Cardiovascular, Pulmonary and Renal Pathology

Distinctive Epidermal Growth Factor Receptor/Extracellular Regulated Kinase-Independent and -Dependent Signaling Pathways in the Induction of Airway Mucin 5B and Mucin 5AC Expression by Phorbol 12-Myristate 13-Acetate

Daphne Yuan-chen Wu,* Reen Wu,*
Sekhar P. Reddy,[†] Yong Chan Lee,* and
Mary Mann-Jong Chang*

From the Center for Comparative Respiratory Biology and Medicine,* University of California, Davis, California; and the Department of Environmental Health Sciences,[†] School of Public of Health, The Johns Hopkins University, Baltimore, Maryland

Elevated expression of gel-forming mucin (MUC) genes *MUC5AC* and *MUC5B* is a major pathological feature in various airway diseases. In this study, we show that phorbol 12-myristate 13-acetate (PMA) is a potent stimulator for *MUC5B* gene expression under air-liquid interface conditions in three airway epithelial cell systems: primary cultures of normal human bronchial epithelial cells, the immortalized normal bronchial epithelial cell line HBE1, and the human lung adenocarcinoma cell line A549. Stimulation was time- and dose-dependent, could be demonstrated by promoter-reporter gene transfection, and was sensitive to mithramycin A, suggesting the involvement of a specificity protein 1-based transcriptional mechanism in the stimulation. PMA-induced *MUC5B* message and promoter-reporter gene activity were specifically sensitive to inhibition of protein kinase C δ , which was further confirmed by the forced expression of dominant-negative mutant of protein kinase C δ . Regarding downstream transduction, PMA-induced *MUC5B* expression was sensitive to inhibitors and dominant-negative expression of signaling molecules involved in Ras/mitogen-activated protein kinase/extracellular signal-regulated kinase kinase1-mediated c-Jun N-terminal kinase and p38 pathways. This contrasted with the inhibition of PMA-induced *MUC5AC* expression by inhibitors of the Ras/epidermal growth factor receptor/extracellular regulated kinase signaling pathway. These results demonstrate

for the first time that PMA-stimulated *MUC5AC* and *MUC5B* expressions are regulated through distinctive epidermal growth factor receptor/extracellular regulated kinase-dependent and -independent signaling pathways. (Am J Pathol 2007, 170:20–32; DOI: 10.2353/ajpath.2007.060452)

Mucus secretion is essential for proper mucociliary function and homeostatic control in the airways.¹ Mucus lining the airways traps inhaled dust particles, chemicals, and microbes.² However, airway mucus hypersecretion and accumulation in the airway lumen are pathological symptoms associated with various chronic airway diseases.^{3,4} Mucus obstruction of the airway is the major cause of morbidity and mortality in patients with chronic airway diseases.⁵ To date, 20 different mucin (MUC) genes have been identified. Among these MUC genes, at least nine of them (*MUC1*, -2, -4, -5AC, -5B, -7, -8, -13, and -19) are expressed in human airways. *MUC2*, *MUC5AC*, *MUC5B*, and the newly found *MUC19* are gel-forming mucin genes^{6–8} expressed by the airway epithelium, but only *MUC5AC* and *MUC5B* gene products have been convincingly demonstrated in human airway secretions.^{2,6,9,10}

In normal human airways, *MUC5AC* is mainly expressed by surface goblet epithelial cells, whereas *MUC5B* is predominantly expressed by mucous cells of submucosal glands.¹¹ Cumulative studies have demonstrated the aberrant elevation and accumulation of

Supported by National Institute of Health grant HL73160-02 (to M.M.-J.C.) and in part by National Institute of Health grant HL077902 (to R.W.).

Accepted for publication September 14, 2006.

Address reprint requests to Mary Mann-Jong Chang, Ph.D., Center for Comparative Respiratory Biology and Medicine, Genome and Biomedical Science Facility, Suite 6510 University of California, Davis, 451 East Health Sciences Dr., Davis, CA 95616. E-mail: mjchang@ucdavis.edu.

MUC5AC and *MUC5B* in airway secretions from patients with lung diseases such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis.^{12,13} However, *MUC5B* gene products in diseased airways are also found in the surface epithelium, rather than just being limited to the submucosal glands. Using an ovalbumin-induced mouse asthma model, our laboratory has shown expression of the glandular *MUC5B* message in surface airway epithelial cells.¹⁴ A similar disease-related gene *trans*-expression has also been demonstrated in patients with emphysema and diffuse panbronchiolitis.^{15,16} Thus, a change in cell type-specific *MUC5B* gene expression is a significant feature associated with the pathogenesis of airway diseases.

Phorbol 12-myristate 13-acetate (PMA) can induce protein kinase C (PKC) activation by acting as an alternative stimulus to diacylglycerol. PMA has been demonstrated as a model inflammatory stimulus that can modulate a variety of cellular events including gene transcription,¹⁷ cell growth, and differentiation.¹⁸ It has also been used as a tumor-promoting agent.¹⁹ PKC activation in airway epithelial cells occurs frequently in airways after cigarette smoking, oxidant exposure, and microorganism infections and during various inflammatory process.^{17,20} The role of PMA in the induction of mucins has been demonstrated for *MUC2* and *MUC5AC* using NCI-H292 and HM3 colon cell lines.^{17,21} The results have suggested a PKC δ -, epidermal growth factor receptor (EGFR)-, Ras/Raf-, extracellular regulated kinase (ERK)-mediated specificity protein 1 (Sp1)-based transcriptional mechanism. Unlike for *MUC2* and *MUC5AC*, there is very little information in regard to the effect of PMA on *MUC5B* expression. Recent completion of the *MUC5B* gene cloning and the characterization of its promoter sequence make it feasible to define molecular mechanisms that regulate the transcription of *MUC5B*.²² In this communication, we show that PMA is a potent mediator for the expression of *MUC5B* in primary human bronchial epithelial cell cultures and in two cell lines: an immortalized normal bronchial epithelial cell line, HBE1, and a lung adenocarcinoma cell line, A549. In contrast to the signaling cascade of *MUC5AC* induction, PMA-enhanced *MUC5B* expression occurs through an EGFR/ERK-independent but PKC δ -, Ras-, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEKK) 1-mediated, c-Jun N-terminal kinase (JNK)/p38-dependent signaling pathway. These are the first data to identify the molecular signaling mechanism involved in the regulation of *MUC5B* expression in airway epithelial cells.

Materials and Methods

Cell Culture

Normal human primary tracheobronchial epithelial cells (NHBE) were isolated from human bronchi and trachea obtained from organ donors or autopsy at the University of California, Davis, Medical Center (Sacramento, CA). Tissue procurement and utilization were approved and

periodically reviewed by the University of California Davis Human Subject Research Review Committee. Two airway epithelial cell lines were used in this study: HBE1, a papilloma virus-immortalized bronchial epithelial cell line, generated by Dr. James Yankaskas (University of North Carolina, Chapel Hill, NC),²³ and A549, a human lung adenocarcinoma cell line obtained from the American Type Culture Collection (Manassas, VA).

Cell isolation and culture methods were performed as described previously,^{24,25} with some modifications. NHBE cells (1×10^4 cells/cm²) were plated on a Costar Transwell chamber (25 mm²) in Ham's F12/Dulbecco's modified Eagle's medium (1:1) supplemented with insulin (5 μ g/ml), transferrin (5 μ g/ml), epidermal growth factor (EGF) (10 ng/ml), dexamethasone (0.1 μ mol/L), cholera toxin (10 ng/ml), bovine hypothalamus extract (15 μ g/ml), and bovine serum albumin (0.5 mg/ml).^{26,27} All-*trans*-retinoic acid (0.03 μ mol/L) was added approximately 2 days after plating when the cells reached confluence. Approximately 1 week after plating, the immersed primary NHBE cells were changed to an air-liquid interface. After 3 weeks in culture (2 weeks under an air-liquid interface), NHBE cells underwent mucociliary differentiation, including formation of cilia and mucus-secreting granules.²⁴ HBE1 cells were cultured under the same air-liquid interface (biphasic) condition as described for primary NHBE cells. In the presence of retinoic acid, HBE1 cells expressed comparable levels of *MUC5AC* and *MUC5B* gene products, except they did not undergo ciliogenesis. A549 cells, which constitutively expressed *MUC5AC* and *MUC5B* gene products, were also cultured under an air-liquid interface in RPMI (GIBCO, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. Most experiments, except the transient transfection studies, were done 21 days after passage.

