

# Calcium-induced Matrix Metalloproteinase 9 Gene Expression Is Differentially Regulated by ERK1/2 and p38 MAPK in Oral Keratinocytes and Oral Squamous Cell Carcinoma\*

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Subhendu Mukhopadhyay<sup>‡§</sup>, Hidayatullah G. Munshi<sup>¶\*\*</sup>, Suman Kambhampati<sup>¶</sup>,  
Antonella Sassano<sup>¶</sup>, Leonidas C. Plataniotis<sup>¶</sup>, and M. Sharon Stack<sup>‡‡‡</sup>

From the <sup>‡</sup>Department of Cell and Molecular Biology and the <sup>¶</sup>Division of Hematology/Oncology,  
Department of Medicine, Feinberg School of Medicine, and the <sup>‡‡‡</sup>Robert H. Lurie Comprehensive Cancer Center,  
Northwestern University, Chicago, Illinois 60611

**Matrix metalloproteinases (MMPs) play an important role in the invasive behavior of a number of cancers including oral squamous cell cancer (OSCC), and increased expression of MMP-9 is correlated with invasive and metastatic OSCC. Because calcium is an important regulator of keratinocyte function, the effect of modulating extracellular calcium on MMP-9 expression in OSCC cell lines was evaluated. Increasing extracellular calcium induced a dose-dependent increase in MMP-9 expression in immortalized normal and premalignant oral keratinocytes, but not in two highly invasive OSCC cell lines. Differential activation of MAPK signaling was also induced by calcium. p38 MAPK activity was down-regulated, whereas ERK1/2 activity was enhanced. Pharmacologic inhibition of p38 MAPK activity or expression of a catalytically inactive mutant of the upstream kinase MAPK kinase 3 (MKK3) increased the calcium induced MMP-9 gene expression, demonstrating that p38 MAPK activity negatively regulated this process. Interestingly blocking p38 MAPK activity enhanced ERK1/2 phosphorylation, suggesting reciprocal regulation between the ERK1/2 and p38 MAPK pathways. Together these data support a model wherein calcium-induced MMP-9 expression is differentially regulated by the ERK1/2 and p38 MAPK pathways in oral keratinocytes, and the data suggest that a loss of this regulatory mechanism accompanies malignant transformation of the oral epithelium.**

Oral squamous cell carcinoma (OSCC)<sup>1</sup> is the most common malignancy of the oral cavity causing more deaths than any

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<sup>‡‡</sup> To whom correspondence should be addressed: Northwestern University Medical School, Dept. of Cell and Molecular Biology, 303 E. Chicago Ave., Tarry 8-715, Chicago, IL 60611. Tel.: 312-908-8216; Fax: 312-503-7912; E-mail: mss130@northwestern.edu.

<sup>1</sup> The abbreviations used are: OSCC, oral squamous cell carcinoma;

other oral disease (1, 2). Although OSCC is characterized by local, regional, and distant spread, the biochemical factors that underlie dissemination are poorly understood (1, 3). OSCC tumors demonstrate a progressive lack of basement membrane staining, more diffuse invasion, and a higher frequency of lymph node metastasis, suggesting that basement membrane loss reflects metastatic potential (4–7). This is consistent with immunohistochemical and *in situ* hybridization studies of human OSCC tumors that implicate enzymes belonging to the matrix metalloproteinase (MMP) family, including MMP-9 (92-kDa gelatinase B), in basement membrane proteolysis and tissue invasion. The presence of gelatinolytic activity attributable to activated MMP-9 in OSCC biopsy specimens correlates with immunohistochemical staining of frozen sections and is enhanced in both highly invasive and metastatic cases, suggesting a correlation between MMP-9 activity and metastatic potential (8, 9). Additional studies of human tumors have shown that MMP-9 is up-regulated in diffuse invasive OSCC specimens, with predominant localization to the invasive front (4, 10). At the cellular level, modulation of MMP-9 expression via phorbol ester treatment leads to enhanced invasive behavior (8–10), whereas blocking MMP-9 expression *in vivo* reduces invasion in an orthotopic murine model of OSCC (11). These data support the hypothesis that MMP-9 is an important determinant of the invasive phenotype in OSCC.

Oral keratinocytes express a variety of differentiation markers, the expression of which is influenced by calcium-induced changes in the transcription of target genes (12). There is a steep calcium gradient within the oral mucosa, with higher calcium present in the uppermost differentiated layers, and altering extracellular calcium has been used to effectively model keratinocyte differentiation *in vitro* (13–16). Immunohistochemical analysis of human keratinocytes indicates a correlation between MMP-9 expression and differentiation. Furthermore, recent studies have demonstrated concomitant calcium-regulated expression of MMP-9 with the keratinocyte terminal differentiation marker involucrin, providing additional evidence for the association of MMP-9 transcriptional regulation and cellular differentiation (17).

Because tight regulation of MMP-9 expression plays a piv-

MMP, matrix metalloproteinase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MKK, mitogen-activated protein kinase kinase; RT, reverse transcription; MAPKAPK, mitogen-activated protein kinase-activated protein kinase; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; CaR, extracellular calcium-sensing receptor.

otal role in keratinocyte behavior and because MMP-9 expression is correlated with invasive and metastatic OSCC, factors controlling MMP-9 gene expression in OSCC were evaluated. Results of the current study demonstrate that increasing extracellular calcium induced a dose-dependent increase in MMP-9 expression in immortalized normal and premalignant oral keratinocytes, but not in highly invasive OSCC cells. Differential activation of mitogen-activated protein kinase (MAPK) signaling was also induced by calcium. p38 MAPK activity was down-regulated, whereas extracellular signal-regulated kinase-1/2 (ERK1/2) activity was enhanced. Pharmacologic inhibition of p38 MAPK activity or expression of a catalytically inactive mutant of the upstream kinase MKK3 increased the calcium-induced MMP-9 gene expression, demonstrating that p38 MAPK activity negatively regulated this process. Interestingly blocking p38 MAPK activity enhanced ERK1/2 phosphorylation, suggesting reciprocal regulation between the ERK1/2 and p38 MAPK pathways. Together these data support a model wherein calcium-induced MMP-9 expression is differentially regulated by the ERK1/2 and p38 MAPK pathways in oral keratinocytes, and the data suggest that a loss of this regulatory mechanism accompanies malignant transformation of the oral epithelium.

