PMA Stimulates *MUC5B* Gene Expression through an Sp1-Based Mechanism in Airway Epithelial Cells

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We previously showed that the *MUC5B* gene expression was elevated by phorbol 12-myristate 13-acetate (PMA) through an epidermal growth factor receptor-independent Ras/MEKK1/JNK and P38 signaling-based transcriptional mechanism. In the current study, we elucidated the molecular basis of this transcriptional regulation using promoter-reporter gene expression and chromatin immunoprecipitation (ChIP) assays with primary human bronchial epithelial cells that are cultured at the air-liquid interface. We have observed that PMA-induced *MUC5B* promoter activity is blocked by the Sp1-binding inhibitor, mithramycin A, in a dose-dependent manner. Deletion analysis with the *MUC5B* promoter construct demonstrated that both basal and PMA-induced promoter-reporter activities reside within the -222/-78 bp region relative to the transcriptional start site. NoShift transcriptional factor assays demonstrated that PMA stimulated Sp1 binding, but not STAT1 and c-Myc binding. Immunoprecipitation studies also verified the enhanced phosphorylation of Sp1 after PMA treatment. Site-directed mutagenesis and transfection studies demonstrated the involvement of Sp1-1 (-122/-114) and the Sp1-2 (-197/-186) cis elements in the basal and PMA-induced *MUC5B* promoter activity. The ChIP assay with anti-RNA polymerase II reconfirmed the PMA-induced *MUC5B* promoter activity by showing enhanced RNA polymerase II-DNA complex containing putative *MUC5B* Sp1-1, Sp1-2, or Sp1-3 sites. However, the ChIP assay using anti-Sp1 antibody demonstrated that the PMA-stimulated binding is only at Sp1-2. These results suggested an Sp1-based transcriptional mechanism with Sp1-1 as the regulator of basal *MUC5B* promoter activity and Sp1-2 as the regulator of PMA-induced *MUC5B* gene expression in the human airway epithelial cells.

Keywords: *MUC5B*; Sp-1; transcription; site-directed mutagenesis; ChIP

Respiratory mucus secretion is essential for protecting the lungs and airways from inhaled particles, hazardous chemicals, and microorganisms (1). In many diseases, such as asthma, cystic fibrosis, and chronic obstructive pulmonary disease (2, 3), excessive mucus production impairs the mucociliary function, leading to respiratory compromise and, ultimately, death (4). This mucus hypersecretion is primarily caused by an increase in mucin gene expression.

At least 20 human mucin (MUC) genes have been identified. Among these, at least nine of them (*MUC1*, 2, 4, 5A, 5B, 7, 8, 13, and 19) are expressed in human airways. The large, gel-forming mucins—*MUC5AC*, *MUC5B*, and *MUC2*—have been identified immunochemically in secretions and bronchial washings from

CLINICAL RELEVANCE

Mucin overproduction is the hallmark of most airway diseases. It is important to understand its gene regulation.

normal airways, while *MUC5AC* (5, 6), *MUC5B* (7–9), *MUC8* (10), and *MUC2* (7) have been identified in sputum samples and bronchial washings from patients with chronic airway diseases. Among them, *MUC5B* and *MUC5AC* are the most prominent mucins in the airway. Normally, *MUC5AC* expression is limited to surface goblet epithelial cells, while *MUC5B* is predominantly expressed in the mucous cells of submucosal glands (11). *MUC5B* expression can also be detected, however, in the surface goblet cells of lung tissues, mostly in diseased conditions. Our laboratory has shown the expression of glandular *MUC5B* in the lining airway epithelial cells with an ovalbumin-induced mouse asthma model (12). A similar trans-expression phenomenon has also been seen in patients who suffered from emphysema and diffuse panbronchiolitis (13, 14). This kind of aberrant trans-expression was only observed with *MUC5B*, but not with *MUC5AC*, suggesting that there is a close relationship between *MUC5B* gene expression and airway diseases.

Phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, has been used as a model inflammatory stimulant that can modulate a variety of cellular events, including gene transcription (15), cell growth, and differentiation (16). PMA also plays a significant role in the induction of both *MUC5AC* and *MUC2* gene expression in NCI-H292 and HM3 colon cell lines (15, 17). In our previous study, we showed that PMA is a potent inducer for *MUC5B* expression in differentiated primary human bronchial epithelial cell cultures, as well as in two cell lines—an immortalized normal bronchial epithelial cell line, HBE1, and a lung adenocarcinoma cell line, A549 (18). In contrast to the epidermal growth factor (EGF) receptor (EGFR)-dependent ERK signaling pathway responsible for PMA-induced *MUC5AC* expression, our signal transduction studies have demonstrated a persistent, EGFR-independent Ras/MEKK1/JNK and p38-mediated transcriptional mechanism for PMA-induced *MUC5B* expression with the above three airway epithelial cell systems. To further extend our study, we have examined the transcriptional mechanism involved in PMA-induced *MUC5B* expression. Transcriptional regulation of airway mucin genes is important for understanding the pathogenesis that leads to mucus overproduction in chronic airway diseases. Data from the current literature implicates the involvement of NF- κ B (17), Sp1, and AP1 transcription factors in the up-regulation of *MUC2* and *MUC5AC* expression (19, 20). There is very little information, however, about which transcription factors are involved in *MUC5B* gene regulation.

In this study, we used the promoter-reporter gene expression and chromatin immunoprecipitation assays to show that Sp1 and its binding sites in the *MUC5B* 5'-flanking regions are involved in PMA-induced *MUC5B* expression using two cell types: differentiated primary human bronchial epithelial cells (NHBE) and an

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immortalized normal bronchial epithelial cell line (HBE1). Sp1 is a member of the zinc finger transcription factor family, which also includes at least four other Sp transcription factors (21). These transcription factors are involved in a variety of physiologic processes and have been shown to be able to bind more than 1,000 different promoters for transcriptional regulation (22). The Sp proteins have several conserved domains, including N-terminal transcription activation domains and C-terminal zinc finger DNA-binding domains (23, 24). Sp1 plays a role in transcription activation, transcription repression, and basal transcriptional activity of both viral and cellular genes (23).