Enzyme-Linked Immunosorbent Assay for *MUC5B* Protein

An antibody to *MUC5B* protein (5B#19-2E) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The monoclonal antibody recognizes a 19-amino acid sequence (QREELPYSRTGLLVEQSGD) that is unique to the N terminus of human *MUC5B* protein. The antibody has been used previously to detect *MUC5B* secretion in primary airway epithelial cell cultures.²⁸ We confirmed the authenticity of the antibody by Western blot analysis; the antibody reacted with salivary secretion and purified *MUC5B* protein but not *MUC5AC* protein as provided by Dr. D. Thornton⁷ (data not included). With this antibody, an enzyme-linked immunosorbent assay (ELISA) method, similar to the work of Rousseau et al²⁹ and Park et al,²⁸ was developed with a slight modification, using the synthetic 19-amino acid peptide as a referenced standard. In brief, day-21 cultures of NHBE, HBE1, and A549 cells were treated with or without 10 nmol/L PMA. Transwell cultures were lysed in 100 μ l of radioimmunoprecipitation assay buffer, (1% NP-40, 0.25% deoxycholic acid, 1 mmol/L ethylenediamine tetraacetic acid, 150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4, 1 mmol/L phenylmeth-

ylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L NaF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin at 0, 8, 16, and 24 hours after PMA treatment. Diluted lysates (1:10 in water) and different amounts of referenced 19-amino acid peptide standards were added (100 μ l/well) to Maxisorp microtiter plates (Nunc, Rochester, NY) and dried overnight at 37°C. The plates were briefly exposed to UV light for 1 minute (UV Stratalinker 2400; Stratagene, La Jolla, CA) to ensure that the proteins were cross-linked to the substratum and were retained in the well during subsequent washings. The plates were deglycosylated with 200 mmol/L NaIO₄ and sodium-acetate buffer (0.33 mol/L NaCl and 0.1 mol/L glacial acetic acid, pH 4.5) and kept overnight at 4°C. On the following day, a sodium-thiosulfate solution (0.133 mol/L Na₂S₂O₃, 0.033 mol/L NaI, and 0.033 mol/L NaHCO₃, pH 7.6) was added for 30 minutes at 4°C. The wells were blocked with 1× Blocker BSA (Pierce, Rockford, IL) for 30 minutes, followed by three washes with phosphate-buffered saline (PBS) (100 μ l/well for 10 minutes). Next, wells were washed three times with PBS/0.05% Tween 20 and incubated for 1 hour with MUC5B antibody diluted 1:30 in PBS/0.05% Tween 20. After five washes with PBS/0.05% Tween 20, an anti-mouse antibody conjugated with horseradish peroxidase was added to the wells and incubated for 30 minutes at room temperature. After five washes with PBS/0.05% Tween 20, a color reaction was developed with 100 μ l of 3,3',5,5'-tetramethylbenzidine substrate (Novagen, San Diego, CA), which was read at 450 nm.

A standard curve was generated with known amounts of serially diluted synthetic 19-amino acid peptide. Cell lysate data were converted according to the standard curve and expressed as moles of MUC5B equivalent per milligram of protein. Experiments were performed in three separate, independent primary NHBE cultures from cells derived from different sources and with two separate passages of HBE1 and A549 cells. Each cell extract was assayed in triplicate wells of ELISA.

MUC5B Promoter-Reporter Transfection Study and Luciferase Assay

A luciferase reporter construct, pGL3-MUC5B, containing 4169 bp of 5'-flanking region of MUC5B previously constructed in our laboratory³⁰ was used for the MUC5B promoter study. For transient transfection studies, NHBE, HBE1, and A549 cells were plated on 24-well plates at 5×10^4 cells/well. The cells were transfected with 0.2 μ g of pGL3-MUC5B clone plus 0.2 μ g of pSV- β -galactosidase (β -gal) plasmid for normalizing transfection efficiency. Transfection was performed by FuGENE 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. Cells were transfected in Opti-MEM reduced serum media (Invitrogen). Sixteen hours after transfection, cells were treated with 10 nmol/L PMA in Ham's F12/Dulbecco's modified Eagle's medium (1:1) supplemented with 5 μ g/ml insulin, 10 ng/ml EGF, 0.1 μ mol/L dexamethasone, 5 μ g/ml transferrin, 20 ng/ml cholera toxin, and 15 μ g/ml bovine hypothalamus extract.

Cells were harvested at 0, 8, 16, 24, and 48 hours after PMA treatment for reporter assays. Luciferase activity was determined using LucLite (PerkinElmer, Boston, MA) and counted in a luminometer. β -Galactosidase activities were assayed and read at OD₄₀₅. For each transfection, relative luciferase activity was normalized to β -galactosidase activity.

For the forced mutant protein expression studies, various dominant-negative (dn) and constitutively active (ca) clones were co-transfected with pGL3-MUC5B. All dn and ca expression clones used were as previously described.³¹ dn-Ras (Ras-N17) was generated by introducing a point mutation at the 17-position of Ha-Ras. Ca-Ras (Ha-Ras-V12) was cloned in a pBCMG vector.³² dn-MEKK1 (Δ 367MEKK1-KR), ca-MEKK1 (Δ 367MEKK1), and dn-SEK1/mitogen-activated protein kinase kinase (MKK) 4 (SEK1-AL, serine 220, and threonine 224 mutated to alanine and leucine, respectively) cloned in pEECMV were generously provided by Dr. Dennis Templeton (Case Western Reserve University, Cleveland, OH).^{33,34} dn-MKK7 (F.MKK7), dn-JNK1 (APF), dn-JNK2, and dn-MKK6 (ala) cloned in pCDNA3 vector; dn-p38 α (AGF) cloned in pCMV5 vector; and dn-MKK3 (ala) cloned in pRSV vector were kindly provided by Dr. Roger Davis (University of Vermont, Burlington, VT).³⁵⁻³⁷ dn-ERK1 (Lys⁷¹→Arg) and dn-ERK2 (Lys⁵³→Arg) mutants, each cloned in a pCEP4 vector, were generously provided by Dr. Melanie Cobb (University of Texas, Austin, TX).³⁸ dn-c-Raf (dn-cRaf-C4) mutant and dn-MKK3 (ala), each cloned in a pRSV vector, were kindly provided by Dr. Stephan Ludwig (Universitat Wurzburg, Wurzburg, Germany).³⁹ dn-PKC α and dn-PKC β , each cloned in a SRD vector,⁴⁰ and dn-PKC δ cloned in pCMV vector were kindly provided by Drs. Motoi Ohba, Toshio Kuroki, and Shigeo Ohno (Showa University, Tokyo, Japan).^{41,42}

Inhibitor Studies

To conduct inhibitor studies, 21-day-old cultures of NHBE, HBE1, and A549 cells were starved 1 day in serum- and hormone-free medium, except for the supplementation of retinoic acid. After starvation, cells were pretreated with various inhibitors or vehicles 1 hour before PMA treatment. RNA and protein extract were prepared from these cultures 24 hours after PMA treatment for real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and luciferase quantification, respectively.

Mithramycin A was purchased from Sigma (St. Louis, MO). Manumycin A, radicicol, JNK inhibitor II (SP600125), SB203580, U0126, AG9, 4-[(3-bromophenyl)amino]-6,7-diaminoquinazoline (BPDQ), and AG1478 were purchased from EMD Biosciences-Calbiochem (San Diego, CA). Calphostin C, Rottlerin, and Gö6979 were purchased from Biomol International (Plymouth Meeting, PA). Inhibitor concentrations were based on the manufacturer's suggested ranges, and at these concentrations, inhibitor toxicity to the cultured cells was minimal as determined by trypan blue exclusion testing (>95% of cultured cells excluding this dye).

Figure 1. Stimulation of *MUC5B* mRNA by PMA. **A:** Dose-response study of PMA effect on *MUC5B* mRNA expression. HBE1, A549, and NHBE cultures grown in air-liquid interface for 2 weeks supplemented with retinoic acid (day 21) were starved for 16 hours (without growth factors) before being treated with different concentrations of PMA (0 to 10 nmol/L) or 4α-PMA (0 to 10 nmol/L). Twenty-four hours after PMA or 4α-PMA treatment, total RNA was harvested, and *MUC5B* mRNA was analyzed using SYBR Green quantitative real-time RT-PCR as described in Materials and Methods. Triplicate dishes were used for each concentration, and experiments were repeated at least three times for different cultures derived from different donors and passages of cell lines. Statistical significance: **P* < 0.05 and ***P* < 0.01, respectively, compared with the unstimulated control. **B:** Time course study of PMA effects on *MUC5B* mRNA expression. Day-21 cultures of HBE1, A549, and NHBE cells as described in **A** were treated with either 10 nmol/L PMA or 10 nmol/L 4α-PMA and harvested at different time points (0, 8, 12, 16, 24, and 48 hours). Samples were measured with SYBR Green quantitative PCR. Triplicate dishes were used for each time point, and experiments were repeated at least three times in different cultures derived from different donors and passages of cell lines. Statistical significance: **P* < 0.05 and ***P* < 0.01, respectively, compared with the unstimulated control.

