAF were immunoprecipitated with Sp1 antibody and tested for the expression of Sp1 (green) and either phosphorylated threonine (Fig. 7B, top; pThr) or phosphorylated serine (Fig. 7B, bottom; pSer) residues. As indicated in Figure 7B, the lower band corresponds to the nonphosphorylated form and the higher band is positive for both pThr and pSer. Stimulation of HCECs by PAF in the presence of PD98059, however, did not cause the phosphorylation of Sp1 (Fig. 7A).

Contribution of MEK/ERK Signaling Cascade to the Expression and Activation of MMP-9 by PAF

To explore the role of the MEK/ERK signaling pathway in MMP-9 expression and activity, we examined the effect of the MEK inhibitor PD98059 on the expression and activity of MMP-9 by Western blot analysis of whole cell extracts and zymography of conditioned media, respectively. MMP-9 expression was upregulated after 24 hours of stimulation with PAF (Fig. 7C, WB). Although PD98059 treatment increased the basal level of *MMP-9* expression in HCECs, PAF stimulation in the presence of PD98059 did not cause any upregulation of MMP-9 expression. Stimulation of HCECs with PAF induced more than a twofold increase in the activity of MMP-9 (Fig. 7C, Zym and bar graph). Pretreatment of the cells with PD98059 resulted in an approximately 58% increase in basal MMP-9 activity. This increased MMP-9 activity may be attributed to the removal of the Sp1 repressor effect and to an increase in the transcriptional activity of the *MMP-9* promoter. However, the stimulation of HCECs with PAF in the presence of the MEK inhibitor did not cause any changes in the activity of MMP-9. These results suggest that the MEK/ERK pathway plays an essential role in the upregulation of MMP-9 activity in HCECs treated with PAF.