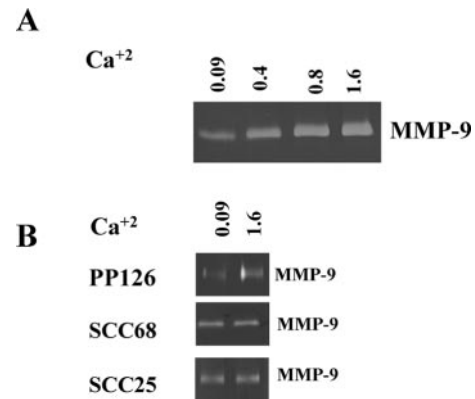
#### EXPERIMENTAL PROCEDURES

**Materials**—Cell culture reagents and peroxidase-conjugated secondary antibodies were purchased from Sigma. The MEK inhibitor PD98059 and the p38 MAPK inhibitor SB203580 were from Calbiochem (La Jolla, CA). Antiphosphorylated p42/p44 (MAPK/ERK), antiphosphorylated p38 MAPK, and anti-p38 MAPK were obtained from Cell Signaling Technology (Beverly, MA). Anti-ERK1/2 (anti-p42/p44) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Supersignal enhanced chemiluminescence (ECL) reagent was obtained from Pierce, and polyvinylidene difluoride membranes were purchased from Millipore (Bedford, MA). TransIT keratinocyte transfection reagent was purchased from Mirus (Madison, WI). A luciferase reporter assay system was from Promega (Madison, WI). Protease inhibitor mixture was purchased from Roche Diagnostics. One-step RT-PCR kits were purchased from Invitrogen, and DNase I was from Ambion (Austin, TX). MAPKAPK2 antibody was obtained from Upstate Biotechnology (Waltham, MA).

**Cell Cultures**—Premalignant oral keratinocytes (pp126 cells) were a gift from Dr. D. Oda (University of Washington, Seattle, WA) (18). Telomerase reverse transcriptase-immortalized normal oral keratinocytes (OKF6/T cells) and malignant OSCC cell lines (SCC25 and SCC68) were provided by Dr. J. Rheinwald (Brigham & Women's Hospital, Harvard Institutes of Medicine, Boston, MA) (19). SCC4 and CAL27 cells were obtained from ATCC (American Tissue Culture Collection, Manassas, VA). pp126 cells were maintained in keratinocyte serum-free medium (Invitrogen) supplemented with 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 5 ng/ml epidermal growth factor, 50  $\mu$ g/ml bovine pituitary extract supplied with the medium, and 0.09 mM  $\text{CaCl}_2$ . All other cells were maintained in keratinocyte serum-free medium supplemented with 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 25  $\mu$ g/ml bovine pituitary extract (supplied with the medium), 0.2 ng/ml epidermal growth factor, and 0.31 mM  $\text{CaCl}_2$ . Cells were plated in low calcium (0.09 mM) medium and serum-starved for 24 h prior to treatment with high calcium (1.6 mM). In additional experiments, inhibitors or other chemical reagents were added 30 min prior to treatment with calcium.

**Analysis of MMP-9 Expression**—Gelatinase activities in the conditioned media at 36 h were determined using SDS-PAGE gelatin zymography as described previously (20). Briefly, SDS-polyacrylamide gels (9% acrylamide) were co-polymerized with 0.1% gelatin, and samples were electrophoresed without reduction or boiling using 5 $\times$  Laemmli sample buffer (21). SDS was removed through a 30-min incubation in 2.5% Triton X-100, and gels were incubated in 20 mM glycine (pH 8.3), 10 mM  $\text{CaCl}_2$ , 1  $\mu$ M  $\text{ZnCl}_2$  at 37  $^\circ\text{C}$  for 24–36 h. The gels were stained with Coomassie Blue to visualize zones of gelatinolytic activity. Levels of MMP-9 protein in the conditioned media were also quantified by ELISA (Oncogene Research Products) according to the manufacturer's specifications.

**MMP-9 RNA Levels**—Total RNA was isolated using TRIzol reagent according to manufacturer's instructions. Following digestion with RQ1



**FIG. 1. Extracellular calcium regulates MMP-9 expression.** OKF6 (A) and pp126, SCC68, and SCC25 (B) cells were plated in low calcium (0.09 mM) medium. Following serum starvation for 24 h, cells were treated with medium containing increasing calcium (0.09–1.6 mM) as indicated. The conditioned media were collected 24 h later, and MMP-9 activity was analyzed by gelatin zymography as described under “Experimental Procedures.”

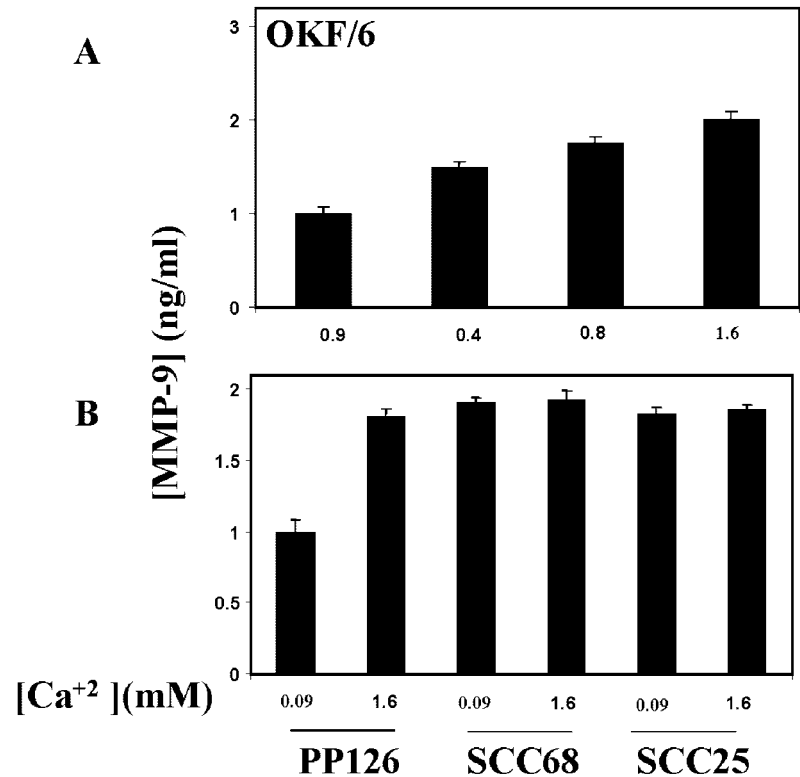
DNase for 30 min at 37  $^\circ\text{C}$ , the total RNA concentration was determined by spectrophotometric measurement. Primer pairs for human MMP-9 and human GAPDH were as follows: forward primer 5'-AAGATGCTGCTGTTTCAGCGGG-3' and reverse primer 5'-GTCCTCAGGGCACTGCAGGAT-3' for MMP-9 and forward primer 5'-CGGAGTCAACGGATTGGTCTGAT-3' and reverse primer 5'-GCAGGTCAGGTCCACCACTGAC-3' for GAPDH (22). The length of the MMP-9 and GAPDH amplicons was 256 and 680 base pairs, respectively. RT-PCR was performed using the one-step RT-PCR kit where reverse transcription and DNA amplification occur in the same reaction. Briefly, 1  $\mu$ g of total RNA was used as template in a reaction that included the appropriate primers in the presence of both reverse transcriptase and *Taq* polymerase. The mixture was incubated at 45  $^\circ\text{C}$  for 30 min and cycled 30 times at 94  $^\circ\text{C}$  for 30 s, 55  $^\circ\text{C}$  for 30 s, and 72  $^\circ\text{C}$  for 2 min. Appropriate negative controls of amplification included reactions without reverse transcriptase. PCR products were visualized by UV transillumination of 1.5% agarose gels stained with ethidium bromide.