RNA Isolation and Quantitative RT-PCR

RNA was extracted from NHBE, HBE1, and A549 cells after PMA and inhibitor treatments by using RNA Trizol reagent (Invitrogen) according to the manufacturer's protocol. Extracted RNA (2 μg) was converted to cDNA by adding MMLV-reverse transcriptase (Promega, Madison, WI) and oligo-dT primers for a total volume of 20 μl. The reaction was further diluted to 40 μl with water and used for real-time PCR analysis. The PCR reaction was performed in a 96-well optical PCR plate, and each reaction contained a 50-μl mixture consisting of 25 μl of 2× SYBR Green PCR Master mix (Applied Biosystems, Inc., Foster City, CA), 2 μl of cDNA sample, 1 μl of 5 μmol/L each of forward and reverse primer, and 21 μl of RNase-free water. Results were analyzed by an ABI PRISM 7900HT Sequence Detection system (Applied Biosystems, Inc.). The following PCR primers were used: β-actin forward, 5'-CTGGAACGGTGAAGGTGACA-3'; β-actin reverse, 5'-AAGGGACTTCCTGTAACAATGCA-3'; *MUC5B* forward, 5'-GCTGCTGCTACTCCTGTGAGG-3'; *MUC5B* reverse, 5'-AGGTGATGTTGACCTCGGTCTC-3'; *MUC5AC* forward, 5'-GGAAGTGTGGGGACAGCTCTT-3'; and *MUC5AC* reverse, 5'-GTCACATTCTCAGCGAGGTC-3'. The relative mRNA amount in each sample was calculated based on its Ct value normalized with the Ct value of housekeeping gene (β-actin). The calculation formula is $2^{-(Ct \text{ of } MUC5B \text{ or } MUC5AC - Ct \text{ of } \beta\text{-actin})}$. Results are the arbitrary unit of PMA-treated samples compared with the arbitrary unit of no treatment controls and presented as induction percentage or fold induction.

Western Blot Analysis

Fifty micrograms of protein lysate prepared in radioimmunoprecipitation assay solution was subjected to electrophoresis on a sodium dodecyl sulfate/12.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). The polyvinylidene difluoride membrane was blocked with 5%

milk in Tris-buffered saline/Tween 20 (TBST) and then probed with anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-stress-activated protein kinase/JNK, anti-stress-activated protein kinase/JNK, anti-phospho-p38 mitogen-activated protein kinase (MAPK), and anti-p38 MAPK antibodies (Cell Signaling Technology, Inc., Danvers, MA). The membrane was washed with TBST and then probed for 1 hour with secondary antibodies conjugated with horseradish peroxidase. After four intensive washes with TBST, immunoreactive bands were visualized by chemiluminescence with the use of Western blotting luminal reagent (Santa Cruz Biotechnology).

Results

PMA Stimulates *MUC5B* Mucin Production

PKC activation has been suggested as one of the major pathways involved in the regulation of *MUC2* and *MUC5AC* gene expression.^{21,43,44} There are very few studies related to the effect of PMA on *MUC5B* expression. In this experiment, we examined whether PMA could stimulate *MUC5B* mucin gene expression under a long-term air-liquid interface condition in NHBE, HBE1, and A549 cells. As shown in Figure 1, PMA induced *MUC5B* mRNA in all primary NHBE, HBE1, and A549 cells, but 4α-PMA, a negative control for phorbol ester activation of PKC, failed to do any induction. *MUC5B* mRNA induced by PMA occurred in a dose- and time-dependent manner (Figure 1, A and B, respectively). Maximum stimulation of *MUC5B* expression occurred 24 hours after the addition of 10 nmol/L PMA. The induction of *MUC5B* mRNA also resulted in an increase in *MUC5B* secretion as detected by an ELISA for *MUC5B* mucin (Figure 2). The concentration of *MUC5B*-equivalent mucin protein in NHBE, A549, and HBE1 cells was 37.03, 33.05, and 53.14 pmol/mg cell lysate, respectively, under basal conditions without PMA. However, after incubation with PMA (10 nmol/L) for 24 hours, *MUC5B*-equivalent

Figure 2. Regulation of MUC5B secretion by PMA. Cells were treated with or without 10 nmol/L PMA. Cell lysates were harvested and prepared at 8, 16, and 24 hours after treatment. MUC5B protein was measured by ELISA as described in Materials and Methods. The quantity of MUC5B protein was presented as picomoles of MUC5B-equivalent/milligram of total protein, using the synthetic 19-amino acid peptide immunogen as the reference. Triplicate dishes were used for each protein ELISA, and experiments were repeated at least three times in different cultures derived from different donors and passages of cell lines, with similar results. Significance: * $P < 0.05$ and ** $P < 0.01$, respectively, compared with unstimulated control at each time point. **A:** NHBE cells. **B:** A549 cells. **C:** HBE1 cells.

protein increased two- to threefold compared with the protein levels in unstimulated cells. These results confirm that PMA, a protein kinase C activator, is a potent stimulator for *MUC5B* gene and protein expression in cultured airway epithelial cells.

PMA Stimulates MUC5B Promoter Activity, and the Promoter Activity Is Sensitive to Mithramycin A

To elucidate the molecular basis of PMA-stimulated *MUC5B* expression, we used pGL3-*MUC5B* promoter-luciferase chimeric constructs for transient transfections.

Freshly passaged cultures of NHBE, A549, and HBE1 cell systems were used. Despite the low-level expression of *MUC5B* message in the freshly passaged cultures, reasonably high promoter activity, at least 10 times higher than the promoterless pGL3 construct, was found in these three cell systems (data not included). As shown in Figure 3A, there was a three- to fourfold induction of *MUC5B* promoter-luciferase activity 24 hours after PMA treatment in all three cell systems. The data are in agreement with the results from the PMA-stimulated mRNA (Figure 1) and protein expression (Figure 2) experiments, suggesting that the stimulation of *MUC5B* expression by PMA is transcriptionally regulated.

Figure 3. PMA induced *MUC5B* expression at the promoter level, and the induction was sensitive to mithramycin. **A:** Effects of PMA on *MUC5B* promoter-mediated reporter gene expression activity. HBE1, A549, and NHBE cells were transiently transfected with full-length pGL3-*MUC5B* luciferase chimeric construct (4.17 kb) and co-transfected with pSV- β -galactosidase to normalize the transfection efficiency. Six to 12 hours after transfection, 10 nmol/L PMA was added to the cells, and cells were then harvested at 0, 8, 16, 24, and 48 hours after PMA treatment for luciferase and β -galactosidase assays. *MUC5B* promoter activity was expressed as luciferase/ β -galactosidase activity (%). Triplicate dishes were used for each time point, and experiments were repeated at least three times in different cultures derived from different donors and passages of cell lines. Statistical significance: * $P < 0.05$ and ** $P < 0.01$, respectively, compared with control (time 0). **B:** Effects of mithramycin A on PMA-induced *MUC5B* promoter activity. HBE1 cells were plated on 24-well plates at 5×10^4 cells/well. Cells were transfected with full-length (4.17-kb) pGL3-*MUC5B* and pSV- β -galactosidase as described in A. On the 2nd day, the cells were treated with 10 nmol/L PMA for 24 hours before being harvested for a reporter assay. Different concentrations of mithramycin A (0.1, 1, and 10 μ M/L) were added 1 hour before PMA treatment. Statistical significance: * $P < 0.05$ and ** $P < 0.01$, respectively, compared with PMA-treated, uninhibited controls. # $P < 0.05$ compared with no PMA-treated, uninhibited control. **C:** Effects of mithramycin A on PMA-induced *MUC5B* mRNA. Day-21 cultures of NHBE, A549, and HBE1 cells were prepared as described in Materials and Methods. One hour before PMA (10 nmol/L) treatment, cultures were pretreated with 1 μ M/L mithramycin A. Cultures were harvested 24 hours after PMA treatment for RNA isolation. *MUC5B* message and β -actin were quantified by the SYBR Green quantitative PCR analysis. PMA-induced and noninduced *MUC5B* mRNA was normalized with β -actin. Results are expressed as fold of induction (PMA-induced *MUC5B* mRNA/non-PMA-induced *MUC5B* mRNA), which are the mean $\pm$ SD of triplicates from two separate primary cultures and different passages of cell line cells. Statistical significance: * $P < 0.05$.