This report is not only the first that identified the regulatory role of Sp1 in PMA-induced *MUC5B* expression in human airway epithelial cells, but also shed light on our understanding of the molecular mechanism underlying the specificity of Sp1 and its post-translationally modified forms and their different biological functions.

MATERIALS AND METHODS

Cell Culture

Normal human primary tracheobronchial epithelial cells (NHBE) were isolated from human bronchi and trachea obtained from organ donors or autopsies through the University of California at Davis (UC Davis) Medical Center (Sacramento, CA). Tissue procurement and utilization were approved and periodically reviewed by the UC Davis Human Subject Research Review Committee. The HBE1 cell line—a papilloma virus-immortalized bronchial epithelial cell line developed by Dr. J. Yankaskas at the University of North Carolina, Chapel Hill (25)—was also used in this study.

Cell isolation and culture methods were carried out as described previously, with some modifications (26, 27). NHBE cells (1×10^4 cells/cm²) were plated on a Costar Transwell chamber (25 mm) in Ham's F12/Dulbecco's modified Eagle's medium (DMEM) (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 M), cholera toxin (10 ng/ml), bovine hypothalamus extract (15 μ g/ml), and bovine serum albumin (0.5 mg/ml) (28, 29). All-trans-retinoic acid (0.03 μ M) was added when the cells reached confluence about 2 days after plating. Under retinoid influence, NHBE cells underwent mucous-cell differentiation. These primary NHBE cells in immersion were shifted to an air-liquid interface 1 week after plating. Under the air-liquid interface (biphasic) culture conditions, further mucociliary differentiation of human TBE cells was observed. After 3 weeks in culture (2 weeks under the biphasic culture condition), NHBE cells underwent mucociliary differentiation, with cilia and mucus-secreting granule formation (26). At this well-differentiated stage, including the transfection studies, cells were treated with PMA (0–10 nM) or vehicle. Cultures were harvested for RNA or cell lysate preparations at various times after the treatment (0–48 h), as indicated.

RNA Isolation and Quantitative Real-Time RT-PCR

RNA was extracted from NHBE cells after PMA or 4 α -PMA treatment using RNA Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Two micrograms of extracted RNA was converted to cDNA by adding MMLV-reverse transcriptase (Promega, Madison, WI) and oligo-dT primers in a total volume of 20 μ l. The reaction was further diluted to 40 μ l with water and used for real-time PCR analysis. The PCR reaction was carried out in a 384-well optical PCR plate. Each reaction contained a 10 μ l mixture containing 5 μ l of 2 $\times$ SYBR Green PCR Master Mix (Applied Biosystems, Inc., Foster City, CA), 1 μ l of cDNA sample, 0.5 μ l each of 5 μ M forward and reverse primers, and 3 μ l of RNase-free water. Results were analyzed by the ABI PRISM 7900HT Sequence Detection system (Applied Biosystems). The following PCR primers were used: β -actin forward, CTGG AACGGTGAAGGTG ACA; β -actin reverse, AAGGGACTTCTCTG TAACAATGCA; *MUC5B* forward, GCTGCTGCTACTCTCTG GAGG; *MUC5B* reverse, AG GTGATGTTGACCTCGGTCTC. The relative mRNA amount in each sample was calculated by normalizing its Ct value to the Ct value of the housekeeping gene β -actin. The calculation formula is $2^{-(Ct \text{ of } MUC5B - Ct \text{ of } \beta\text{-actin})}$. PMA-treated samples

were compared to non-PMA-treated controls, with results presented as fold induction.

Construction of *MUC5B* Promoter–Luciferase Clones: Deletion and Site-Directed Mutagenesis Constructs

The *MUC5B* 5'-flanking region (4,169 base pairs)–luciferase reporter construct was previously constructed in our laboratory (30) and was used for the *MUC5B* promoter study. The clones containing systematically deleted *MUC5B* 5'-flanking region were generated by PCR amplification with pairs of primers bearing restriction sites KpnI and NheI at their 5' and 3' ends, respectively. The resulting DNA fragments were digested, resolved, and extracted from agarose gels, then purified and re-cloned into a promoterless pGL3 vector at the same restriction enzyme sites. The clone pGL3-*MUC5B* (2.6 kb) extends from site –2,561 to +6, pGL3-*MUC5B* (1.62 kb) from –1,610 to +6, pGL3-*MUC5B* (1.14 kb) from –1,136 to +6, pGL3-*MUC5B* (0.66 kb) from –652 to +6 and pGL3-*MUC5B* (0.22 kb) from –222 to +6. The smallest clone is pGL3-*MUC5B* (0.08 kb), which extends from –78 to +18. All clones were sequenced on both strands for sequence verification.

Site-directed mutagenesis of the Sp1 transcription factor-binding sites on the *MUC5B* promoter was carried out according to the manufacturer's protocol using the Transformer Site Directed Mutagenesis kit (BD Biosciences-Clontech, Mountain View, CA). Briefly, a 4.17-kb pGL3-*MUC5B* luciferase reporter construct was used as a template. Specific primers were designed to introduce mutations into the Sp1 binding sites, as detailed below: selection primer, CGATAAGGGACCGTCG ACCGATG; mutagenic primers—Sp1-1m, CCACAGCCCCCTAACC GAGAGCAAAC; Sp1-2m, GCTGCTGGGTTTGGGGGCGTCTCTG; Sp1-3m, GCAGTG GTGGTTGGGGAGCTCCAG. After synthesis, NruI restriction enzyme digestion was used for primary selection. Mutagenic plasmids were amplified by transformation using mutS71-18 *Escherichia coli*. Mutated promoters were sequenced to confirm the inclusion of site-directed base changes and the exclusion of random mutations. Successfully mutated pGL3-*MUC5B*Sp1-1m, pGL3-*MUC5B*Sp1-2m, and pGL3-*MUC5B*Sp1-3m clones were generated. All clones were sequenced on both strands to verify the sequences.