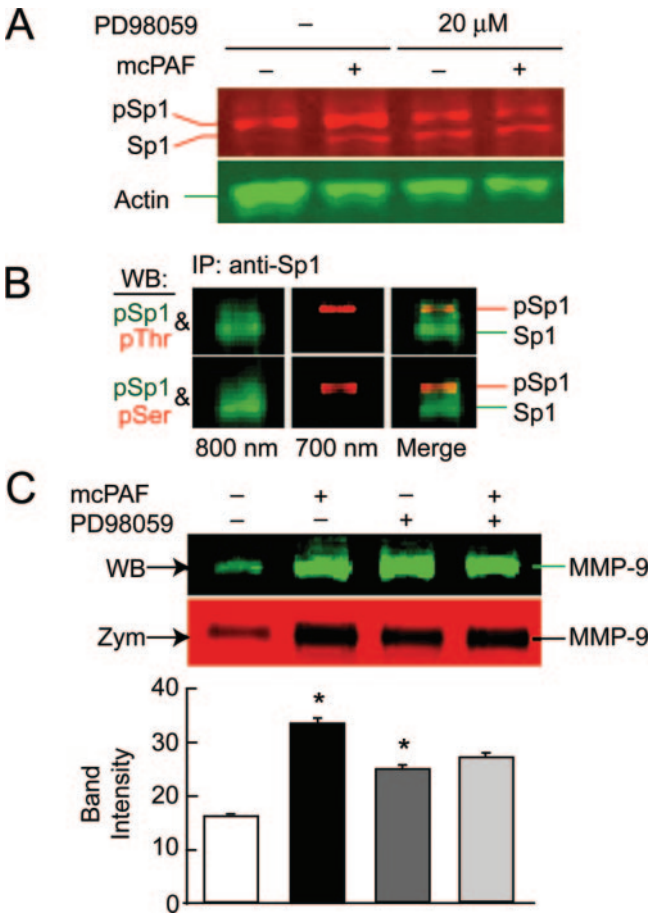

Figure 7. PAF induced the phosphorylation of Sp1 and the upregulation of *MMP-9* expression and activity in HCECs. (**A**) HCECs were left untreated or were stimulated with 100 nM mcPAF for 24 hours in the absence or presence of 20 μM PD98059. Whole cell extracts were subjected to Western blot analysis. Images were captured using the 700-nm (*top*) or 800-nm (*bottom*) channel on an infrared imaging system. (**B**) HCECs were stimulated with 100 nM mcPAF for 24 hours. Whole cell extract was subjected to immunoprecipitation with anti-Sp1 antibody, and membranes were immunoblotted for Sp1 and either phosphothreonine (*top*) or phosphoserine (*bottom*). Images were captured using the 800-nm (*left*) or 700-nm (*middle*) channel on an infrared imaging system and merged together (*right*). In the merged captures, *orange* represents the binding of both antibodies to the same band, indicating that these are phosphorylated Sp1. (**C**) HCECs were left untreated or were stimulated overnight with 100 nM mcPAF in the absence or presence of 20 μM PD98059. Whole cell extracts were subjected to Western blotting, and conditioned media were subjected to zymography, to detect the expression level and the activity of MMP-9. Images were captured on 800 nm channel (*WB*) and 700 nm channel (*Zym*) on an infrared imaging system. Bands on the zymography gels from three independent experiments were measured for the intensity of reactions, and the averages (±SEM) were graphed to indicate the significant differences (*) between PAF-stimulated or PD98059-treated and untreated conditions ($P < 0.05$).

Discussion

Although our previous studies have shown that PAF strongly induces the gene and protein expression of MMP-9 and two of its inhibitors, TIMP-1 and TIMP-2, with an imbalance in favor of MMP-9,[21–23] the regulatory mechanism of such induction is unknown. With the use of HCECs, we found that PAF stimulates the DNA-binding activity of NFκB, Sp1, and AP-1 in the cells. Activation of these transcription factors is known to be involved in the expression of *MMP-9* by other stimu-

lants,[25,26,28–33] indicating that an orchestrated collaboration of various transcription factors may be responsible for the upregulation of *MMP-9* promoter activity.

To uncover the significance of their role in the expression profile of *MMP-9* in HCECs, we studied the contribution of various segments of the regulatory sequence of the human *MMP-9* gene by implementing sequential deletions at the 5′ end of a construct spanning −2172 to +54 bp relative to the transcriptional start site of the *MMP-9* gene. We found that the basal expression of the gene was predominantly regulated by a segment spanning −1112 to −670 bp upstream of the transcription start site, which we designated BES. The elements participating in the PAF-induced expression of the *MMP-9* gene in HCECs were, however, primarily accumulated in a region positioned immediately downstream of the BES, from −670 to −460, which was designated PRS. We further confirmed our findings by performing truncation studies in which the PRS was cut out from two of the constructs carrying the 5′-deleted *MMP-9* promoter sequence, which resulted in the elimination of PAF-induced activation of the promoter. We also carried out fusion studies by the insertion of PRS or BES upstream of the promoter sequence of thymidine kinase and found that PAF enhanced the promoter activity of HSV-TK adjoined by the PRS and not the BES. These findings demonstrate that PRS has the capacity to enhance the activity of various promoter sequences in PAF-stimulated cells, and they signified the undisputable contribution of the elements integrated in the PRS of the *MMP-9* regulatory sequence in the gene expression induced by PAF in HCECs.

PRS consists of consensus sequences for NFκB at −600 and Sp1 at −558,[25] indicating a possible role for these transcription factors in the upregulation of *MMP-9* gene expression in HCECs stimulated with PAF. It has been reported that the mutation or deletion of −600NFκB, −558Sp1, or −79AP-1 motifs reduced or abolished the ability of tumor necrosis factor-α to stimulate the *MMP-9* promoter in OST osteosarcoma and HepG2 hepatoma cells.[25] Moreover, activation of the *MMP-9* promoter by v-*src* in HT 1080 fibrosarcoma cells was attributed to a binding site for AP-1 (−79) and an Sp1-binding GT box at −52.[26] We therefore extended our investigation by using the wild-type and mutant constructs of *MMP-9* promoter sequence, with the −600NFκB, −558Sp1, or −79AP-1 site knocked down by site-directed mutagenesis. We found that though −600NFκB site was partially involved in basal and PAF-induced expression of the *MMP-9* gene, the −558Sp1 site is a negative regulator in the expression of *MMP-9* in quiescent HCECs but contributes to the upregulation of gene expression in cells stimulated with PAF. We also showed that mutation of the −79AP-1 site did not entirely abolish basal expression of the *MMP-9* gene and had only a diminishing effect on PAF-induced expression. In a previous study, the proximal −79AP-1 site was identified as an essential element for TPA- and TNFα-induced promoter activity. It was also shown that the −79AP-1 motif is indispensable but insufficient for induction of the gene by TPA or TNF-α, which also requires the two upstream binding sites for NFκB (−600) and Sp-1 (−558).[25] We found that the proximal −79AP-1 site is not essential for the response of HCECs to PMA, whereas it is a crucial element for the PAF-induced activity of the *MMP-9* promoter. We also found that though the −79AP-1 site contributes to the induction of *MMP-9* promoter activity by PAF, the NFκB (−600) and Sp1 (−558) binding sites were also essential and played a major role in the elevated activity of the *MMP-9* promoter in PAF-stimulated HCECs. Removal of the NFκB (−600) and Sp1 (−558) motifs by deletion or truncation of the PRS abolished the upregulation of *MMP-9* promoter activity by PAF, indicating the collaborative and synergistic role of the two transcription factors as the most important element in the PAF-induced expression of *MMP-9* in HCECs.