**MMP-9 Promoter Activity**—pGL3 luciferase reporter vector (Promega) containing MMP-9 promoter (–573 to +30 from the start site) was kindly provided by Dr. L. Hudson (University of New Mexico, Albuquerque, NM). pRSV vector containing either the constitutively active MKK3Glu or the dominant negative mutant of MKK3Ala was generously provided by Dr. R. J. Davis (University of Massachusetts Medical School, Worcester, MA) (23). Subconfluent cells grown in low calcium medium were transfected with 2  $\mu$ g of MMP-9 promoter/luciferase reporter construct using TransIT keratinocyte (Mirus) according to the manufacturer's instructions. In additional experiments, 1  $\mu$ g of either the dominant negative or the constitutively active mutant of MKK3 was co-transfected with the MMP-9 luciferase construct. Six h later, the cells were washed with serum-free medium to remove the unbound DNA-lipid complexes and shifted to medium containing increasing calcium for 36 h. The cells were then lysed, and luciferase activities were determined by luminometry. The results were normalized to protein concentration and expressed relative to control cells maintained in low calcium condition.

**ERK1/2 Phosphorylation**—Following treatment with calcium, the cells were lysed at the indicated times in modified radioimmune precipitation assay buffer containing proteinase and phosphatase inhibitors. The samples were analyzed by SDS-PAGE (9% gels), and the blots were probed with anti-ERK1/2 antibody (1:2000) to detect total ERK1/2 expression or probed with antiphosphorylated ERK1/2 (1:1000) antibody to detect active forms of ERK as reported previously (24).

**p38 MAPK Activity Assay**—OKF6 cells were serum-starved overnight and treated with 10  $\mu$ M SB203580 for 30 min prior to treatment with medium containing low (0.09 mM) or high (1.6 mM) calcium. The cells were lysed in phosphorylation lysis buffer (0.5% Triton X-100, 150 mM NaCl, 200  $\mu$ M sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM EDTA, 50 mM Hepes, 1.5 mM magnesium chloride, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10  $\mu$ g/ml aprotinin) for 60 min at 4  $^\circ\text{C}$ , and lysates were immunoprecipitated with antibodies to MAPK-activated protein kinase 2 as detailed previously (25, 26). The immunoprecipitated proteins were washed three times in phosphorylation lysis buffer and two times in kinase

FIG. 2. **ELISA analysis of calcium-induced MMP-9 expression.** OKF6 (A) and pp126, SCC25, and SCC68 (B) cells were plated in low calcium (0.09 mM) medium. Following serum starvation for 24 h, cells were treated with medium containing increasing calcium (0.09–1.6 mM) as indicated. The conditioned media were collected 24 h later, and MMP-9 expression was quantified by ELISA as detailed under “Experimental Procedures.” Results represent mean  $\pm$  S.E. of three independent experiments.



buffer (25 mM Hepes, pH 7.4, 25 mM MgCl<sub>2</sub>, 25 mM  $\beta$ -glycerophosphate, 100  $\mu$ M sodium orthovanadate, 2 mM dithiothreitol, 20  $\mu$ M ATP), and the immunocomplex kinase assays were initiated by the addition of 30  $\mu$ l of kinase buffer containing 5  $\mu$ g of Hsp-25 protein (Stress Gen Laboratories) as a substrate and 25  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. The reaction was incubated for 30 min at room temperature and was terminated by the addition of SDS-sample buffer. Proteins were subsequently analyzed by 10% SDS-PAGE, and the phosphorylated form of Hsp-25 was detected by autoradiography.

## RESULTS

**Extracellular Calcium Regulates MMP-9 Expression**—Because calcium-mediated changes in MMP-9 expression have been linked to keratinocyte differentiation, the effect of calcium on MMP-9 expression in immortalized normal (OKF6) or premalignant (pp126) oral keratinocytes and malignant OSCC cells (SCC4, CAL27, SCC25, and SCC68) was evaluated. Cells were cultured in low calcium (0.09 mM), serum-starved overnight, and incubated with fresh medium containing increasing concentrations of calcium (0.09–1.6 mM) for 24 h, followed by analysis of MMP-9 by gelatin zymography and ELISA. Whereas normal and premalignant oral keratinocytes (OKF6 and pp126) increased MMP-9 expression in response to calcium (Fig. 1), MMP-9 expression was not calcium-inducible in SCC68 and SCC25 cells (Fig. 1B). No MMP-9 expression was detectable in SCC4 and CAL27 cells (not shown). This was confirmed by ELISA, showing an increase in MMP-9 antigen levels in response to calcium in OKF6 and pp126 cells (Fig. 2), whereas the MMP-9 level in SCC68 and SCC25 cells remained unaltered (Fig. 2B). To determine whether the calcium-induced MMP-9 expression was due to enhanced transcriptional activation, luciferase reporter gene assays were performed using the sequence of –573 to +30 of the MMP-9 promoter. Cells lines were transfected with MMP-9 promoter luciferase fusion constructs, serum-starved overnight, and treated with a gradient of calcium (0.09–1.6 mM) for 24 h. Similar to results obtained by gelatin zymography and ELISA, a dose-dependent increase in transcriptional activation of the MMP-9 promoter was observed with increasing calcium in normal and premalignant

oral keratinocytes, resulting in a 2–5-fold increase in promoter activity (Fig. 3, A and B), whereas calcium did not induce MMP-9 promoter activity in SCC68 and SCC25 cells (Fig. 3, C and D). As these data indicated a calcium-induced increase in MMP-9 mRNA levels, representative RT-PCR analysis was performed using OKF6 and SCC68 cells. These data support the results of promoter/luciferase assays, showing an increase in MMP-9 mRNA in response to calcium in OKF6 cells (Fig. 4A) but not SCC68 cells (Fig. 4B). GAPDH controls were unaltered in response to extracellular calcium (Fig. 4, A and B, lower panels). Together these data suggest that normal or premalignant oral keratinocytes respond to extracellular calcium signals via changes in MMP-9 expression.