Transient Transfection and Luciferase Assay

NHBE or HBE1 cells were plated (1×10^4 cells/cm²) on a 6-well Costar Transwell chamber (25 mm). Under the culture conditions described in the "cell culture" section, NHBE cells underwent mucociliary differentiation after 3 weeks. These cells were transfected with 1 μ g of the pGL3-*MUC5B* clone, plus 0.5 μ g of the pSV- β -galactosidase (β -gal) plasmid to allow for normalization of transfection efficiency. Transfection was carried out using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol in Opti-MEM reduced serum media (Invitrogen). Sixteen hours after transfection, cells were treated with 10nM PMA in Ham's F12/Dulbecco's modified Eagle's medium (DMEM) (1:1) supplemented with 5 μ g/ml insulin, 10 ng/ml EGF, 0.1 μ M dexamethasone, 5 μ g/ml transferrin, 20 ng/ml cholera toxin, and 15 μ g/ml bovine hypothalamus extract. Cells were harvested for reporter assays after 24 hours of incubation. Luciferase activity was determined using Lucite (PerkinElmer, Wellesley, MA) and quantified in a luminometer. β -galactosidase activities were assayed and read at OD₄₀₅. For each transfection, relative luciferase activity was normalized to β -galactosidase activity. In this report, only NHBE transfections are shown.

Despite the historical difficulties expected for transfecting cells cultured at an air-liquid interface, this method allowed us to obtain luciferase readings from transfected cells 10 to 100 times higher than the untransfected control; the transfection efficiency is about 20%.

Nuclear Extract Preparation and NoShift Transcription Factor Assay

The nuclear extracts from NHBE or HBE1 cells cultured for 2 weeks in retinoic acid (RA)-supplemented media at an air-liquid interface were prepared according to the manufacturer's protocol with the Panomics Nuclear Extraction kit (Panomics, Redwood City, CA). The enzyme-linked immunosorbent assay–based electrophoretic mobility shift assay was carried out using NoShift transcription factor assay kits from EMD

Biosciences (San Diego, CA) according to the manufacturer's protocol. Fifteen micrograms of PMA-treated and untreated nuclear extract were incubated on ice for 30 minutes with either biotinylated consensus Sp1, STAT1, or c-Myc DNA (10 pmole), together with 500 ng salmon sperm DNA and 0.01U Poly(dI-dC) in 1× NoShift binding buffer. Simultaneously, a competition assay was performed with a 50-fold molar excess (500 pmole) of nonbiotinylated Sp1-1, Sp1-2, Sp1-3, or mutant nonbiotinylated Sp1-1m, Sp1-2m, Sp1-3m, added before the addition of extracts.

After 1 hour of incubation at 37°C on streptavidin-coated microassay plates, samples were incubated with anti-Sp1, anti-STAT1 (BD Biosciences, San Jose, CA), or anti-c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies and further incubated at 37°C for 1 hour. The bound transcription factor was then detected with a specific secondary horseradish peroxidase conjugate antibody. After intensive washing to remove the secondary antibody, TMB substrate and 1 N HCl (stop solution) were added before the plates were read at 450 nm wavelength. The sequences of the biotinylated oligonucleotides are as follows. Consensus Sp1: forward, ATTCGATCGGGGCGGGGCGAG; reverse, TAAGCTAGCCCC GCCCGCTC. STAT1: forward, TCC CTTCCGATTCTCAGAAGCT; reverse, AGCTTCTG AGAATCG GAAGGA. C-Myc: forward, AACACACGTGGCTGGAGCGGGG; reverse, CCCC GCTCCAGCCACGTGTGTT.

Inhibitor Studies

For the inhibitor studies, NHBE and HBE1 cells were cultured in RA-supplemented media at an air-liquid interface for 2 weeks and then starved for 1 day in medium containing no supplement except RA. After the starvation period, cells were pretreated with mithramycin A or vehicle for 1 hour before PMA treatment. RNA and protein extracts were prepared from these cultures 24 hours after PMA treatment for real-time RT-PCR and luciferase quantification, respectively. For the reporter gene activity assay, cells were transfected with pGL3-*MUC5B* after the inhibitor treatments.

Mithramycin A, purchased from Sigma (Sigma-Aldrich, St. Louis, MO), was used at dose ranges based on the manufacturer's suggestion. The toxicity toward our cultured cells was negligible, as observed by the trypan blue exclusion test.