Several studies have shown the involvement of Sp1 in the regulation of mucin genes, including *MUC5AC*⁴⁵ and *MUC5B*.²² In the study of Van Seuning et al,²² the Sp1 *cis*-elements were present not only in the promoter region but also in the first intron; the Sp1 sites in both regions appeared to account for the *MUC5B* transcription activity. Therefore, mithramycin A, a pharmacological agent that can interfere with Sp1-DNA binding, was used to examine its effect on the PMA-induced *MUC5B* gene expression.⁴⁶ As shown in Figure 3B, mithramycin A at 1 and 10 $\mu\text{mol/L}$ inhibited the PMA-induced *MUC5B* gene activity. Inhibition is consistent with the sensitivity of PMA-induced *MUC5B* mRNA expression toward this drug. As shown in Figure 3C, PMA-induced *MUC5B* expression in the air liquid interface cultures of NHBE, A549, and HBE1 cells was suppressed in the presence of mithramycin A. These results further confirm that an Sp1-based transcriptional mechanism is involved in the regulation of PMA-induced *MUC5B* expression.

Requirement of PKC δ Isotype for PMA-Induced MUC Gene Expression

To reveal the signaling mechanism involved in PMA-induced mucin gene expression, two approaches were adapted. One approach was to use inhibitors on day-21 cultures to attenuate various signaling pathways. This approach was complemented with transient transfection to examine the effects of forced expression of dn and ca mutant proteins of various signaling molecules on gene expression. Because it was quite difficult to carry out the transient transfection on day-21 cultures, we had to rely on the use of freshly passaged cultures.

PMA is known as an activator of PKC. To investigate which specific PKC is involved in the PMA-induced mucin gene expression, inhibitors of Pan-PKC and different PKC isotypes were used. As shown in Figure 4, A and B, PMA-induced *MUC5AC* and *MUC5B* expression in these three cell systems was inhibited by the pan inhibitor of protein kinase C, Calphostin C, and the specific inhibitor of PKC δ and θ isotypes, Rottlerin. However, *MUC5AC* and *MUC5B* expression was not inhibited by Gö6979, a specific inhibitor of the calcium-sensitive PKC isoforms α and β 1. Because NHBE cells do not contain PKC θ ,²⁸ the fact that Rottlerin caused inhibition suggested that only PKC δ is involved in PMA-induced *MUC5B* activity. These results are consistent with the previous work of PMA-induced *MUC5AC* expression in the NCI-H292 cell line.^{17,20}

Consistent with the data obtained from day-21 cultures, PMA elevated *MUC5B* promoter two- to threefold in the promoter-reporter gene transfection assay conducted in the HBE1 cell system (Figure 4C). This stimulation was blocked by Calphostin C and Rottlerin but not by Gö6979. Inhibition was further supported by co-transfection with forced expression of dn-PKC isotypes. As shown in Figure 4D, PMA-induced *MUC5B* luciferase activity was reversed in cells co-transfected with the dn-

PKC δ isotype, whereas co-transfection with the dn-PKC α , β , or ϵ isotypes had no effect on basal or PMA-induced promoter activities.

Requirements for PKC Downstream of EGFR, Ras, Raf, and MEKK1 Signaling in PMA-Induced MUC Gene Expression

To elucidate downstream signaling of PKC, various inhibitors, including the ones previously used for PMA-induced *MUC2* and *MUC5AC* expression,^{47,48} were used. PMA-treated cultures were treated with manumycin A, a specific inhibitor of farnesyltransferase in the Ras signaling pathway ($\text{IC}_{50} = 5 \mu\text{mol/L}$); radicicol, an inhibitor that disrupts K-Ras-activated signaling pathways by selectively depleting Raf kinase ($\text{IC}_{50} = 5 \mu\text{mol/L}$); AG1478, an inhibitor for EGFR kinase ($\text{IC}_{50} = 3 \text{ nmol/L}$); AG9, a negative control for the inhibition of EGFR; and BPDQ, a potent and specific inhibitor of the tyrosine kinase activity of EGFR. As shown in Figure 5, A and B, PMA-stimulated *MUC5AC* and *MUC5B* messages were all sensitive to manumycin A in day-21 cultures. However, inhibitors targeting Raf and EGFR signaling, namely, radicicol, BPDQ, and AG1478, showed quite different results. All of those inhibitors attenuated PMA-induced *MUC5AC* expression yet failed to affect the PMA-induced *MUC5B* expression.

To confirm further the inhibitor experiments done with day-21 cultures, transient transfection with pGL3-*MUC5B* construct was performed. As shown in Figure 5C, PMA-induced *MUC5B* promoter activity was diminished to basal level by 0.2 $\mu\text{mol/L}$ manumycin A, whereas AG1478 and radicicol produced no effect. These results support the significant role of Ras in mediating PMA-induced *MUC5B* gene expression. The lack of any effect of AG1478 or radicicol on PMA-induced luciferase activity suggests that neither Raf- nor EGFR-mediated signaling is required for *MUC5B* expression. Because MEKK1 is another Ras effector,³¹ we conducted co-transfection studies with dn and ca clones of Ras, Raf, and MEKK1 in *MUC5B* promoter-luciferase transfected cells. As shown in Figure 5D, both dn-Ras and dn-MEKK1, but not dn-c-Raf-1, were able to reverse PMA-induced promoter activity. Furthermore, when non-PMA-treated HBE1 cells were co-transfected with constitutively active ca-Raf and ca-MEKK1, *MUC5B* basal promoter activity was induced with ca-MEKK1 but not ca-Raf. Therefore, Ras/MEKK1 but not Ras/Raf appeared to be involved in PMA-induced *MUC5B* promoter activity. To investigate whether MEKK1 is truly the downstream effector of Ras for *MUC5B* gene induction, we further transfected the HBE1 cells with ca-Ras along with either dn-c-Raf-1 or dn-MEKK1. As shown in Figure 5E, dn-MEKK1 was able to reverse *MUC5B* promoter activity induced by ca-Ras, whereas dn-c-Raf-1 failed to do so. These results collectively demonstrated the PKC/Ras/MEKK1 signaling cascade involved in PMA-induced *MUC5B* expression, whereas PMA-induced *MUC5AC* involved the PKC/EGFR/Ras/Raf signaling pathway.

Figure 4. Effects of PKC signaling on PMA-stimulated *MUC* gene expression. **A:** Effects of PKC inhibitors on PMA-stimulated *MUC5AC* expression. Day-21 cultures of NHBE (unfilled bars), HBE1 (partially filled bars), and A549 (filled bars) cells were prepared as described in Figure 1 and were treated with 3 μ mol/L Rotterlin, 70 nmol/L Gö6976, or 0.5 μ mol/L Calphostin C 1 hour before PMA (10 nmol/L) treatment. Cultures were harvested 24 hours after PMA treatment for RNA isolation. *MUC5AC* message, normalized to β -actin, was quantified by SYBR Green quantitative PCR analysis as described in Materials and Methods. Results are expressed as the mean $\pm$ SD of triplicates from two separate primary cultures and different passages of cell line cells. Statistical significance: * P < 0.05 compared with the vehicle-treated cells. **B:** Effects of PKC inhibitors on PMA-stimulated *MUC5B* expression. Day-21 cultures of NHBE (unfilled bars), HBE1 (partially filled bars), and A549 (filled bars) cells were prepared as described in Figure 1 and were treated with 3 μ mol/L Rotterlin, 70 nmol/L Gö6976, or 0.5 μ mol/L Calphostin C 1 hour before PMA (10 nmol/L) treatment. Cultures were harvested 24 hours after PMA treatment for RNA isolation. *MUC5B* message, normalized to β -actin, was quantified by the SYBR Green quantitative PCR analysis as described in Materials and Methods. The results are expressed as the mean $\pm$ SD of triplicates from two separate primary cultures and different passages of cell line cells. Statistical significance: * P < 0.05 compared with the vehicle-treated cells. **C:** Effects of PKC inhibitors on PMA-stimulated *MUC5B* promoter activity. HBE1 cells were transfected and treated with 10 nmol/L PMA as described in Figure 3B. Cell lysates were harvested for reporter assay 24 hours after PMA treatment. Inhibitors for a variety of PKC isoforms were added 1 hour before PMA treatment at the following concentrations: Calphostin C, a pan inhibitor of PKC, at 0.2 and 0.5 μ mol/L; Gö6976, an inhibitor of the calcium-sensitive isoform α and β 1, at 7 and 70 nmol/L; and Rotterlin, a specific inhibitor of PKC δ and θ , at 3 and 30 μ mol/L. The results were the mean $\pm$ SD of triplicate samples from two separate cultures. Statistical significance: * P < 0.05 compared with PMA-induced promoter activity in control vehicle-treated condition; # P < 0.05 compared with the basal promoter activity in vehicle-treated cultures. **D:** Effects of forced dn PKC isoforms expression on the regulation of *MUC5B* promoter activity. HBE1 cells were transfected with pGL3-*MUC5B* and co-transfected with dn-PKC α , dn-PKC β , dn-PKC δ , pBRL, dn-PKC ϵ , SRD, or CMV10. Ten nmol/L PMA was added after transfection, and cell lysates were harvested for luciferase reporter assays after 24 hours of incubation. β -Galactosidase activities were used to normalize the transfection efficiency. Results are mean $\pm$ SD of quadruplicate samples. Statistical significance: * P < 0.05 compared with PMA-induced promoter activity in cells co-transfected with vector DNA.