**Calcium Regulates ERK1/2 and p38 MAPKs**—Numerous studies have demonstrated that MMP-9 expression can be regulated by ERK1/2 (27, 28), and pharmacological inhibitors that block ERK1/2 phosphorylation down-regulate MMP-9 gene expression (28). To determine whether the calcium-induced MMP-9 expression was mediated via an ERK1/2-dependent pathway, the effect of calcium on ERK1/2 activation was examined. Cells were serum-starved for 24 h in low calcium followed by treatment with calcium (final [Ca<sup>2+</sup>] of 1.6 mM) and then were lysed at the indicated time points and analyzed for phosphorylated ERK1/2 by Western blot analysis. A rapid induction of ERK1/2 phosphorylation occurred in response to extracellular calcium in OKF6 and pp126 cells, peaking at 15 min and decaying to near basal levels by 60 min (Fig. 5, A and B). Densitometric analysis indicated that calcium enhanced ERK1/2 phosphorylation by ~2–3-fold, with maximal effect seen at 15 min (Fig. 5, A and B). Total ERK1/2 expression levels remained constant (Fig. 5, A–D, T-ERK blots). In contrast, no further increase in ERK1/1 phosphorylation was observed in response to calcium in SCC68 and SCC25 cells (Fig. 5, C and D).

In addition to ERK1/2, p38 MAPK signaling has also been implicated in the regulation of MMP-9 expression (29). p38 MAPK phosphorylates a wide array of downstream effectors

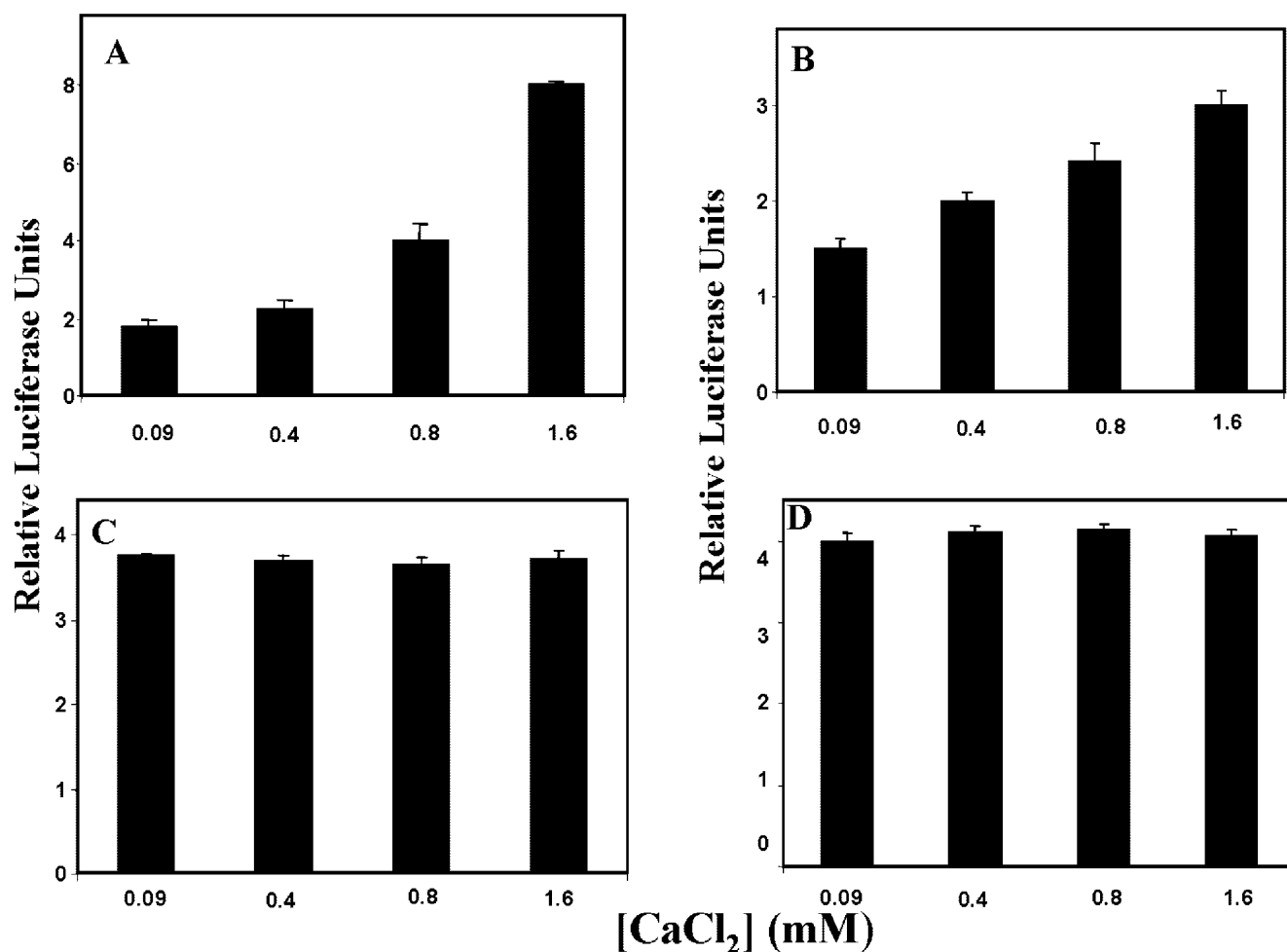


FIG. 3. **Extracellular calcium increases MMP-9 promoter activity.** OKF6 (A), pp126 (B), SCC25 (C), and SCC68 (D) cells were transiently transfected with a MMP-9 promoter/luciferase reporter construct. Following serum starvation for 24 h, the cells were treated with increasing calcium as indicated. Twenty-four h later, the cells were lysed, and luciferase activities were determined by luminometry. Results are normalized for protein concentration and represent the mean  $\pm$  S.E. of three different experiments.