Immunoprecipitation and Western Blot Analysis

NHBE cells were cultured in RA-supplemented media at an air-liquid interface for 2 weeks and then starved for 1 day in medium containing no supplement except RA. Cells were then treated with vehicle or 10 nM PMA for 24 hours before being lysed in RIPA buffer (1% NP-40; 0.5% deoxycholic acid; 1 mM EDTA; 150 mM NaCl; 50 mM Tris-HCl; pH 8.0, 1 mM PMSF; 1 mM sodium orthovanadate; 1 mM NaF; 1 µg/ml aprotinin; 1 µg/ml leupeptin; 1 µg/ml pepstatin). Phosphorylated Sp1 was immunoprecipitated using anti-phospho-serine antibody conjugated agarose (Sigma-Aldrich). Eight hundred micrograms of lysate was incubated with 50 µl agarose at 4°C overnight. Immunoprecipitated complexes were subjected to electrophoresis on an SDS/10% polyacrylamide gel and transferred to a PVDF membrane (BioRad

Laboratories, Hercules, CA). Sp1 was detected using Sp1 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Chromatin Immunoprecipitation Assays

NHBE cells were cultured in RA-supplemented media at an air-liquid interface for 2 weeks and then starved for 1 day in medium containing no supplement except RA. After the starvation period, cells were treated with vehicle or 10 nM PMA for 24 hours. Chromatin immunoprecipitation (ChIP) assays were performed using the chromatin immunoprecipitation assay kit (Millipore, Chicago, IL) according to the manufacturer's protocol, with some modifications. Briefly, NHBE cells were treated with formaldehyde (1%) for 20 minutes at room temperature to cross-link proteins to DNA. The reaction was then quenched with 137.5 mM glycine for 5 minutes at room temperature. Next, cells were lysed and sonicated to shear DNA to a length between 200 and 1,000 bp. The sonicated supernatant was pre-cleared using protein A agarose slurry containing salmon sperm DNA and 1% bovine serum albumin. After centrifugation, the pre-cleared supernatant was diluted 10-fold with ChIP dilution buffer and incubated with either anti-Sp1 or anti-RNA polymerase II antibody (Santa Cruz Biotechnology) overnight at 4°C. After intensive washing, the immunoprecipitated complex was eluted and reverse cross-linked by adding NaCl (final concentration 200 µM) and heated at 65°C overnight. The recovered DNA was used for PCR amplification with the following *MUC5B* promoter-specific primers: Sp1-1: -171/-27, forward, ACAGAGCTGCAAATCCTTCCTGA; reverse, ATGGCCGCGAGAGCCAACA. Sp1-2: -246/-141, forward, CAGGTGGGGTAGGCCCTTCTCTC; reverse, CCTGGATCAGG AAGGATTGCA. Sp1-3: -569/-297, forward, TGGAAATAGAG CCTCTCCAG GGA; reverse, AGGGTCCATGGAAACAGTG GTCAG.

Statistical Analysis

Experiments were carried out in triplicate and at least in two independent cultures. Representative data from these independent cultures are presented. Significance of the difference in means between treated and control samples were determined by Student's *t* test; *P* < 0.05 was considered as a significant difference.

RESULTS

PMA Stimulates *MUC5B* Mucin Production

We previously showed that PMA induced both *MUC5B* mRNA and protein synthesis in primary NHBE, HBE1, and A549 cells (1). In this study, we examined whether the induction is time- and dose-dependent. As shown in Figure 1, using primary NHBE cells maintained under air-liquid interface conditions, PMA induced *MUC5B* mRNA in a dose- and time-dependent manner (Figures 1A and 1B, respectively), whereas 4α-PMA, a negative control for phorbol ester, failed to elicit any induction. Induction was seen at PMA levels as low as 2 nM.

Figure 1. Stimulation of *MUC5B* mRNA by PMA. (A) Dose study of PMA effects on *MUC5B* mRNA expression. Normal human bronchial epithelial (NHBE) cells cultured for 2 weeks in retinoic acid (RA)-supplemented media at an air-liquid interface were starved for 16 hours with growth factor-free medium before treating with varying dosages of PMA or 4α-PMA (0–10 nM). Twenty-four hours after 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 dosage and experiments were repeated at least three times in cultures derived from

different donors or different passages of cell line. Significance: **P* < 0.05 compared with unstimulated controls. (B) Time course study of PMA effects on *MUC5B* mRNA expression. NHBE cells cultured in RA-supplemented media at an air-liquid interface for 2 weeks were treated with 10 nM PMA or 4α-PMA and harvested at different time points (0, 8, 16, 24, 48 h). Samples were measured with SYBR Green quantitative real-time PCR. Triplicate dishes were used for each time point and experiments were repeated at least three times in different cultures derived from different donors or different passages of cell line. Significance: **P* < 0.05 compared with unstimulated controls.

More than 2-fold induction was seen 16 hours after PMA treatment, and the induction reached to more than 3-fold 48 hours after PMA treatment. These results confirm that PMA, a protein kinase C activator, is a potent stimulator for *MUC5B* gene expression in cultured human airway epithelial cells.

Deletion Analysis of PMA-Induced *MUC5B* Promoter Activity

Systematically deleted *MUC5B* 5'-flanking regions spanning from +6 bp to -4,169 bp, -2,561 bp, -1,610 bp, -1,136 bp, -652 bp, -222 bp, and -78 bp relative to the transcription initiation site (Figure 2A) were generated and cloned into the promoterless pGL3 vector. Those clones were used for transient transfection studies. As shown in Figure 2B, the basal activity of the pGL3-*MUC5B* (0.22 kb) construct was increased 4- to 5-fold compared with the activity of pGL3-*MUC5B* (0.08 kb), the smallest construct. Furthermore, PMA-induced *MUC5B* promoter activity only existed for constructs with the *MUC5B* 5' flanking region longer than the proximal 78 base pairs. These results suggest that the PMA-induced promoter activity resides in the 5'-flanking sequence between -222 and -78 bp. Promoter activity was not increased between the pGL3-*MUC5B* (1.14 kb) and pGL3-*MUC5B* (1.62 kb)-transfected cells, suggesting that the distal TATA Box-like sequence at -1,185/-1,193, which was implicated in the basal promoter activity in a colon cancer cell line (31), does not play a role in airway epithelial cells.