Requirements for MAPK Signaling Pathways in PMA-Induced Mucin Gene Expression

To identify which MAPK signaling pathway is involved in *MUC5B* gene expression regulation, we examined

whether PMA could induce the phosphorylation of ERK1/2, JNKs, and p38 in HBE1 cells. Protein lysates of HBE1 cells at day 21 were prepared at 0, 0.5, 1, 2, 4, and 8 hours after PMA treatment. As shown in the Western blots of Figure 6, phosphorylation of ERK, JNK, or p38

Figure 5. Effects of PKC downstream signaling pathways on PMA-stimulated MUC gene expression. **A:** Effects of PKC downstream signaling inhibitors on PMA-stimulated *MUC5AC* expression. Day-21 cultures of NHBE (unfilled bars), HBE1 (partially filled bars), and A549 (filled bars) cells were prepared as described in Figure 1 and were treated with 1 μ mol/L radicalcol, 0.2 μ mol/L manumycin A, 5 μ mol/L AG1478, 120 pmol/L BPDQ, or 10 μ mol/L AG9 1 hour before PMA (10 nmol/L) treatment. Cultures were harvested 24 hours after PMA treatment for RNA isolation. *MUC5AC* message, normalized to β -actin, was quantified by the SYBR Green quantitative PCR analysis as described in Materials and Methods. Results are expressed as the mean $\pm$ SD of triplicates from two separate primary cultures and different passages of cell line cells. Statistical significance: * P < 0.05 compared with the vehicle-treated cells. **B:** Effects of PKC downstream signaling inhibitors on PMA-stimulated *MUC5B* expression. Day-21 cultures of NHBE (unfilled bars), HBE1 (partially filled bars), and A549 (filled bars) cells were prepared as described in Figure 1 and were treated with 1 μ mol/L radicalcol, 0.2 μ mol/L manumycin A, or 5 μ mol/L AG1478, 120 pmol/L BPDQ, or 10 μ mol/L AG9 1 hour before PMA (10 nmol/L) treatment. Cultures were harvested 24 hours after PMA treatment for RNA isolation. *MUC5B* message, normalized to β -actin, was quantified by the SYBR Green quantitative PCR analysis as described in the text. The results are expressed as the mean $\pm$ SD of triplicates from two separate primary cultures and different passages of cell line cells. Statistical significance: * P < 0.05 compared with the vehicle-treated cells. **C:** Effects of Ras, Raf, and EGFR inhibitors on *MUC5B* promoter activity. Experiments were performed with HBE1 cells as described in Figure 4C. Inhibitors, as indicated, were added 1 hour before PMA treatment, and cell lysates were harvested for reporter gene assays 24 hours after PMA treatment. The inhibitors used were AG1478, an inhibitor for EGFR tyrosine kinase; manumycin A, an inhibitor of farnesyltransferase in the Ras signaling pathway; and Radicalcol, an inhibitor that disrupts K-Ras-activated signaling pathways by selectively depleting Raf kinase. **D:** Effects of forced expression of dn and ca clones of Ras, MEKK1, and Raf on *MUC5B* promoter activity. HBE1 cells transfected with pGL3-*MUC5B* were co-transfected with the following empty vectors: pEECMV (lane 1), pCDNA3 (lane 2), PRSV (lane 6), and pEECMV (lane 8); or co-transfected with the following dominant-negative mutants: dn-MEKK1 (lane 3), dn-Ras (lane 4), and dn-c-Raf-1 (lane 5); or co-transfected with the following constitutively active mutants: ca-Raf (lane 7) and ca-MEKK1 (lane 9). One day after transfection, PMA (10 nmol/L) was added. Cell lysates were harvested 24 hours after PMA treatment for reporter gene assays. Statistical significance: * P < 0.05 compared with PMA-induced promoter activity in empty vector co-transfected controls; # P < 0.05 compared with empty vector co-transfected controls. **E:** Effects of dn-c-Raf-1 and dn-MEKK1 mutants on ca-Ras-enhanced *MUC5B* promoter activity. HBE1 cells were transfected with pGL3-*MUC5B* and pSV- β -galactosidase constructs along with either vector or ca-Ras plus dn-c-Raf-1 or dn-MEKK1 as described in Figure 4C. Forty-eight hours after transfection, cells were harvested for reporter gene assays. Statistical significance: * P < 0.05 compared with empty vector co-transfected controls; # P < 0.05 compared with ca-Ras-enhanced promoter activity.

Figure 6. Western blot analysis of PMA-induced phosphorylation of ERK1/2, JNKs, and p38 of MAPK pathways. Day-21 cultures of HBE1 cells were prepared as described in Figure 1. Cells were treated with PMA (10 nmol/L) and harvested at various times as indicated for protein lysate preparation. Protein lysates (30 mg/lysate) were electrophoresed on a sodium dodecyl sulfate polyacrylamide gel and blotted to a nitrocellulose membrane for Western blot analysis. Blot membranes were treated with anti-phospho-ERK1/2 and anti-total ERK1/2 antibodies (A), anti-phospho-JNKs and anti-total JNK antibodies (B), and anti-phosphorylated p38 and anti-p38 antibodies (C), as indicated. Similar experiments with identical results were repeated in day-21 primary NHBE cultures and the freshly passaged day 3 cultures of HBE1 cells (data not included).

could be induced by PMA, although the induction was modest for JNK.

Using inhibitors, we assessed the effect of various MAPK pathways on PMA-induced mucin gene expression. As shown in Figure 7, A and B, respectively, PMA-induced *MUC5AC* was sensitive to U0126 but not SP600125 or SB203580, whereas PMA-induced *MUC5B* expression had an opposite response to these inhibitors in day-21 cultures. Transient transfection assays with pGL3-*MUC5B* promoter-reporter gene assay were consistent with the data obtained from day-21 cultures. As shown in Figure 7C, JNK and p38 inhibitors (SP600125 and SB203580, respectively) inhibited PMA-induced promoter activity in a dose-dependent manner. In contrast, U0126, an inhibitor of the ERK pathway, had no effect regardless of the concentration used.

To confirm further the significance of p38 and JNK pathways, dn expression clones of various MAPK signaling molecules were used. As shown in Figure 7D, PMA failed to induce *MUC5B* luciferase activity in dn-JNK1, dn-JNK2, and dn-p38 co-transfected cells. However, this was not the case for cells co-transfected with dn-ERK1, dn-ERK2, or empty vectors. Consistent with these findings, dn expression clones of MKK3, MKK4, MKK6, and MKK7, all upstream kinases of p38 and JNK, effectively inhibited PMA-induced *MUC5B* promoter activity (Figure 7D). These results further confirm the signaling cascade

of PKC, Ras, MEKK1, and p38/JNK in the regulation of PMA-induced *MUC5B* promoter activity.

To show that the inhibitory effect on the promoter activity is reflected in the *MUC5B* mRNA production, steady-state mRNA level was measured with SYBR quantitative PCR after HBE1 cells were transfected with the dn form of various signaling molecules. As shown in Figure 7E, forced expression of dn-JNK1, dn-JNK2, or dn-p38 but not dn-ERK1 or dn-ERK2 was able to reduce PMA-induced *MUC5B* message. These results further support the significant roles of p38 and JNK signaling in PMA-induced *MUC5B* expression.

Discussion

MUC2, *MUC5AC*, and *MUC5B* are the three major gel-forming mucin genes found in human airways and are clustered on a single band of chromosome 11p15.5.⁴⁹ Structurally, these three genes show extensive similarity in their 5' and 3' regions. Despite the similarities at the genomic level and the pathological significance of the gene products, most research has been done on the molecular mechanisms of *MUC2* and *MUC5AC* gene expression.^{6,17,20,21,47} There are very few studies on the regulation of *MUC5B* gene expression. A major reason for the scarcity of studies may be due to a late completion of *MUC5B* genomic structure^{22,30} and the low level of *MUC5B* expression in the widely used NCI-H292 cell line.⁴⁷ The NCI-H292 cell line has been the major cell system used to understand the regulation of *MUC2*/*MUC5AC* gene expression. However, mucin genes are expressed in a cell- and tissue-specific manner,^{11,48} and therefore, the information generated for *MUC2*/*MUC5AC* expression may not be suitable for *MUC5B*.