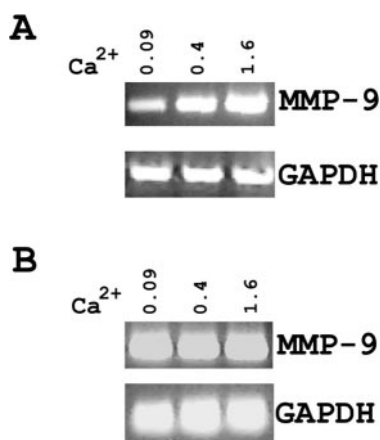
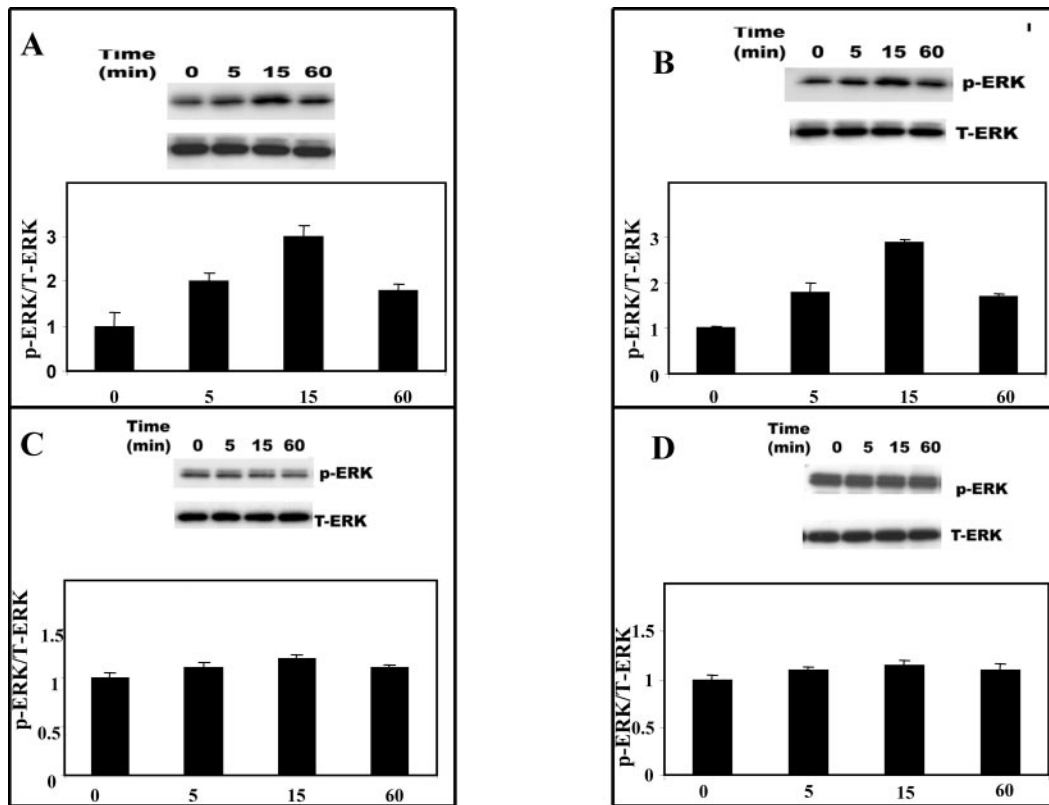


FIG. 4. **Extracellular calcium increases MMP-9 message levels.** OKF6 (A) and SCC68 (B) cells were serum-starved for 24 h and then treated for an additional 24 h with increasing calcium concentrations as indicated. Total RNA was isolated using TRIzol reagent, and RT-PCR reactions for MMP-9 and GAPDH messages were performed using primers and conditions detailed under "Experimental Procedures." The samples were electrophoresed on 1.5% agarose gel and visualized by UV transillumination.

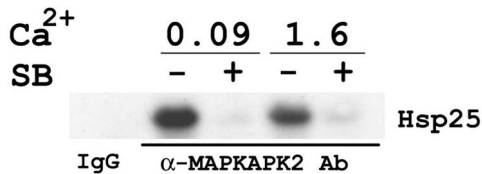
including ATF-2 (30, 31), ELK (32), and MAPKAPK2 (33–35). To evaluate the effect of extracellular calcium on p38 MAPK activity, the ability of the p38 MAPK-activated kinase MAPKAPK2 to phosphorylate a substrate (Hsp-25) was ana-

lyzed using an immunocomplex kinase assay. OKF6 cells were serum-starved for 24 h followed by culture in low (0.09 mM) or high (1.6 mM) calcium for 1 h. Harvested cells were then lysed and subjected to immunoprecipitation with anti-MAPKAPK2 or control IgG as detailed under "Experimental Procedures." The immunoprecipitates were utilized to catalyze *in vitro* phosphorylation of the MAPKAPK2 substrate Hsp-25 using radiolabeled [ $\gamma$ - $^{32}$ P]ATP. A calcium-dependent decrease in MAPKAPK2 activity was observed, as evidenced by a 30% decrease in phosphorylation of Hsp-25 by cells grown in high calcium (Fig. 6, *second* and *fourth* lanes). In control experiments, a pharmacologic inhibitor of p38 MAP kinase (SB203580) abolished Hsp-25 phosphorylation (Fig. 6, *third* and *fifth* lanes). No Hsp-25 phosphorylation was observed in samples precipitated with control IgG (Fig. 6, *first* lane).

*Calcium-mediated MMP-9 Expression Is Regulated by MAPKs*—As ERK1/2 and p38 MAPKs have been implicated in regulation of MMP-9 expression (36) and as changes in extracellular calcium differentially modulate ERK1/2 and p38 MAPK activity (Figs. 5 and 6), the contribution of MAPK activity to calcium-induced MMP-9 expression was evaluated. Cells were transiently transfected with the MMP-9 promoter/luciferase reporter construct as described under "Experimental Procedures," serum-starved, and pretreated for 1 h either with the MEK inhibitor PD98059 to block ERK1/2 activation by the upstream kinase MEK or with the direct p38 MAPK inhibitor SB203580 prior to induction with 1.6 mM calcium for 24 h. In



**FIG. 5. Extracellular calcium promotes ERK1/2 phosphorylation only in normal and premalignant oral keratinocytes.** OKF6 (A), pp126 (B), SCC25 (C), and SCC68 (D) cells were serum-starved overnight in low calcium (0.09 mM) medium followed by treatment with medium containing 1.6 mM calcium for the indicated time periods. Cells were lysed, and lysates (50  $\mu$ g of protein) were electrophoresed on 9% SDS-polyacrylamide gels and electroblotted to polyvinylidene difluoride membranes. The lysates were probed with antiphospho-ERK1/2 antibody (1:1000 dilution) to detect the phosphorylated, active form of ERK1/2 (*upper blots*) or with anti-ERK1/2 antibody (1:2000 dilution) to detect total ERK1/2 (*T-ERK*) expression (*lower blots*), followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. The results are representative of three independent experiments. Semiquantitative densitometric analysis of the blots was performed using an Amersham Biosciences PhosphorImager Storm 860.



**FIG. 6. Extracellular calcium inhibits p38 MAPK activity.** OKF6 cells were serum-starved for 24 h and then incubated with low or high calcium in the presence or absence of SB203580 (SB) (10  $\mu$ M) as indicated. Cells were lysed and immunoprecipitated with an antibody against MAPKAPK2 ( $\alpha$ -MAPKAPK2 Ab), and *in vitro* kinase assays were carried out using Hsp-25 as an exogenous substrate as detailed under "Experimental Procedures." Phosphorylated Hsp-25 was detected by autoradiography.

OKF6 and pp126 cells, inhibition of ERK1/2 activation significantly reduced MMP-9 promoter activity (Fig. 7A), whereas blocking p38 MAPK activity led to a dramatic increase in transcription of the reporter gene (Fig. 7B), indicating that p38 MAPK is a negative regulator of MMP-9 transcription in normal and premalignant oral keratinocytes. Inhibition of ERK1/2 activation caused a similar decrease in MMP-9 promoter activity in SCC68 and SCC25 cells (Fig. 7A); however, no transcriptional activation was observed in response to p38 MAPK inhibition (Fig. 7B). In control experiments, inhibition of p38 MAPK activity had no effect on activation of the MMP-9 promoter under low calcium conditions (not shown).