Sp1 cis Elements Are Important for Basal and PMA-Induced *MUC5B* Transcription

To further study the potential transcription factors involved in PMA-induced *MUC5B* expression, the proximal -247 bp of *MUC5B* promoter was analyzed for putative transcription factor binding sites with the TRANSFAC database (<http://transfac.gdf.de/TRANSFAC/>) (32). The results of this analysis are listed in Figure 3A. There are two putative Sp1 (-197/-186 and -122/-114), one STAT (-214/-206), and one c-Myc (-105/-98) binding sites on the 5'-flanking region of the *MUC5B* promoter. Using the NoShift transcriptional factor

assay with biotinylated consensus DNA sequences corresponding to these binding sites and the cell lysates obtained from both PMA-treated and control NHBE cultures, we observed that PMA stimulated Sp1-binding affinity significantly by 75, 133, and 111% in cells after 16, 24, and 48 hours of treatment, respectively, whereas the binding of both c-Myc and STAT 1 remains the same with or without PMA treatment (Figure 3B).

To further confirm that Sp1 binding is directly involved in the regulation of *MUC5B* gene transcription, NHBE cells were pretreated with mithramycin A to inhibit Sp1 binding. A dose-dependent inhibition of *MUC5B* promoter activity was observed (Figure 3C). With 0.1 μ M mithramycin A, there was 30% inhibition of PMA-induced promoter activity with no change in the basal promoter activity. At 1 μ M mithramycin A, however, there was a significant reduction of PMA-induced activity, in addition to the abrogation of basal promoter activity. The reduction in basal promoter activity is not due to toxicity, since no apparent cell death was detected at this dose according to the trypan blue dye-exclusion assay (unpublished data). Similar results were seen in HBE1 cells (right panel of Figure 3C), suggesting the involvement of an Sp1-based transcriptional mechanism in the regulation of both basal and PMA-induced *MUC5B* gene expression.

Sp1 Phosphorylation Is Activated by PMA Treatment

Since phosphorylation has been implicated in changes of Sp1 transcriptional activity (33, 34), we asked whether PMA stimulated Sp1 phosphorylation using immunoprecipitation with whole protein lysates of NHBE cells with or without PMA treatment. Because phosphorylation of Sp1 is predominantly on serine residues, with less than 5% on threonine and none on tyrosine (35), the phosphorylated form of Sp1 was immunoprecipitated with anti-phospho-serine antibody-conjugated agarose, followed by Western blot analyses with anti-Sp1 antibody. Simultaneously, nonimmunoprecipitated samples were loaded on the same gel and probed with anti-Sp1 antibody to allow for protein normalization (Figure 4, lower panel). As shown in Figure 4, PMA treatment significantly enhanced the phosphorylation

Figure 2. The proximal region at -222/-78 of *MUC5B* promoter is responsible for basal and PMA-induced *MUC5B* gene activation. (A) Deletion map of *MUC5B* promoter-luciferase chimeric constructs. The 5'-flanking region of the *MUC5B* promoter was serially truncated and cloned into promoterless pGL3 vector. This map represents the schematically truncated DNA fragments in the 5'-flanking region spanning from +6 to -2,561 (2.6 kb), -1,610 (1.62 kb), -1,136 (1.14 kb), -652

(0.66 kb), -222 (0.22 kb) and from +18 to -78 (0.08 kb) upstream of the transcription start site (+1). The TATA box (-32/-26) and putative second TATA box (-1,193/-1,185) are also indicated. (B) Relative *MUC5B* promoter activity in transfected cells. NHBE cells cultured in RA-supplemented media at an air-liquid interface for 2 weeks were transfected with serially deleted mutants along with pSV- β -galactosidase for normalization of the transfection efficiency. Cells were stimulated with or without 10 nM PMA for 24 hours before harvesting. The diagram shows *MUC5B* promoter luciferase activity normalized by β -galactosidase activity. Triplicate dishes were used for each transfection assay and experiments were repeated twice with different culture passages. Significance: * $P < 0.05$ compared with the control case, without PMA treatment.

Figure 3. Sp1 cis elements are important for basal and PMA-induced *MUC5B* transcription. (A) Nucleotide sequence of the proximal 247 base pairs of the *MUC5B* promoter. The TATA box (TACATAA) at $-32/-26$ is indicated in this graph. The transcription start site is designated as +1, with the first ATG bolded. Potential binding sites of known transcription factors based on the TRANSFAC database (<http://tansfac.gdf.de/TRANSFAC/>) are underlined (32). We named the Sp1 binding site at $-122/-114$ as Sp1-1, and the Sp1 site at $-197/-186$ as Sp1-2. (B) Binding of transcription factors to the *MUC5B* promoter in NHBE cells. A NoShift transcription factor assay was carried out using well-differentiated biphasic-cultured cells. Fifteen micrograms of 10 nM PMA-treated or untreated nuclear protein were incubated with the biotin-labeled nucleotides of interest, and antibodies of interest were added to detect DNA-protein interaction, as described in the manufacturer's protocol for the NoShift transcription factor assay kit. The figure shows the binding for consensus DNA sequences specific for Sp1, STAT1, and C-Myc. Significance: * $P < 0.05$ compared with non-PMA-treated control at each time point. (C) The effect of mithramycin A on PMA-induced *MUC5B* expression. In the left panel, NHBE cells cultured in RA-supplemented media at an air-liquid interface for 2 weeks were transfected with full-length pGL3-*MUC5B* (4.17 kb) and pSV- β -galactosidase as described in Figure 2. On the second day, the cells were treated with 10 nM PMA for 24 hours before being harvested for the reporter assay. The inhibitor was added at different doses (0.1, 1 μ M) 1 hour before PMA treatment. Significance: * $P < 0.05$, compared with PMA-treated, uninhibited controls; # $P < 0.05$ compared with non-PMA-treated, uninhibited control. In the right panel, NHBE and HBE1 were cultured at an air-liquid interface for 2 weeks in the presence of RA as described in MATERIALS AND METHODS. One hour before PMA (10 nM) treatment, cultures were pretreated with 1 μ M of mithramycin A, an Sp1-binding inhibitor. Cultures were harvested for RNA isolation 24 hours after PMA treatment. *MUC5B* and β -actin messages were quantified by SYBR Green quantitative real-time RT-PCR analysis as described in MATERIALS AND METHODS. PMA-induced and non-induced *MUC5B* mRNA normalized with β -actin expression were compared and represented in the figure as fold of stimulation. The results show the mean $\pm$ SD for triplicates from two separate primary cultures or different passages of cell line. * $P < 0.05$.

of Sp1, suggesting that PMA can activate Sp1 transcriptional activity through serine phosphorylation.