Among other regulatory elements reported to be involved in the activation of the *MMP-9* promoter, Pax6 was shown to bind directly to a motif that is not located within the PRS and to interact with a consensus sequence within the PRS that also contains the Sp1 motif.[34] The possibility of collaboration between Pax6 and Sp1 in the activation of the *MMP-9* promoter by PAF must be further investigated. AP-2 shares two motifs located within the PRS with other transcription factors, NFκB (−470) and NFκB and Sp1 (−482). Further studies on these two consensus sequences are required to identify the possible collaboration between AP-2 and Sp1 or NFκB with regard to activation of the *MMP-9* promoter by PAF.

Although the inducing effect of PAF on the expression of the *MMP-9* gene in HCECs is regulated by more than one element, we report, for the first time, that a negative regulatory element (Sp1-binding site at −558) has an important role in the upregulation of *MMP-9*. Previous studies suggest that Sp1 can act as either a transcription activator or a repressor.[35–44] These contradictory findings indicate that Sp1 and the Sp1-like transcription factors may exert a bipolar function, depending on the type and intensity of stimulation.[36,43,45] The ultimate course of action taken by the Sp1-like family largely depends on the promoter to which they bind and on their interactions with coregulators.[41,42,44,46–52] It has been shown that the zinc finger DNA-binding domain (ZFDBD) and inhibitory domain (ID) of Sp1 are involved in protein–protein interactions with corepressors[53–55] and that they play an important role in the transcriptional regulation of Sp1-dependent genes.[56] These interactions are apparently regulated by the MEK/ERK signaling pathway, and the activation of MEK was shown to reduce the association between the corepressor/s and Sp1 bound to the regulatory sequence of the gene, resulting in an enhanced transcriptional activity of Sp1.[56] It has also been reported that the activation of ERK1/2 increases the DNA-binding activity of Sp1.[57] Furthermore, recombinant ERK2 stimulates and dephosphorylation reduces the DNA-binding activity of Sp1,[58] indicating that the activity of Sp1 entails its phosphorylation.

ERK1/2 has also been shown to directly phosphorylate Sp1 on threonines 453 and 739 in vitro and in vivo.[59] We found that MEK1/2 and ERK1/2 were rapidly phosphorylated after PAF stimulation. ERK2 activation was more prominent after 24 hours of stimulation with PAF and was accompanied by threonine and serine phosphorylation of Sp1 that could be eliminated by MEK inhibition. The DNA-binding and transcriptional activity of Sp1 were also upregulated by PAF and were abolished by blocking of the activation of the MEK/ERK pathway. This correlates with elimination of the PAF-induced upregulation of *MMP-9* protein expression and gelatinase activity. Our findings indicate that PAF regulates the expression and enzymatic activity of *MMP-9* by activation of the MEK/ERK pathway and phosphorylation of Sp1, resulting in the increased DNA-binding and transcriptional activity of Sp1. Our data also show that though an orchestrated collaboration of NFκB, Sp1, and AP-1 is required for the optimal upregulation of *MMP-9* gene expression by PAF, Sp1 plays a pivotal role by changing its repressor disposition to an inducer element. It may, therefore, be postulated that PAF could disengage the repressor elements from Sp1 (Fig. 8A), enhance the cross talk between the coactivators and Sp1 (Fig. 8B), or use a combination of the two effects (Fig. 8C) through activation of the MEK/ERK pathway. These effects overrule the repressor effect of Sp1 that, in unstimulated HCECs, plays a balancing role in the expression of *MMP-9*. Moreover, Sp1-like proteins, including a class of Krüppel-like factors (KLFs), have recently been identified by the presence of zinc-binding domain structures highly similar to the ZFDBD of Sp1[35,60–62] and were described, along with