To offset studies based on one cell line, the current study used the following three airway epithelial cell systems: primary NHBE cells, the immortalized normal human bronchial epithelial cell line HBE1, and the human lung epithelial carcinoma cell line A549. The three cell systems were cultured for 3 weeks: 1 week immersed in media followed by 2 weeks in an air-liquid interface culture condition with media supplemented with all-*trans*-retinoic acid. Under these conditions, reasonable levels of *MUC5AC* and *MUC5B* gene expression were observed in the three cell systems. We show that PMA is a potent inducer for *MUC5B* expression, in addition to being an inducer of *MUC5AC*. Because promoter-reporter gene expression was elevated in proportion to *MUC5B* gene products in the transfection studies of all three cell types, PMA induction apparently occurs at the transcriptional level.

Remarkably, under the long-term culture condition, all three cell systems had a similar PMA-inducible response, with the responses to various inhibitors relating to the regulation of *MUC5B* and *MUC5AC* expression. Studies done with day-21 cultures of the three cell systems demonstrated that *MUC5B* and *MUC5AC* inductions are sensitive to inhibitors of PKC δ (Figure 4, A and B), inhibitors of Ras (Figure 5, A and B), and mithramycin A (Figure 3). Consistent with the published results in NCI-H292 cell

Figure 7. Effects of MAPK pathways on PMA-induced MUC gene expression. **A:** Effects of MAPK inhibitors on PMA-induced *MUC5AC* expression. Day-21 cultures of NHBE (unfilled bars), HBE1 (partially filled bars), and A549 (filled bars) cells were prepared as described in Figure 1 and were treated with 10 μ mol/L U0126, 1 μ mol/L SP600126 (JNKII inhibitor), and 6 μ mol/L SB203580 as indicated, 1 hour before PMA (10 nmol/L) treatment. Cultures were harvested 24 hours after PMA treatment for RNA isolation. *MUC5AC* message, normalized to β -actin, was quantified by the SYBR Green quantitative PCR analysis as described in Materials and Methods. Results are expressed as the mean $\pm$ SD of triplicates from two separate primary cultures and different passages of cell line cells. Statistical significance: $*P < 0.05$ compared with the vehicle-treated cells. **B:** Effects of MAPK inhibitors on PMA-induced *MUC5B* expression. Day-21 cultures of NHBE (unfilled bars), HBE1 (partially filled bars), and A549 (filled bars) cells were prepared as described in Figure 1 and were treated with 10 μ mol/L U0126, 1 μ mol/L SP600126 (JNKII inhibitor), and 6 μ mol/L SB203580 as indicated, 1 hour before PMA (10 nmol/L) treatment. Cultures were harvested 24 hours after PMA treatment for RNA isolation. *MUC5B* message, normalized to β -actin, was quantified by the SYBR Green quantitative PCR analysis as described in Materials and Methods. The results are expressed as the mean $\pm$ SD of triplicates from two separate primary cultures and different passages of cell line cells. Statistical significance: $*P < 0.05$ compared with the vehicle-treated cells. **C:** Effects of MAPK inhibitors on *MUC5B* promoter activity. HBE1 cells were transfected with *MUC5B* promoter-reporter and β -gal constructs as described in Figure 4C. One hour before PMA (10 nmol/L) treatment, cells were exposed to various MAPK inhibitors, as indicated. Cells were harvested for the reporter gene assays 24 hours after PMA treatment. Statistical significance: $*P < 0.05$ compared with PMA-induced promoter activity in vehicle-treated control. **D:** Effects of forced expression of dominant-negative (dn) clones of MAPK on *MUC5B* promoter activity. Experiments were performed as described in Figure 4C. Cell lysates were harvested for reporter assay 48 hours after PMA treatment. The results were expressed as the mean $\pm$ SD of triplicates from two independent cultures. Statistical significance: $*P < 0.05$ compared with PMA-enhanced promoter activity in vector co-transfected cultures. **E:** Effects of forced expression of dn clones of MAPK on *MUC5B* expression at the mRNA level. HBE1 cells were transfected with various dn clones, as indicated, and treated with PMA as described in **D**. After 24 hours of incubation, RNA was harvested for quantitative RT-PCR. β -Actin message levels were used for normalization of *MUC5B* mRNA. Statistical significance: $*P < 0.05$ compared with PMA-induced message expression in the empty vector-transfected controls.

Figure 8. A schematic summary of signal transduction pathways exerted by PMA involved in the regulation of *MUC5B* and *MUC5AC* expression in airway epithelial cells. Shaded boxes indicate the molecules involved in PMA-induced *MUC5B* expression.

system,⁴⁷ PMA-induced *MUC5AC* was attenuated by inhibitors of Raf and EGFR (Figure 5A) and ERK (Figure 6A) but not by inhibitors of JNKs and p38 (Figure 7A). In contrast, PMA-induced *MUC5B* expression was sensitive to inhibitors of JNKs and p38 (Figure 7B) but not the inhibitors of Raf and EGFR (Figure 5B) and ERK (Figure 7B).

Forced expression of various dn and ca mutants of relevant signaling molecules were used to compensate for the nonspecific features of the inhibitors. However, it was nearly impossible to do gene transfer studies in the highly confluent cultures. For this reason, like many other researchers in the field, we chose to do transient transfections with freshly passaged cultures. Despite the lower expression of *MUC5B* and *MUC5AC* messages in the freshly passaged cultures, the cultures were still responsive to PMA. Most importantly, the freshly passaged cultures allowed high efficiency of gene transfer for the promoter-reporter gene expression analysis and the co-transfection study that examined the effects of the forced expression of dn and ca mutants in the regulation of gene expression.

Data from the co-transfection studies further demonstrate a signal pathway for *MUC5B* that is distinctively different from the documented signaling mechanisms for *MUC2* and *MUC5AC*^{17,20,21,48} (Figure 8). Our study establishes an EGFR/ERK-independent but PKC/Ras/MEKK1-mediated JNK and p38 signaling-dependent regulation through an Sp1-based transcriptional mechanism for PMA-stimulated *MUC5B* expression. This signaling mechanism is in contrast to the prevailing PKC/EGFR/Ras/Raf/ERK/Sp1 signaling pathway for induced *MUC5AC* expression, as studied by others.^{17,20,21,48}

PMA, an analog of diacylglycerol, is known to activate PKC, which in turn initiates signaling cascades that lead to a variety of cell events, including gene expression, cell differentiation, cell proliferation, and respiratory secretions. Adler and colleagues⁵⁰ have identified a PKC-dependent phosphorylation of myristoylated alanine-rich

C-kinase substrate as being the key element in the exocytosis of mucin granules. A similar conclusion has also been reported by Abdullah et al.⁵¹ Studies of PMA-induced *MUC5AC* and *MUC2* expressions have also demonstrated the important role of PKC in mucin gene expression.^{17,20} Using various PKC isoform-specific inhibitors, we have shown that Rotterlin, a specific inhibitor of PKC δ and θ , diminished PMA-enhanced *MUC5B* promoter activity. Because NHBE cells do not contain the θ isoform of PKC,²⁸ the rotillerin effect should be on PKC δ only. This conclusion is supported by the use of dn mutants of various PKC isotypes in a *MUC5B* promoter activity assay. Both studies demonstrated the involvement of PKC δ in the regulation of *MUC5B* expression. Because PKC activation is a prevalent event that is frequently found in inflammatory airways associated with the exposure to smoke, particulates, oxidant air pollutants, and microbial organism infection, our findings further strengthen the role of PKC, especially the PKC δ isoform, in disease-associated pathogenesis involving mucin overproduction and accumulation as is found in various airway diseases.

Using a combination approach of inhibitor treatment and co-transfections with dn and ca expression constructs, the Ras, MEKK1, and p38/JNK signaling cascade downstream of PKC was characterized. The involvement of Ras/MEKK1 rather than Ras/Raf was clearly demonstrated using dn and ca clones. For the MAPK pathway, we have shown that PMA treatment can induce the phosphorylation of ERKs, JNKs, and p38 in cells. However, the inhibitor U0126, which is specific to ERK signaling, had no effect on PMA-enhanced *MUC5B* expression. These results were further supported by the transfection studies with dn-ERK1 and dn-ERK2 clones, which also failed to abrogate the enhanced *MUC5B* expression. On the other hand, dn clones of JNK and p38 were able to abrogate PMA-enhanced *MUC5B* promoter activity as well as mRNA production, suggesting that the MAPK signaling pathways involved in PMA-enhanced *MUC5B* expression are JNK and p38, which are quite different from the known ERK-dependent *MUC5AC* expression.