Characterization of Putative Sp1 Sites by NoShift Transcriptional Factor Assay and Site-Directed Mutagenesis

To further characterize the putative Sp1 sites that are important in the regulation of *MUC5B* expression, we examined the two *MUC5B* putative Sp1 sequences situated in the critical -222 to -78

region, and a third Sp1 site located at -458/-437 bp with lysates obtained from PMA treated and untreated cells. PMA treated lysates induced a more than 3-fold increase in cis- / trans-Sp1 binding when compared to the untreated samples (Figure 5A). Both basal and PMA-induced Sp1 binding were completely blocked in a competition assay with a 50-fold molar excess (500 pM) of wild type synthetic Sp1-1, Sp1-2, and Sp1-3 oligomers. However, the mutated form of those putative Sp1 cis elements, Sp1-1m, Sp1-2m and Sp1-3m, failed to compete, indicating that

Figure 4. Sp1 phosphorylation is activated by PMA treatment. Protein lysates of NHBE cells cultured in RA-supplemented media at an air-liquid interface for 2 weeks were harvested 24 hours after PMA (10 nM) treatment. Eight hundred micrograms of lysate was incubated with 50 μ l anti-phospho-serine antibody-conjugated agarose at 4°C overnight. Immunoprecipitated complexes were subjected to electrophoresis on an SDS/10% polyacrylamide gel and transferred to a PVDF membrane (Bio-Rad). Sp1 was detected using Sp1 polyclonal antibodies (Santa Cruz Biotechnology). To monitor loading consistency, an equal amount of protein lysate with no immunoprecipitation was loaded on the same gel and probed with Sp1 antibody.

the specific mutated base pairs on Sp1-1m, Sp1-2m and Sp1-3m (upper panel of Figure 5A) are critical for Sp1 binding.

Based on the above mutated Sp1 sequences, three Sp1-specific mutants were generated in the pGL3-*MUC5B* (4.17 kb) clone and are named as pGL3-*MUC5B*Sp1-1m, pGL3-*MUC5B*Sp1-2m, and pGL3-*MUC5B*Sp1-3m. Plasmid DNAs obtained from these three mutants and the wild-type pGL3-*MUC5B* were used to transfect NHBE cells. As shown in Figure 5B, both basal and PMA-induced luciferase activities were abrogated in pGL3-*MUC5B*Sp1-1m-transfected cells. Cells transfected with pGL3-*MUC5B*Sp1-2m showed basal-level promoter activity but not PMA-induced activity. Mutation at the distal Sp1 site (pGL3-*MUC5B*Sp1-3m) did not inhibit either basal or PMA-induced promoter activity. These results suggest that either the Sp1-1 site (–122/–114) is involved in both basal and PMA-induced promoter activities, or that it is involved only in the basal activity, and that PMA-induced activity is dependent on the fulfillment of the basal activity. The Sp1-2 site (–197/–186) is clearly involved in PMA-induced activity only, since the activity of *MUC5B* promoter containing the mutated form of SP1-2 was not induced by PMA. The Sp1-3 site (–458/–437) does not appear to be involved in either the basal or the PMA-induced *MUC5B* transcription activity. These results agree with the deletion analysis (Figure 2).

Demonstration of Sp1 Binding to *MUC5B* Promoter *In Situ* by ChIP Assay

To determine if PMA-induced *MUC5B* promoter activity is due to altered interactions between Sp1 and *MUC5B* promoter DNA, the effects of PMA on the binding of endogenous Sp1 to the putative cis element on *MUC5B* promoter *in situ* was examined by the ChIP assay. After the protein and the DNA were cross-linked with formaldehyde, Sp1 and RNA polymerase II complexes in NHBE cells were immunoprecipitated with anti-Sp1 and anti-RNA polymerase II antibodies, respectively. The binding of Sp1 and RNA polymerase II to the putative cis element in the immunoprecipitates was analyzed by SYBR Green quantitative real-time PCR and regular PCR. In these experiments, cell lysates before antibody treatment and immunoprecipitation were used as the total input control. As shown in Figure 6A, when anti-RNA polymerase II antibody was used to pull down the RNA polymerase II–DNA complex, we found that the DNA-protein (RNA polymerase II) complex containing the Sp1 sites was elevated in the PMA-treated samples. This elevation is due to the PMA-induced transcriptional activity

and it is irrelevant to the binding situation of those Sp1 sites. When we look at the binding status of those three Sp1 sites by using Sp1 antibody, we found that PMA treatment of NHBE cells significantly increased the binding of Sp1 protein to the putative *MUC5B* Sp1-2 binding site (–197/–186), whereas the binding of Sp1 protein to the putative *MUC5B* Sp1-1(–122/–114) and Sp1-3 (–458/–437) sites were not increased. These results suggested that PMA induced an additional binding of Sp1 protein to the Sp1-2 cis element on the *MUC5B* promoter and that the Sp1-2 domain is responsible for the PMA-induced transcriptional activity. The lack of PMA-induced Sp1-1 binding suggested that Sp1-1 is not involved in the PMA-induced promoter activity. Therefore, SP1-1 is responsible for the basal activity of *MUC5B* promoter, and the PMA-induced *MUC5B* promoter activity is due to the increased binding on Sp1-2. However, this induction can only happen when the basal activity is fulfilled. In a separate study with regular PCR rather than the real-time PCR, a similar result was also observed (Figure 6B).