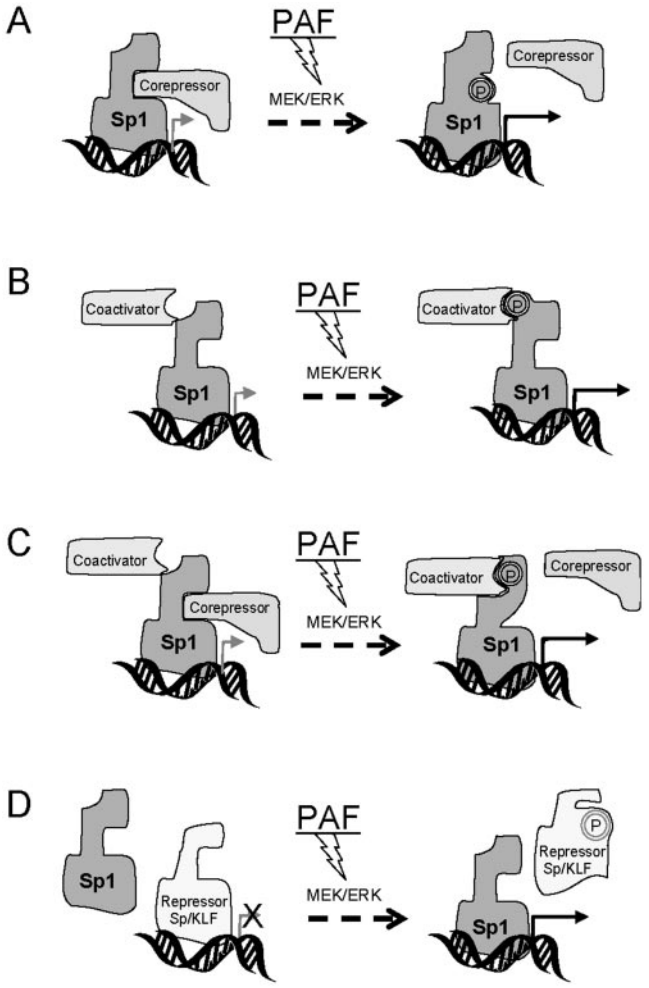

FIGURE 8. Models of postulated mechanisms for PAF-induced overturn of the Sp1-mediated transcriptional repression of *MMP-9* gene expression. Phosphorylation of the MEK/ERK cascade in PAF-stimulated HCECs may lead to modifications in the interactions between Sp1 and its repressors, activators, or both or to changes in the DNA-binding activity of repressor members of the Sp/KLF family. One or more of the following alterations may be an underlying mechanism of PAF-induced upregulation of transcriptional activity of *MMP-9* promoter. Phosphorylation of Sp1 (**A**) weakens the interactions between the transcription factor and its corepressors, resulting in the removal of binding hindrance, transcriptional interruption, or both. Alternatively, phosphorylation of Sp1 (**B**) may promote the interactions between Sp1 and its coactivators, which leads to enhanced DNA-binding or transcriptional activity of Sp1. A third possibility is that the phosphorylation of Sp1 (**C**) may turn down the connections between Sp1 and its corepressors on one hand and may improve the interactions between Sp1 and its coactivators on the other hand, the result of which will be an increased DNA-binding or transcriptional activity of Sp1. Another alternative mechanism is that phosphorylation of a repressor member of the Sp/KLF family (**D**) weakens its DNA-binding activity and allows Sp1 to compete out the repressor protein and to make a stronger interaction with its consensus sequence, which in turn upregulates the transcriptional activity of *MMP-9* promoter.

Sp1-Sp6, as the Sp1-like/KLF (Sp/KLF) family.[36] Some members of the Sp/KLF family have been reported to function as repressor proteins that bind the same consensus sequence as Sp1 and that prevent proper activation of the promoter sequence by Sp1.[36,45] Therefore, it is possible that activation of the MEK/ERK pathway by PAF turns down the DNA-binding activity of a repressor protein, which in turn allows a more compelling interaction between Sp1 and its consensus sequence, resulting in the upregulation of transcriptional activity of an otherwise repressed promoter (Fig. 8D). Further studies are required to determine which of these mechanisms are activated by PAF. Overall, our results support the conclusion that PAF stimulation transposes the suppression effect of Sp1 transcription complex to an activating function through MEK/ERK signaling and increased phosphorylation of Sp1. Activation of Sp1, along with an increase in the DNA-binding activity of other transcription factors (NFκB and AP-1), leads to upregulation in the expression and enzymatic activity of *MMP-9* in HCECs stimulated with PAF. Further studies on the pathways involved in the overturn of Sp1 from a repressor to an inducer in corneal epithelial cells may reveal the corresponding corepressors, coactivators, or both that may be targeted for therapeutic approaches in the regulation of inflammatory responses by PAF.