It is noteworthy that both JNK and p38 pathways, instead of just one single pathway, are required for the transcriptional induction of *MUC5B*. One possible explanation is that the two pathways are controlling two different events for one function, that is, to enhance *MUC5B* expression, especially related to the Sp1-mediated transcriptional mechanism. It is possible that one of those two molecules, JNK or p38, is involved in the activation of Sp1, whereas the other one is responsible for an activation of accessory protein(s). Our study here, along with other documented reports,^{17,47} demonstrates the involvement of the SP1 sites in *MUC5AC* and *MUC5B* gene regulation. We speculate that the difference in the SP1-mediated transcription of those two genes resides on the fact that *MUC5B* transcriptional induction requires an accessory protein for SP1 activation, and the accessory protein involves a second MAPK pathway, either JNK or P38. *MUC5AC* may not require a secondary protein, and therefore, only the ERK pathway is involved.

The EGFR signaling pathway is considered to be a common pathway by which many stimuli induce *MUC5AC* production.^{17,20,52–57} The same outcome has also been demonstrated for tumor necrosis factor- α -induced⁴⁸ and adenosine-induced⁵⁸ *MUC2* gene expression. AG1478, the pharmacological agent that was used frequently in studies related to the inhibition of EGFR tyrosine kinase activity previously,^{17,20,52–57} has been shown to be toxic in certain system. For that reason, another EGFR inhibitor, BPDQ, was also used to inhibit the PMA-induced *MUC5AC* or *MUC5B*. Both inhibitors attenuated *MUC5AC* gene expression but failed to abolish PMA-mediated *MUC5B* expression in all three cell types (Figure 5), indicating that PMA-induced *MUC5B* gene expression is EGFR independent. This is inconsistent with the study of *MUC5B* gene expression by Perrais et al⁵⁹ on colon cancer cell lines. Therefore, our study supports the notion that *MUC5B* expression is independent from EGFR- and ERK-activated pathways, a key step that has been proposed in PMA and various mediator-induced *MUC5AC* expression.¹⁷

In summary, we have identified distinctive signaling pathways involved in the stimulation of *MUC5AC* and *MUC5B* expression by PMA in three airway epithelial cell systems. For *MUC5B* expression, this is the first study to examine the molecular signaling pathway involved in its regulation. We have demonstrated a PKC δ -, Ras-, MEKK1-, and JNK/p38-dependent but EGF receptor/ERK-independent signaling pathway in the regulation of PMA-enhanced *MUC5B* expression. This signaling pathway is different from the known EGF receptor/ERK-dependent pathway for *MUC2* and *MUC5AC* gene expression. Because JNK and p38 pathways are generally activated under stress-related conditions, as in various airway diseases, we propose that such activation may be responsible for the *trans*-differentiation of epithelial cells from non-*MUC5B*-producing cells to *MUC5B*-expressing cells in disease states. Mucins are expressed in a cell- and tissue-specific manner. Therefore, an altered expression of mucin gene products, especially the gel-forming ones, could impair the mucociliary function such that the pathogenesis of various airway diseases is enhanced.

Acknowledgment

We thank Dr. Suzette Smiley-Jewell for her critical reading and editing of this manuscript.

References

- West JB: Respiratory Physiology: The Essentials, ed 6. Philadelphia, PA, Lippincott Williams & Wilkins, 2000
- McDonald JA: Lung growth and development. Lung Biology in Health and Disease vol 100. New York, M. Dekkar, 1997
- Vestbo J, Prescott E, Lange P: Association of chronic mucus hypersecretion with FEV1 decline and chronic obstructive pulmonary disease morbidity: Copenhagen City Heart Study Group. Am J Respir Crit Care Med 1996, 153:1530–1535
- Aikawa T, Shimura S, Sasaki H, Ebina M, Takishima T: Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. Chest 1992, 101:916–921
- Wang J, Lory S, Ramphal R, Jin S: Isolation and characterization of *Pseudomonas aeruginosa* genes inducible by respiratory mucus derived from cystic fibrosis patients. Mol Microbiol 1996, 22:1005–1012
- Hovenberg HW, Davies JR, Herrmann A, Linden CJ, Carlstedt I: *MUC5AC*, but not *MUC2*, is a prominent mucin in respiratory secretions. Glycoconj J 1996, 13:839–847
- Thornton DJ, Howard M, Khan N, Sheehan JK: Identification of two glycoforms of the *MUC5B* mucin in human respiratory mucus: evidence for a cysteine-rich sequence repeated within the molecule. J Biol Chem 1997, 272:9561–9566
- Chen Y, Zhao YH, Kalaslavadi TB, Hamati E, Nehrke K, Le AD, Ann DK, Wu R: Genome-wide search and identification of a novel gel-forming mucin *MUC19/Muc19* in glandular tissues. Am J Respir Cell Mol Biol 2004, 30:155–165
- Wickström C, Davies JR, Eriksen GV, Veerman EC, Carlstedt I: *MUC5B* is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage. Biochem J 1998, 334:685–693
- Meezaman D, Charles P, Daskal E, Polymeropoulos MH, Martin BM, Rose MC: Cloning and analysis of cDNA encoding a major airway glycoprotein, human tracheobronchial mucin (*MUC5*). J Biol Chem 1994, 269:12932–12939
- Reid CJ, Gould S, Harris A: Developmental expression of mucin genes in the human respiratory tract. Am J Respir Cell Mol Biol 1997, 17:592–598
- Groneberg DA, Eynott PR, Lim S, Oates T, Wu R, Carlstedt I, Roberts P, McCann B, Nicholson AG, Harrison BD, Chung KF: Expression of respiratory mucins in fatal status asthmaticus and mild asthma. Histopathology 2002, 40:367–373
- Groneberg DA, Eynott PR, Oates T, Lim S, Wu R, Carlstedt I, Nicholson AG, Chung KF: Expression of *MUC5AC* and *MUC5B* mucins in normal and cystic fibrosis lung. Respir Med 2002, 96:81–86
- Chen Y, Zhao YH, Wu R: In silico cloning of mouse *Muc5b* gene and upregulation of its expression in mouse asthma model. Am J Respir Crit Care Med 2001, 164:1059–1066
- Chen Y, Zhao YH, Wu R: Differential regulation of airway mucin gene expression and mucin secretion by extracellular nucleotide triphosphates. Am J Respir Cell Mol Biol 2001, 25:409–417
- Kamio K, Matsushita I, Hijikata M, Kobashi Y, Tanaka G, Nakata K, Ishida T, Tokunaga K, Taguchi Y, Homma S, Azuma A, Kudoh S, Keicho N: Promoter analysis and aberrant expression of the *MUC5B* gene in diffuse panbronchiolitis. Am J Respir Crit Care Med 2005, 171:949–957
- Hewson CA, Edbrooke MR, Johnston SL: PMA induces the *MUC5AC* respiratory mucin in human bronchial epithelial cells, via PKC, EGF/TGF- α , Ras/Raf MEK, ERK and Sp1-dependent mechanisms. J Mol Biol 2004, 344:683–695
- Park SJ, Kang SY, Kim NS, Kim HM: Phosphatidylinositol 3-kinase regulates PMA-induced differentiation and superoxide production in HL-60 cells. Immunopharmacol Immunotoxicol 2002, 24:211–226
- Busuttill V, Bottero V, Frelin C, Imbert V, Ricci JE, Auberger P, Peyron JF: Blocking NF- κ B activation in Jurkat leukemic T cells converts the survival agent and tumor promoter PMA into an apoptotic effector. Oncogene 2002, 21:3213–3224
- Shao MX, Nadel JA: Neutrophil elastase induces *MUC5AC* mucin production in human airway epithelial cells via a cascade involving protein kinase C, reactive oxygen species, and TNF- α -converting enzyme. J Immunol 2005, 175:4009–4016
- Lee HW, Ahn DH, Crawley SC, Li JD, Gum JR Jr, Basbaum CB, Fan NQ, Szymkowski DE, Han SY, Lee BH, Sleisenger MH, Kim YS: Phorbol 12-myristate 13-acetate up-regulates the transcription of *MUC2* intestinal mucin via Ras ERK, and NF- κ B. J Biol Chem 2002, 277:32624–32631
- Van Seuning I, Perrais M, Pigny P, Porchet N, Aubert JP: Sequence of the 5'-flanking region and promoter activity of the human mucin gene *MUC5B* in different phenotypes of colon cancer cells. Biochem J 2000, 348:675–686
- Verma M, Murthy VV, Mathew S, Banerji D, Kurl RN, Olnes MJ, Yankaskas JR, Blass C, Davidson EA: Promoter of the canine tracheobronchial mucin gene. Glycoconj J 1996, 13:797–807
- Wu R, Zhao YH, Chang MM: Growth and differentiation of conducting airway epithelial cells in culture. Eur Respir J 1997, 10:2398–2403
- Wu R, Martin WR, Robinson CB, St George JA, Plopper CG, Kurland G, Last JA, Cross CE, McDonald RJ, Boucher R: Expression of mucin