DISCUSSION

Extensive work has been done to elucidate the molecular mechanism of *MUC5AC* and *MUC2* gene expression (16, 18, 20, 36), but studies on the regulation of *MUC5B* gene expression are scarce. In this paper, we examined the molecular mechanism of *MUC5B* gene expression in human airway epithelial cells using a potent PKC activator, PMA. Under PMA treatment, *MUC5B* gene expression was enhanced in a time- and dose-dependent manner (Figure 1). This effect of PMA appeared to be at the transcriptional level, since the elevation pattern of *MUC5B* promoter-reporter activity was similar to the elevation pattern of *MUC5B* mRNA and protein resulting from PMA-treated NHBE cells (1). Promoter-reporter and transcription factor analyses revealed significant roles of the Sp1 transcription factor and its interacting sites at the 5'-flanking region of the *MUC5B* gene based on deletion and site-directed mutagenesis analyses of the promoter-reporter construct and the NoShift transcription factor assay, as well as a functional study with the pharmacological inhibitor, mithramycin A. Mithramycin A is a drug that binds to GC-rich regions in chromatin and interferes with the transcription of genes that bear GC-rich motifs in their promoters. Transcriptional inhibition due to mithramycin A treatment indicated the involvement of GC-rich motifs on *MUC5B* promoter. Most importantly, we have demonstrated a physical interaction of the Sp1 nuclear protein with putative Sp1 sites on the *MUC5B* promoter in NHBE and HBE1 cells by ChIP assays. These interactions occurred with both the basal and the PMA-induced conditions. These findings are consistent with the notion that *MUC5B*, like other mucin genes, is regulated by an Sp1-dependent transcriptional mechanism (16, 20). Similar results were reported in colon cancer cells and gastric carcinoma cancer cell lines (31). In gastric carcinoma cells, *MUC5B* expression was governed by a proximal promoter as well as a highly active distal promoter with a TATA-like sequence located at –1,142/–1,134 (TAAATAAAA) (31). In our study of NHBE and HBE1 cells, the 5'-flanking region in the vicinity of the distal TATA box appeared to have no effect on *MUC5B* gene expression, as suggested by our deletion study in Figure 2.

Sp1 is a member of a multigene family that binds DNA through COOH-terminal zinc-finger motifs (37). Although Sp1 activity was initially thought to be constitutive, it has since been shown to be regulated at different levels (38). Sp1 can be O-glycosylated, which affects its turnover (39). Sp1 can also be phosphorylated at several amino acid residues under various circumstances by a variety of kinases (40). Phosphorylated Sp1 has been reported to up- or down-regulate gene transcription

*MUC5B*Sp1-3m) were sequence-verified, as shown in the figure. The mutated sequences are bolded and indicated below each Sp1 site. NHBE cells cultured in RA-supplemented media at an air-liquid interface for 2 weeks were used to do transfection. Cells were transfected with pGL3-*MUC5B*, pGL3-*MUC5B*Sp1-1m, pGL3-*MUC5B*Sp1-2m, pGL3-*MUC5B*Sp1-3m, or pGL3-Basic and treated with 10 nM PMA as described in MATERIALS AND METHODS. Twenty-four hours after PMA treatment, cells were harvested for luciferase and β-galactosidase assays. Significance: **P* < 0.05, compared with PMA-induced stimulation in pGL3-*MUC5B*-transfected cells; #*P* < 0.05 as compared to basal activity in pGL3-*MUC5B*-transfected cells.

(41, 42). It has been shown that a change in the level of Sp1 phosphorylation results in altered DNA-binding activity in various genes (43–45). Here, we show that PMA treatment induces Sp1 phosphorylation (Figure 4), which increases the DNA-binding activity of Sp1 to the *MUC5B* promoter and then increases *MUC5B* mucin expression.

While a significant role of Sp1 in the transcriptional regulation of other mucin genes has been reported (16, 46–49), the physical interaction and functionality of the Sp1-binding sites in these mucin genes were not clear. Here, we have identified two proximal Sp1-binding sites: Sp1-1 and Sp1-2, located at –122/–114 and –196/–184 bp, respectively, on the human *MUC5B* promoter region as the important regulators for basal and PMA-enhanced promoter activities in human airway epithelial cells. Site-directed mutagenesis studies and Chip assays indicated that

Figure 5. Specific Sp1 binding sites on the *MUC5B* promoter are required for basal and PMA-induced *MUC5B* transcription activation.