To confirm the negative regulatory effect of p38 MAPK on MMP-9 expression observed with pharmacologic inhibition (Fig. 7B), constitutively active and dominant negative mutants of the immediate upstream p38 MAPK activator MKK3 were

utilized. OKF6 cells were co-transfected with the MMP-9 promoter/reporter plasmid and the MKK mutant as indicated. Cells were then serum-starved for 24 h followed by treatment with high calcium for 24 h. Consistent with the results observed using the pharmacologic p38 MAPK inhibitor SB203580, overexpression of a dominant negative catalytically inactive MKK3 mutant (MKK3Ala) significantly enhanced calcium-induced MMP-9 gene expression (Fig. 8). In contrast, the constitutively active MKK3 mutant (MKK3Glu) decreased calcium-induced MMP-9 promoter activation, and expression was partially restored using the p38 MAPK inhibitor SB203580 (Fig. 8). These data support the hypothesis that p38 MAPK negatively modulates calcium-induced MMP-9 gene expression in OKF6 cells.

**Reciprocal Regulation of ERK1/2 and p38 MAPK Activity**—As the above results suggest reciprocal regulation of calcium-induced MMP-9 expression via differential activation of the ERK1/2 and p38 MAPK pathways in normal and premalignant oral keratinocytes, the potential for signaling cross-talk between these pathways was evaluated. Following serum starvation, cells were pretreated with increasing concentrations of the p38 MAPK inhibitor SB203580 prior to treatment with 1.6 mM calcium for 30 min. Inhibition of p38 MAPK activity led to a dose-dependent increase in ERK1/2 phosphorylation in OKF6 cells and pp126 cells (Fig. 9, A and B, *upper panels*). The effect of SB203580 on ERK1/2 phosphorylation was mediated by MEK because concomitant treatment with the MEK inhibitor U0126 abrogated the effect (Fig. 9B). In contrast, inhibition of p38 MAPK activity did not considerably alter ERK1/2 phosphorylation in SCC25 and SCC68 cells (Fig. 9, C and D, *upper panels*). However, the constitutive level of ERK1/2 phosphorylation

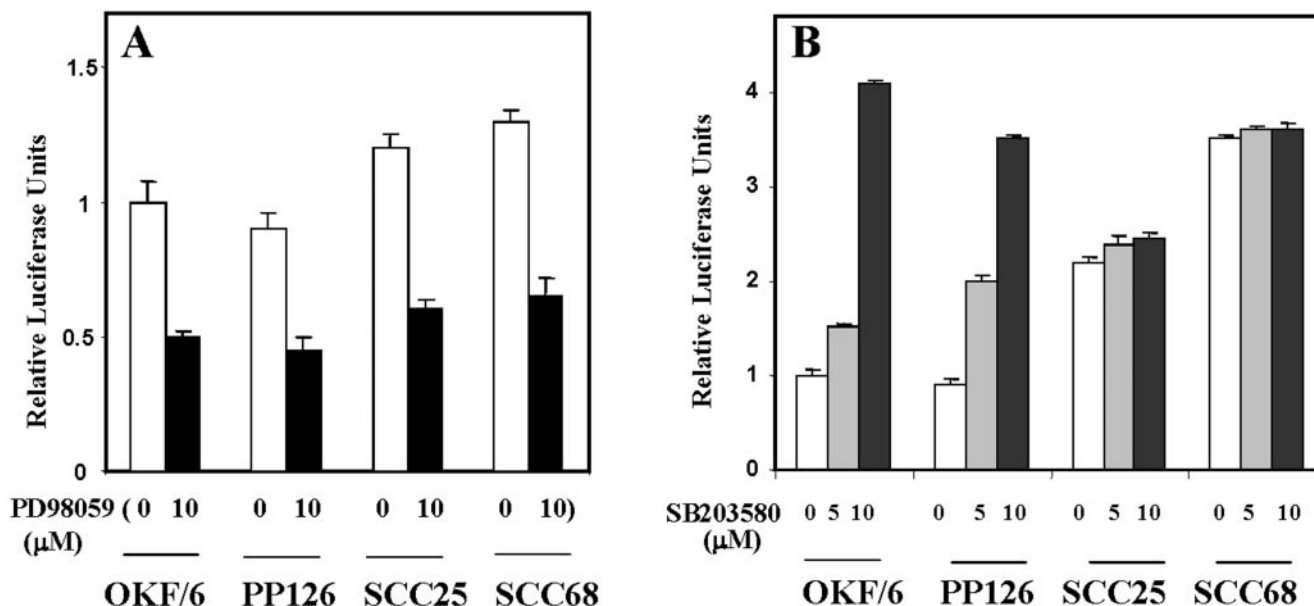


FIG. 7. ERK1/2 and p38 MAPK differentially regulate calcium-induced MMP-9 promoter activity. Cells were transiently transfected with MMP-9 promoter/luciferase reporter construct, serum-starved for 24 h, and pretreated with either PD98059 (10  $\mu$ M) (A) or SB203580 (10  $\mu$ M) (B) for 30 min prior to treatment with extracellular calcium (1.6 mM). Twenty-four h later, the cells were lysed, and luciferase activities were determined by luminometry. Results are normalized for transfection efficiency and expressed relative to the concentration of control cells treated with Me<sub>2</sub>SO alone, designated as 1.0. The results represent the mean  $\pm$  S.E. of three different experiments.

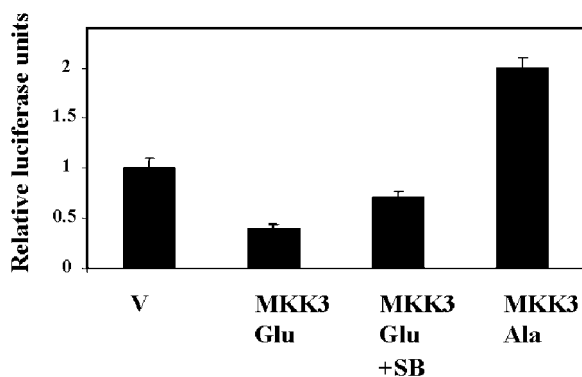


FIG. 8. MKK3 negatively regulates MMP-9 promoter activity. OKF6 cells were transiently transfected with a MMP-9 promoter/luciferase reporter construct and co-transfected with either dominant negative (MKK3Ala) or constitutively active (MKK3Glu) MKK3 mutants. Following serum starvation for 24 h, the cells were treated with high calcium (1.6 mM) for an additional 24 h. The cells were then lysed, and luciferase activities were determined by luminometry. Results are normalized for protein concentration and expressed relative to control cells co-transfected with vector (V), designated as 1.0. The results represent the mean  $\pm$  S.E. of three different experiments. SB, SB203580.