Acknowledgments

The authors thank Joelle Finley for maintaining the HCEC cultures and Azucena Kakazu for technical support.

References

1. Bazan HE. Cellular and molecular events in corneal wound healing: significance of lipid signalling. *Exp Eye Res*. 2005;80:453–463.
2. Woessner JF Jr. MMPs and TIMPs—an historical perspective. *Mol Biotechnol*. 2002;22:33–49.
3. Turpeenniemi-Hujanen T. Gelatinases (MMP-2 and –9) and their natural inhibitors as prognostic indicators in solid cancers. *Biochimie (Paris)*. 2005;87:287–297.
4. Chakraborti S, Mandal M, Das S, et al. Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem*. 2003;253:269–285.
5. Nagase H, Woessner JF Jr. Matrix metalloproteinases. *J Biol Chem*. 1999;274:21491–21494.
6. Vu TH. Don't mess with the matrix. *Nat Genet*. 2001;28:202–203.
7. Massova I, Kotra LP, Fridman R, Mobashery S. Matrix metalloproteinases: structures, evolution, and diversification. *FASEB J*. 1998;12:1075–1095.
8. Vu TH, Werb Z. Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev*. 2000;14:2123–2133.
9. Sivak JM, Mohan R, Rinehart WB, et al. Pax-6 expression and activity are induced in the reepithelializing cornea and control activity of the transcriptional promoter for matrix metalloproteinase gelatinase B. *Dev Biol*. 2000;222:41–54.
10. Matsubara M, Zieske JD, Fini ME. Mechanism of basement membrane dissolution preceding corneal ulceration. *Invest Ophthalmol Vis Sci*. 1991;32:3221–3237.
11. Matsubara M, Girard MT, Kublin CL, et al. Differential roles for two gelatinolytic enzymes of the matrix metalloproteinase family in the remodelling cornea. *Dev Biol*. 1991;147:425–439.
12. Leppert D, Waubant E, Galardy R, et al. T cell gelatinases mediate basement membrane transmigration in vitro. *J Immunol*. 1995;154:4379–4389.
13. Okada S, Kita H, George TJ, et al. Migration of eosinophils through basement membrane components in vitro: role of matrix metalloproteinase-9. *Am J Respir Cell Mol Biol*. 1997;17:519–528.
14. Fini ME, Parks WC, Rinehart WB, et al. Role of matrix metalloproteinases in failure to re-epithelialize after corneal injury. *Am J Pathol*. 1996;149:1287–1302.
15. Gay JC. Mechanism and regulation of neutrophil priming by platelet-activating factor. *J Cell Physiol*. 1993;156:189–197.
16. Shukla SD. Platelet-activating factor receptor and signal transduction mechanisms. *FASEB J*. 1992;6:2296–2301.
17. Buttke TM, Sandstrom PA. Redox regulation of programmed cell death in lymphocytes. *Free Radic Res*. 1995;22:389–397.
18. Braquet P, Touqui L, Shen TY, Vargaftig BB. Perspectives in platelet-activating factor research. *Pharmacol Rev*. 1987;39:97–145.
19. Bazan HE, Reddy ST, Lin N. Platelet-activating factor (PAF) accumulation correlates with injury in the cornea. *Exp Eye Res*. 1991;52:481–491.