- synthesis and secretion in human tracheobronchial epithelial cells grown in culture. *Am J Respir Cell Mol Biol* 1990, 3:467–478
26. Chang MM, Juarez M, Hyde DM, Wu R: Mechanism of dexamethasone-mediated interleukin-8 gene suppression in cultured airway epithelial cells. *Am J Physiol* 2001, 280:L107–L115
27. Wu R, Smith D: Continuous multiplication of rabbit tracheal epithelial cells in a defined, hormone-supplemented medium. *In Vitro* 1982, 18:800–812
28. Park JA, He F, Martin LD, Li Y, Chorley BN, Adler KB: Human neutrophil elastase induces hypersecretion of mucin from well-differentiated human bronchial epithelial cells in vitro via a protein kinase C(δ)-mediated mechanism. *Am J Pathol* 2005, 167:651–661
29. Rousseau K, Wickstrom C, Whitehouse DB, Carlstedt I, Swallow DM: New monoclonal antibodies to non-glycosylated domains of the secreted mucins MUC5B and MUC7. *Hybrid Hybridomics* 2003, 22:293–299
30. Chen Y, Zhao YH, Di YP, Wu R: Characterization of human mucin 5B gene expression in airway epithelium and the genomic clone of the amino-terminal and 5'-flanking region. *Am J Respir Cell Mol Biol* 2001, 25:542–553
31. Vuong H, Patterson T, Shapiro P, Kalvakolanu DV, Wu R, Ma WY, Dong Z, Kleeberger SR, Reddy SP: Phorbol ester-induced expression of airway squamous cell differentiation marker, SPRR1B, is regulated by protein kinase C δ /Ras/MEKK1/MKK1-dependent/AP-1 signal transduction pathway. *J Biol Chem* 2000, 275:32250–32259
32. Kumar CC, Prorock-Rogers C, Kelly J, Dong Z, Lin JJ, Armstrong L, Kung HF, Weber MJ, Afonso A: SCH 51344 inhibits ras transformation by a novel mechanism. *Cancer Res* 1995, 55:5106–5117
33. Yan M, Templeton DJ: Identification of 2 serine residues of MEK-1 that are differentially phosphorylated during activation by raf and MEK kinase. *J Biol Chem* 1994, 269:19067–19073
34. Yan M, Dai T, Deak JC, Kyriakis JM, Zon LI, Woodgett JR, Templeton DJ: Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. *Nature* 1994, 372:798–800
35. Dérjard B, Raingeaud J, Barrett T, Wu IH, Han J, Ulevitch RJ, Davis RJ: Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science* 1995, 267:682–685
36. Dérjard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M, Davis RJ: JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* 1994, 76:1025–1037
37. Raingeaud J, Whitmarsh AJ, Barrett T, Dérjard B, Davis RJ: MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol Cell Biol* 1996, 16:1247–1255
38. Robbins DJ, Zhen E, Owaki H, Vanderbilt CA, Ebert D, Geppert TD, Cobb MH: Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro. *J Biol Chem* 1993, 268:5097–5106
39. Ludwig S, Engel K, Hoffmeyer A, Sithanandam G, Neufeld B, Palm D, Gaestel M, Rapp UR: 3pK, a novel mitogen-activated protein (MAP) kinase-activated protein kinase, is targeted by three MAP kinase pathways. *Mol Cell Biol* 1996, 16:6687–6697
40. Hirai S, Izumi Y, Higa K, Kaibuchi K, Mizuno K, Osada S, Suzuki K, Ohno S: Ras-dependent signal transduction is indispensable but not sufficient for the activation of AP1/Jun by PKC δ . *EMBO J* 1994, 13:2331–2340
41. Ohba M, Ishino K, Kashiwagi M, Kawabe S, Chida K, Huh NH, Kuroki T: Induction of differentiation in normal human keratinocytes by adenovirus-mediated introduction of the ϵ and δ isoforms of protein kinase C. *Mol Cell Biol* 1998, 18:5199–5207
42. Akita Y, Ohno S, Yajima Y, Konno Y, Saido TC, Mizuno K, Chida K, Osada S, Kuroki T, Kawashima S: Overproduction of a Ca(2+)-independent protein kinase C isozyme, nPKC ϵ , increases the secretion of prolactin from thyrotropin-releasing hormone-stimulated rat pituitary GH4C1 cells. *J Biol Chem* 1994, 269:4653–4660
43. Shao MX, Nakanaga T, Nadel JA: Cigarette smoke induces MUC5AC mucin overproduction via tumor necrosis factor- α -converting enzyme in human airway epithelial (NCI-H292) cells. *Am J Physiol* 2004, 287:L420–L427
44. Han SY, Lee MS, Kim HR, Baek SH, Ahn DH, Chae HS, Erickson RH, Sleisenger MH, Kim YS: Phorbol 12-myristate 13-acetate induces alteration in mucin gene expression and biological properties of colon cancer cells. *Int J Oncol* 2000, 17:487–494
45. Li D, Gallup M, Fan N, Szymkowski DE, Basbaum CB: Cloning of the amino-terminal and 5'-flanking region of the human MUC5AC mucin gene and transcriptional up-regulation by bacterial exoproducts. *J Biol Chem* 1998, 273:6812–6820
46. Ray R, Snyder RC, Thomas S, Koller CA, Miller DM: Mithramycin blocks protein binding and function of the SV40 early promoter. *J Clin Invest* 1989, 83:2003–2007
47. Perras M, Pigny P, Copin MC, Aubert JP, Van Seuningen I: Induction of MUC2 and MUC5AC mucins by factors of the epidermal growth factor (EGF) family is mediated by EGF receptor/Ras/Raf/extracellular signal-regulated kinase cascade and Sp1. *J Biol Chem* 2002, 277:32258–32267
48. Hovenberg HW, Davies JR, Carlstedt I: Different mucins are produced by the surface epithelium and the submucosa in human trachea: identification of MUC5AC as a major mucin from the goblet cells. *Biochem J* 1996, 318:319–324
49. Pigny P, Guyonnet-Duperat V, Hill AS, Pratt WS, Galiegue-Zouitina S, d'Hooge MC, Laine A, Van-Seuningen I, Degand P, Gum JR, Kim YS, Swallow DM, Aubert JP, Porchet N: Human mucin genes assigned to 11p15.5: identification and organization of a cluster of genes. *Genomics* 1996, 38:340–352
50. Li Y, Martin LD, Spizz G, Adler KB: MARCKS protein is a key molecule regulating mucin secretion by human airway epithelial cells in vitro. *J Biol Chem* 2001, 276:40982–40990
51. Abdullah LH, Bundy JT, Ehre C, Davis CW: Mucin secretion and PKC isoforms in SPOC1 goblet cells: differential activation by purinergic agonist and PMA. *Am J Physiol* 2003, 285:L149–L160
52. Takeyama K, Dabbagh K, Lee HM, Agusti C, Lausier JA, Ueki IF, Grattan KM, Nadel JA: Epidermal growth factor system regulates mucin production in airways. *Proc Natl Acad Sci USA* 1999, 96:3081–3086
53. Takeyama K, Dabbagh K, Jeong Shim J, Dao-Pick T, Ueki IF, Nadel JA: Oxidative stress causes mucin synthesis via transactivation of epidermal growth factor receptor: role of neutrophils. *J Immunol* 2000, 164:1546–1552
54. Lee HM, Malm L, Dabbagh K, Dao-Pick T, Ueki IF, Kim S, Shim JJ, Nadel JA: Epidermal growth factor receptor signaling mediates regranulation of rat nasal goblet cells. *J Allergy Clin Immunol* 2001, 107:1046–1050
55. Shim JJ, Dabbagh K, Ueki IF, Dao-Pick T, Burgel PR, Takeyama K, Tam DC, Nadel JA: IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils. *Am J Physiol* 2001, 280:L134–L140
56. Takeyama K, Fahy JV, Nadel JA: Relationship of epidermal growth factor receptors to goblet cell production in human bronchi. *Am J Respir Crit Care Med* 2001, 163:511–516
57. Takeyama K, Jung B, Shim JJ, Burgel PR, Dao-Pick T, Ueki IF, Protin U, Kroschel P, Nadel JA: Activation of epidermal growth factor receptors is responsible for mucin synthesis induced by cigarette smoke. *Am J Physiol* 2001, 280:L165–L172
58. McNamara N, Gallup M, Khong A, Sucher A, Maltseva I, Fahy J, Basbaum C: Adenosine up-regulation of the mucin gene, MUC2, in asthma. *FASEB J* 2004, 18:1770–1772
59. Perras M, Pigny P, Buisine MP, Porchet N, Aubert JP, Van Seuningen-Lempire I: Aberrant expression of human mucin gene MUC5B in gastric carcinoma and cancer cells: identification and regulation of a distal promoter. *J Biol Chem* 2001, 276:15386–15396