ation was catalyzed by MEK because concomitant treatment with MEK inhibitor U0126 abolished endogenous ERK1/2 phosphorylation (Fig. 9, C and D). To confirm that blocking p38 MAPK activity enhances MMP-9 transcription through reciprocal activation of ERK1/2, cells were transiently transfected with the MMP-9 promoter/luciferase construct and pretreated with the p38 MAPK inhibitor SB203580 in the presence or absence of the MEK inhibitor PD98059 prior to treatment with high calcium (Fig. 10). As observed previously (Fig. 7), inhibition of p38 MAPK activity enhanced MMP-9 transcription in OKF6 and pp126 cells (Fig. 10, A and B). However, pretreatment with the MEK inhibitor blocked this stimulatory effect, demonstrating that inhibiting p38 MAPK activity induces MMP-9 gene expression via an ERK1/2-dependent pathway. In contrast to normal and premalignant oral keratinocytes, p38 MAPK inhibition had no effect on MMP-9 promoter activation

in SCC25 and SCC68 cells (Fig. 10, C and D). As shown previously (Fig. 7), inhibition of ERK1/2 activation decreased MMP-9 promoter activity, and this effect was retained in the presence of the p38 MAPK inhibitor (Fig. 10, C and D). Together these data support a model for control of MMP-9 gene expression via reciprocal cross-talk between MAPK signaling pathways, such that a calcium-induced decrease in p38 MAPK activity enhances MMP-9 gene expression through reciprocal up-regulation of ERK1/2 activation.

#### DISCUSSION

Stringent regulation of MMP-9 expression plays an important role in normal keratinocyte behavior. Dysregulation of MMP-9 expression is prevalent in human squamous cell carcinoma of the oral cavity, with enhanced expression observed in areas of increased invasion (4–7, 10). The contribution of MMP-9 to the invasive phenotype is supported by studies in a murine orthotopic OSCC model, showing MMP-9-dependent invasion of the floor-of-mouth musculature (11). Furthermore, therapeutic modulation of MMP-9 expression via pharmacologic inhibition of ERK1/2 activation reduces invasion, demonstrating the importance of this MAPK to MMP-9 regulation *in vivo* (11). As microenvironmental factors such as alterations in extracellular calcium have been shown to influence differentiation of the oral mucosa and to modulate MAPK signaling, the effect of calcium on MMP-9 gene expression was investigated. Results of the current study demonstrate that increasing extracellular calcium induces a dose-dependent increase in MMP-9 expression in premalignant oral keratinocytes, but not in highly invasive OSCC cells. Calcium also induced activation of ERK1/2 with a concomitant down-regulation of p38 MAPK activity. Inhibition of p38 MAPK activity using pharmacologic or genetic approaches enhanced the calcium-induced MMP-9 gene expression, suggesting that p38 MAPK is a negative regulator of MMP-9 transcription.

Recent reports have indicated that calcium can affect MMP gene expression or activity. We recently demonstrated that calcium promotes proMMP-2 activation in SCC25 (derived from a squamous cell carcinoma of the oral cavity) via modu-

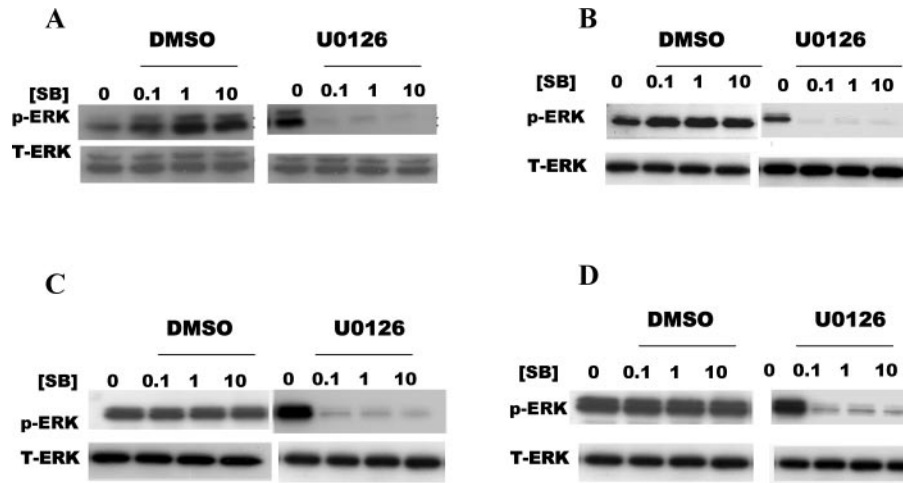


FIG. 9. Effect of p38 MAPK inhibition on calcium-induced ERK1/2 activity. OKF6 (A), pp126 (B), SCC25 (C), and SCC68 (D) cells were serum-starved overnight and then treated with SB203580 (0.1–10  $\mu\text{M}$ ) (SB) alone (left-hand blots) or in combination with 10  $\mu\text{M}$  U0126 (right-hand blots) for 30 min prior to treatment with 1.6 mM calcium for 15 min. Cells were lysed, and 50  $\mu\text{g}$  of lysate was electrophoresed on 9% SDS-polyacrylamide gels and electroblotted to polyvinylidene difluoride membranes. The blots were probed with antiphospho-ERK1/2 antibody (1:1000) to detect the phosphorylated, active form of ERK1/2 or with anti-ERK1/2 antibody (1:2000) to detect total ERK1/2 (T-ERK) expression. DMSO, Me<sub>2</sub>SO.