20. Chandrasekher G, Ma X, Lallier TE, Bazan HE. Delay of corneal epithelial wound healing and induction of keratocyte apoptosis by platelet-activating factor. *Invest Ophthalmol Vis Sci*. 2002;43: 1422–1428.
21. Tao Y, Bazan HE, Bazan NG. Platelet-activating factor induces the expression of metalloproteinases-1 and –9, but not –2 or –3, in the corneal epithelium. *Invest Ophthalmol Vis Sci*. 1995;36:345–354.
22. Ottino P, Bazan HE. Corneal stimulation of MMP-1, –9 and uPA by platelet-activating factor is mediated by cyclooxygenase-2 metabolites. *Curr Eye Res*. 2001;23:77–85.
23. Ottino P, Taheri F, Bazan HE. Platelet-activating factor induces the gene expression of TIMP-1, -2, and PAI-1: imbalance between the gene expression of *MMP-9* and TIMP-1 and -2. *Exp Eye Res*. 2002;74:393–402.
24. Huhtala P, Tuuttila A, Chow LT, et al. Complete structure of the human gene for 92-kDa type IV collagenase: divergent regulation of expression for the 92- and 72-kilodalton enzyme genes in HT-1080 cells. *J Biol Chem*. 1991;266:16485–16490.
25. Sato H, Seiki M. Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. *Oncogene*. 1993;8:395–405.
26. Sato H, Kita M, Seiki M. v-Src activates the expression of 92-kDa type IV collagenase gene through the AP-1 site and the GT box homologous to retinoblastoma control elements: a mechanism regulating gene expression independent of that by inflammatory cytokines. *J Biol Chem*. 1993;268:23460–23468.
27. Bazan HE, Varner L. A mitogen-activated protein kinase (MAP-kinase) cascade is stimulated by platelet activating factor (PAF) in corneal epithelium. *Curr Eye Res*. 1997;16:372–379.
28. Gum R, Lengyel E, Juarez J, et al. Stimulation of 92-kDa gelatinase B promoter activity by ras is mitogen-activated protein kinase kinase 1-independent and requires multiple transcription factor binding sites including closely spaced PEA3/ets and AP-1 sequences. *J Biol Chem*. 1996;271:10672–10680.
29. Himelstein BP, Lee EJ, Sato H, et al. Transcriptional activation of the matrix metalloproteinase-9 gene in an H-ras and v-myc transformed rat embryo cell line. *Oncogene*. 1997;14:1995–1998.
30. Yan C, Wang H, Boyd DD. KiSS-1 represses 92-kDa type IV collagenase expression by down-regulating NF-κB binding to the promoter as a consequence of IκBα-induced block of p65/p50 nuclear translocation. *J Biol Chem*. 2001;276:1164–1172.
31. Crowe DL, Brown TN. Transcriptional inhibition of matrix metalloproteinase 9 (*MMP-9*) activity by a c-fos/estrogen receptor fusion protein is mediated by the proximal AP-1 site of the *MMP-9* promoter and correlates with reduced tumor cell invasion. *Neoplasia*. 1999;1:368–372.
32. Bond M, Fabunmi RP, Baker AH, Newby AC. Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: an absolute requirement for transcription factor NF-κB. *FEBS Lett*. 1998;435:29–34.
33. Esteve PO, Chicoine E, Robledo O, et al. Protein kinase C-zeta regulates transcription of the matrix metalloproteinase-9 gene induced by IL-1 and TNF-alpha in glioma cells via NF-kappa B. *J Biol Chem*. 2002;277:35150–35155.
34. Sivak JM, West-Mays JA, Yee A, et al. Transcription factors Pax6 and AP-2α interact to coordinate corneal epithelial repair by controlling expression of matrix metalloproteinase gelatinase B. *Mol Cell Biol*. 2004;24:245–257.
35. Philipsen S, Suske G. A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. *Nucleic Acids Res*. 1999;27: 2991–3000.
36. Kaczynski J, Cook T, Urrutia R. Sp1- and Kruppel-like transcription factors. *Genome Biol*. 2003;4:206.
37. Bouwman P, Philipsen S. Regulation of the activity of Sp1-related transcription factors. *Mol Cell Endocrinol*. 2002;195:27–38.
38. Lin SY, Black AR, Kostic D, et al. Cell cycle-regulated association of E2F1 and Sp1 is related to their functional interaction. *Mol Cell Biol*. 1996;16:1668–1675.
39. Lee DK, Suh D, Edenberg HJ, Hur MW. POZ domain transcription factor, FBI-1, represses transcription of ADH5/FDH by interacting with the zinc finger and interfering with DNA binding activity of Sp1. *J Biol Chem*. 2002;277:26761–26768.