(A) Site-directed mutagenesis of three Sp1 transcription factor binding sites and its effects on transcriptional factor binding demonstrated by the NoShift transcription factor binding assay. Nuclear extractions were harvested from biphasic-cultured HBE1 cells. Two Sp1 binding sites on the proximal 220 base of the *MUC5B* promoter and a nearby Sp1 binding site at –458/–437, named Sp1-1, Sp1-2 and Sp1-3, were synthesized. The mutated form of the above Sp1 sequences, designated as Sp1-1m, Sp1-2m and Sp1-3m were also synthesized. The biotinylated Sp1-1 (lanes 1–3), Sp1-2 (lanes 4–6), and Sp1-3 (lanes 7–9) nucleotides were incubated with 15 μg of nuclear proteins from PMA (10 nM) treated (shaded bars) or untreated (solid bars) HBE1 cultures in the presence of a 50-fold molar excess of competitor DNA from the corresponding Sp1-1, Sp1-2, and Sp1-3 wild-type or mutated sequences. The control with no competitor DNA was also included (lanes 1, 4, and 7). Experiments were carried out in triplicate dishes and repeated at least twice with different culture passages. (B) Effects of Sp1 binding site mutations on *MUC5B* promoter activity. The pGL3-*MUC5B* promoter was subjected to a site-directed mutagenesis to generate a mutation at each Sp1 sites, as shown in the mutated sequences of these three sites (A). These clones (pGL3-*MUC5B*Sp1-1m, pGL3-*MUC5B*Sp1-2m, and pGL3-*MUC5B*Sp1-3m) were sequence-verified, as shown in the figure. The mutated sequences are bolded and indicated below each Sp1 site. NHBE cells cultured in RA-supplemented media at an air-liquid interface for 2 weeks were used to do transfection. Cells were transfected with pGL3-*MUC5B*, pGL3-*MUC5B*Sp1-1m, pGL3-*MUC5B*Sp1-2m, pGL3-*MUC5B*Sp1-3m, or pGL3-Basic and treated with 10 nM PMA as described in MATERIALS AND METHODS. Twenty-four hours after PMA treatment, cells were harvested for luciferase and β-galactosidase assays. Significance: **P* < 0.05, compared with PMA-induced stimulation in pGL3-*MUC5B*-transfected cells; #*P* < 0.05 as compared to basal activity in pGL3-*MUC5B*-transfected cells.

Sp1-1 is pivotal for basal activity of the *MUC5B* promoter, while the Sp1-2 site is needed for PMA-induced *MUC5B* expression (Figure 5B). Although the third Sp1 site (Sp1-3, at –458/–437) can bind Sp1 transcription factor in the cell-free NoShift transcription factor assay, as well as the Chip assay, mutation at this site showed no effect on *MUC5B* promoter activity. These results are consistent with our deletion study, indicating that the promoter region affecting PMA-induced *MUC5B* expression is limited to the region of –222/–78 bp relative to the transcriptional start site (Figure 2B).

The ChIP assay has recently been used to determine the physical interactions between transcriptional factors and putative binding sites at the chromatin level *in situ*. Using this approach, we have shown increased RNA polymerase II binding with the flanking regions of all three Sp1 sites on the *MUC5B* promoter in

containing the different Sp1 sites. The PCR products were then run on 2% agarose gels with ethidium bromide staining and the image is analyzed with the LAS-3000 image analysis system (Fujifilm Life Science USA, Stamford, CT). The results shown represent data obtained from at least three independent experiments.

NHBE cells after PMA treatment. This is consistent with the notion that PMA induced *MUC5B* transcriptional activity, and therefore more *MUC5B* promoter–RNA polymerase II complex was formed in cells treated with PMA. However, ChIP assays using anti-Sp1 antibody revealed that the PMA-induced Sp1 DNA-protein complex is only limited to the Sp1-2 (–197/–186) domain. Apparently, Sp1-2 is the only site that responds specifically to PMA treatment. The ChIP assay data are consistent with the site-directed mutagenesis data suggesting that Sp1-2 is the important site in the induction of PMA-mediated *MUC5B* gene expression. The ChIP assay also demonstrated the noninvolvement of the Sp1-1 domain in the PMA induction mechanism. Together these data verified the *in situ* interactions of RNA polymerase II and Sp1 transcriptional factor with the *MUC5B* promoter in NHBE cells under both basal and PMA-stimulated conditions.

In summary, we have identified the two proximal Sp1 binding sites, Sp1-1 and Sp1-2, as the significant cis elements in the regulation of *MUC5B* gene expression. The Sp1-1 (–122/–114) site is pivotal for the basal activity of *MUC5B* promoter, while the Sp1-2 site (–197/–186) is needed for the PMA-induced *MUC5B* gene expression, whereas Sp1-3 has no effect on either the basal or the PMA-induced *MUC5B* activity. These observations suggest that these different Sp1 sites on the *MUC5B* promoter perform different functions. Apparently, each Sp1 site is recognized differently based upon its specific physical position, its flanking region, and the binding status of its nearby sequences. A functionally significant distinction between Sp1 and other Sp

Figure 6. ChIP assays show that Sp1 binds to *MUC5B* promoter *in vivo* NHBE cells cultured in RA-supplemented media at an air–liquid interface for 2 weeks were treated with or without 10 nm of PMA for 24 hours before the proteins were cross-linked to DNA as described in MATERIALS AND METHODS. Endogenous Sp1–DNA hybrid was then immunoprecipitated from the sonicated lysates of PMA-treated and -untreated samples using an antibody recognizing Sp1 protein. The interaction of RNA polymerase II with *MUC5B* promoter DNA was also examined using an antibody recognizing RNA polymerase II protein. (A) After uncross-linking the DNA-protein hybrid, SYBR Green quantitative real-time PCR was performed using primers to amplify the *MUC5B* promoter DNA, which contains different Sp1 sites. Results are the arbitrary unit of either PMA-treated or untreated samples compared to its own input control taken from each sample before addition of specific antibodies. Significance: * $P < 0.05$ compared with the non-PMA-treated control at each condition. (B) Simultaneously, the PCR reactions were performed using the same set of primers to amplify the *MUC5B* promoter DNA

family members is that Sp1 has the capacity to form multimers (50). When organized as a multimer, Sp1 presents multiple docking sites for protein interaction. On the *MUC5B* promoter, the two Sp1 sites involved in gene expression regulation are relatively close together; thus, it is possible to have the Sp1 multimer and its associated proteins involved in the binding. Further studies may be needed to reveal the interaction between Sp1 and other associated proteins in PMA-induced *MUC5B* gene regulation.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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