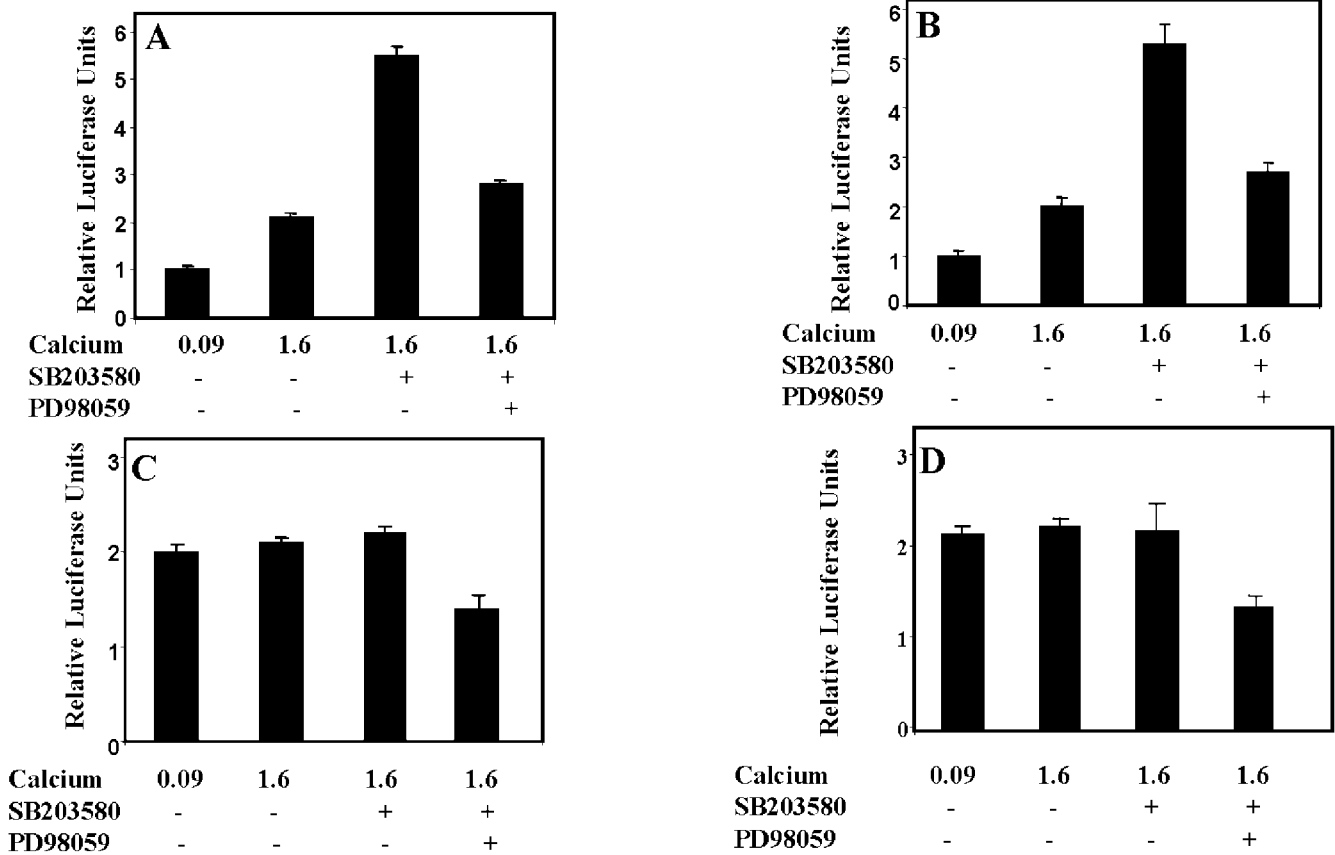


FIG. 10. Effect of p38 MAPK inhibition on calcium-induced MMP-9 promoter activity. OKF6 (A), pp126 (B), SCC25 (C), and SCC68 (D) cells were transiently transfected with a MMP-9 promoter/luciferase reporter construct. After serum starvation for 24 h, cells were pretreated with SB203580 (10  $\mu\text{M}$ ) or with both SB203580 (10  $\mu\text{M}$ ) and PD98059 (10  $\mu\text{M}$ ) for 30 min prior to treatment with high calcium. The cells were then lysed, and luciferase activities were determined by luminometry. Results are normalized for protein concentration and expressed relative to control cells (0.09 mM Ca<sup>2+</sup>, Me<sub>2</sub>SO), designated as 1.0. The results represent the mean  $\pm$  S.E. of three different experiments.

lation of membrane type 1 MMP (MT1-MMP) activity (37). However, MMP-9 expression in SCC25 and SCC68 cells was unaltered in response to calcium, consistent with the current data that calcium regulation of MMP-9 is limited to normal or premalignant cells. Although the current study has evaluated only four oral cell lines (two normal/premalignant and two malignant), it is nevertheless interesting to speculate that ma-

lignant OSCC cells may escape normal differentiation-induced controls on MMP-9 expression. In premalignant cells, calcium regulated MMP-9 expression at the level of gene transcription. In support of this observation, it was shown recently that calcium promotes MMP-9 expression in normal skin keratinocytes. Induction was at the level of gene transcription, with promoter analysis demonstrating that the effect of calcium is

mediated through a promoter element designated KRE-M9 (17).

Calcium-mediated MMP-9 induction was differentially regulated by MAPKs. MMP-9 expression was correlated with enhanced ERK1/2 phosphorylation in premalignant oral keratinocytes, and transfection of a dominant negative mutant of ERK blocked calcium-induced MMP-9 promoter activity (data not shown). This is in agreement with a number of recent reports implicating ERK1/2 as the key mediator of MMP-9 gene expression. Epidermal growth factor- and scatter factor/hepatocyte growth factor-induced MMP-9 expression in keratinocytes was mediated by ERK1/2 activation and could be inhibited by treatment with MEK inhibitor PD98059 (38). Moreover, transfection of constitutively active MEK increased MMP-9 expression in NIH3T3 cells, whereas catalytically inactive ERK1 blocked MMP-9 expression in glioblastoma cells (39). In contrast, in the current study a negative correlation was observed between p38 MAPK activity and calcium-induced MMP-9 gene expression in normal/premalignant oral keratinocytes. Furthermore, our results support a model for cross-talk between the ERK1/2 and p38 MAPK pathways, as evidenced by data showing that pharmacologic or genetic inhibition of p38 activity results in sustained ERK1/2 activation (40, 41). Together these data support a model for control of MMP-9 gene expression via reciprocal cross-talk between MAPK signaling pathways, such that a calcium-induced decrease in p38 MAPK activity enhances MMP-9 gene expression through reciprocal up-regulation of ERK1/2 activation. This hypothesis is consistent with published data showing that lipopolysaccharide-induced MMP-9 activity in monocytes is positively regulated by ERK1/2 and negatively regulated by p38 MAP kinase activity (36). Moreover, treatment of monocytes with the p38 MAPK inhibitor SB203580 increased ERK1/2 activation, and this phosphorylation was blocked by the MEK inhibitor PD98059, indicating that there is also cross-talk between ERK1/2 and p38 MAPK in monocytes (42). Changes in extracellular calcium are monitored by the ubiquitously expressed extracellular calcium-sensing receptor (CaR) (43, 44). Calcium- or agonist-induced activation of this G protein-coupled receptor has been linked to activation of various MAP kinases, including ERK1/2 (45–47). Our preliminary data suggest that CaR activation with the polycationic agonist  $Gd^{3+}$  can also induce MMP-9 gene expression (data not shown), suggesting that signaling through the CaR may control MMP-9 expression in premalignant oral keratinocytes. As calcium is a key regulator of keratinocyte function, these data suggest that localized changes in calcium in the extracellular milieu may also function to regulate MMP activity. Loss of this regulatory mechanism, through alteration of CaR and/or its downstream effectors, may accompany malignant transformation of the oral epithelium.

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