40. Doetzlhofer A, Rotheneder H, Lagger G, et al. Histone deacetylase 1 can repress transcription by binding to Sp1. *Mol Cell Biol*. 1999;19:5504–5511.
41. Zaid A, Hodny Z, Li R, Nelson BD. Sp1 acts as a repressor of the human adenine nucleotide translocase-2 (ANT2) promoter. *Eur J Biochem*. 2001;268:5497–5503.
42. Ogra Y, Suzuki K, Gong P, et al. Negative regulatory role of Sp1 in metal responsive element-mediated transcriptional activation. *J Biol Chem*. 2001;276:16534–16539.
43. Li L, He S, Sun JM, Davie JR. Gene regulation by Sp1 and Sp3. *Biochem Cell Biol*. 2004;82:460–471.
44. Briggs MR, Kadonaga JT, Bell SP, Tjian R. Purification and biochemical characterization of the promoter-specific transcription factor, Sp1. *Science*. 1986;234:47–52.
45. Lomberk G, Urrutia R. The family feud: turning off Sp1 by Sp1-like KLF proteins. *Biochem J*. 2005;392:1–11.
46. Kadonaga JT, Carner KR, Masiarz FR, Tjian R. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell*. 1987;51:1079–1090.
47. Suzuki T, Kimura A, Nagai R, Horikoshi M. Regulation of interaction of the acetyltransferase region of p300 and the DNA-binding domain of Sp1 on and through DNA binding. *Genes Cells*. 2000; 5:29–41.
48. Hagen G, Muller S, Beato M, Suske G. Sp1-mediated transcriptional activation is repressed by Sp3. *EMBO J*. 1994;13:3843–3851.
49. Dennig J, Hagen G, Beato M, Suske G. Members of the Sp transcription factor family control transcription from the uteroglobin promoter. *J Biol Chem*. 1995;270:12737–12744.
50. Kwon HS, Kim MS, Edenberg HJ, Hur MW. Sp3 and Sp4 can repress transcription by competing with Sp1 for the core cis-elements on the human ADH5/FDH minimal promoter. *J Biol Chem*. 1999;274:20–28.
51. Shou Y, Baron S, Poncz M. An Sp1-binding silencer element is a critical negative regulator of the megakaryocyte-specific αIIb gene. *J Biol Chem*. 1998;273:5716–5726.
52. Murata Y, Kim HG, Rogers KT, et al. Negative regulation of Sp1 trans-activation is correlated with the binding of cellular proteins to the amino terminus of the Sp1 trans-activation domain. *J Biol Chem*. 1994;269:20674–20681.
53. Huynh KD, Fischle W, Verdin E, Bardwell VJ. BCoR, a novel corepressor involved in BCL-6 repression. *Genes Dev*. 2000;14: 1810–1823.
54. Chen JD, Evans RM. A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature*. 1995;377:454–457.
55. Zamir I, Dawson J, Lavinsky RM, et al. Cloning and characterization of a corepressor and potential component of the nuclear hormone receptor repression complex. *Proc Natl Acad Sci USA*. 1997;94: 14400–14405.
56. Lee JA, Suh DC, Kang JE, et al. Transcriptional activity of Sp1 is regulated by molecular interactions between the zinc finger DNA binding domain and the inhibitory domain with corepressors, and this interaction is modulated by MEK. *J Biol Chem*. 2005;280: 28061–28071.
57. Milanini J, Vinals F, Pouyssegur J, Pages G. p42/p44 MAP kinase module plays a key role in the transcriptional regulation of the vascular endothelial growth factor gene in fibroblasts. *J Biol Chem*. 1998;273:18165–18172.
58. Merchant JL, Du M, Todisco A. Sp1 phosphorylation by Erk 2 stimulates DNA binding. *Biochem Biophys Res Commun*. 1999; 254:454–461.
59. Milanini-Mongiat J, Pouyssegur J, Pages G. Identification of two Sp1 phosphorylation sites for p42/p44 mitogen-activated protein kinases: their implication in vascular endothelial growth factor gene transcription. *J Biol Chem*. 2002;277:20631–20639.
60. Turner J, Crossley M. Mammalian Kruppel-like transcription factors: more than just a pretty finger. *Trends Biochem Sci*. 1999; 24:236–240.
61. Black AR, Black JD, Azizkhan-Clifford J. Sp1 and Kruppel-like factor family of transcription factors in cell growth regulation and cancer. *J Cell Physiol*. 2001;188:143–160.
62. Cook T, Gebelein B, Urrutia R. Sp1 and its likes: biochemical and functional predictions for a growing family of zinc finger transcription factors. *Ann NY Acad Sci*. 1999;880:94–102.