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AP2α Is Essential for *MUC8* Gene Expression in Human Airway Epithelial Cells

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ABSTRACT
Mucins are high molecular weight proteins that make up the major components of mucus. Hypersecretion of mucus is a feature of several chronic inflammatory airway diseases. *MUC8* is an important component of airway mucus, and its gene expression is upregulated in nasal polyp epithelium. Little is known about the molecular mechanisms of *MUC8* gene expression. We first observed overexpression of activator protein-2alpha (AP2α) in human nasal polyp epithelium. We hypothesized that AP2α overexpression in nasal polyp epithelium correlates closely with *MUC8* gene expression. We demonstrated that phorbol 12-myristate 13-acetate (PMA) treatment of the airway epithelial cell line NCI-H292 increases *MUC8* gene and AP2α expression. In this study, we sought to determine which signal pathway is involved in PMA-induced *MUC8* gene expression. The results show that the protein kinase C and mitogen-activating protein/ERK kinase (MAPK) pathways modulate *MUC8* gene expression. PD98059 or ERK1/2 siRNA and RO-31-8220 or PKC siRNA significantly suppress AP2α as well as *MUC8* gene expression in PMA-treated cells. To verify the role of AP2α, we specifically knocked down AP2α expression with siRNA. A significant AP2α knock-down inhibited PMA-induced *MUC8* gene expression. While dominant negative AP2α decreased PMA-induced *MUC8* gene expression, overexpressing wildtype AP2α increased *MUC8* gene expression. Furthermore, using lentiviral vectors for RNA interference in human nasal polyp epithelial cells, we confirmed an essential role for AP2α in *MUC8* gene expression. From these results, we concluded that PMA induces *MUC8* gene expression through a mechanism involving PKC, ERK1/2, and AP2α activation in human airway epithelial cells. J. Cell. Biochem. 110: 1386–1398, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: MUC8; AP2α; PMA; ERK1/2

Mucins are high molecular weight glycoproteins that comprise the major components of mucus. Virtually all forms of airway inflammation are associated with mucus overproduction, which can lead to airway obstruction. Mucins are responsible for the viscoelastic properties of secreted mucus while lubricating and protecting mucus membranes. Mucus hypersecretion is commonly observed in many respiratory diseases, such as rhinitis, sinusitis, otitis media, nasal allergy, chronic bronchitis, and cystic fibrosis [Rose and Voynow, 2006]. To date, 20 distinct mucin genes have been identified. The mucins are usually subdivided into two groups based on domain: membrane-bound and secreted mucins. MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC9, and MUC19 are secreted mucins, whereas MUC1, MUC3, MUC4, MUC11, MUC12, MUC13, MUC17, MUC18, and MUC20 are membrane-bound mucins [Kufe, 2009]. The other mucin genes, including *MUC8*, have not been fully characterized.

MUC8 mRNA levels are upregulated in middle ear effusions, chronic sinusitis, and endometrial adenocarcinomas [Yoon et al., 2002; Takeuchi et al., 2003; Hebbar et al., 2005]. In our previous study, we found that *MUC8* mRNA levels were up-regulated in human nasal polyps epithelium whereas MUC5AC gene was not [Seong et al., 2002; Yoon et al., 2002]. This is coincident with

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recently reported studies in which MUC8 expression was highly increased in all studied epithelia and glands of nasal polyp samples compared with the expression of the other major mucin genes, MUC5AC and MUC5B, which are moderately expressed in nasal polyps [Martinez-Anton et al., 2006, 2008]. These studies raised the possibility that MUC8 plays a major role in the airway mucosa and its over-expression is related to mucus hypersecretion. Because only a small part of the C-terminal domain has been published for MUC8, further molecular studies have been limited. Although the importance of MUC8 expression has been investigated in airway mucosal, its potential role has not been well-established.

In this study, we observed that AP2α was overexpressed in nasal polyp epithelium. The transcription factor AP2α has been implicated as a cell-type-specific regulator of gene expression. Several studies suggest that AP2α may control proliferation, apoptosis, and mammary epithelial cell differentiation [Schorle et al., 1996; Zhang et al., 2003]. An important regulator of a number of epithelium-specific genes, AP2α mediates transcriptional E-cadherin [Hennig et al., 1996; Batsche et al., 1998] and hST3Gal IV regulation [Taniguchi and Matsumoto, 1999] in epithelial cells. Retinoic acid, cAMP, and phorbol esters regulate AP2α activity [Imagawa et al., 1987; Gravel et al., 2000]. PMA can substitute for diacylglycerol, the endogenous PKC activator, and it has been used in an inflammatory stimulus model to study mucin signaling mechanisms [Lee et al., 2002; Shao et al., 2003; Hewson et al., 2004; Shao and Nadel, 2005; Wu et al., 2007; Yuan-Chen Wu et al., 2007]. Activation of PKC is regulated by LPS [Comalada et al., 2003; Lee et al., 2008]. and cytokines such as IL-1β [Varley et al., 2001] and TNF-α [Chang and Tepperman, 2003], [Suzuki et al., 2001] and these inflammatory mediators increase *MUC8* expression [Yoon et al., 1999; Ishinaga et al., 2002; Song et al., 2003]. In our previous study, *MUC8* gene expression was induced by prostaglandin E_2, while Tomas Sitter et al. reported that prostaglandin E_2 synthesis was increased by PMA through PKC activation [Sitter et al., 1998; Cho et al., 2005]. These results indicate that PMA, an artificial PKC activator, can be used to stimulate *MUC8* gene expression.

PMA has been shown to upregulate several mucin genes, including MUC2, MUC5B, and MUC5AC [Nagle and Blumberg, 1980; Lee et al., 2002; Hewson et al., 2004; Wu et al., 2007; Yuan-Chen Wu et al., 2007]. A detailed analysis of the signaling pathways involved in PMA-induced upregulation of *MUC8* has not been demonstrated.

A recent study revealed that nasal polyp epithelium overexpresses AP2α protein compared with normal nasal mucosa. In addition, previous studies have shown that *MUC8* is clearly also upregulated in nasal polyp epithelium indicating that this transcription factor might be important for regulating *MUC8* gene expression in nasal polyp epithelium. This study examined the functional significance of AP2α for *MUC8* gene expression by exploring the biochemical mechanisms involved in AP2α and *MUC8* overexpression in nasal polyp cells.

We used PMA to evaluate the signaling mechanism of PMA-induced *MUC8* expression by AP2α. We found that extracellular signal-regulated kinase mitogen-activated protein kinase (ERK MAPK) and AP2α are essential for PMA-induced *MUC8* gene expression in NCI-H292 cells. In addition, using lentiviral vectors for RNA interference in human nasal polyp epithelial cells, we confirmed an essential role for AP2α in *MUC8* gene expression. These pathways provide insight into the molecular mechanisms of *MUC8* overexpression in nasal polyps.

MATERIALS AND METHODS

MATERIALS

Anti phospho-p44/42 MAP kinase (Thr202/Tyr204), anti-phospho-p38 MAP kinase (Thr180/Tyr182), anti-phospho-SAPK/c-Jun NH2-terminal kinase, MAP kinase (Thr183/Tyr185), and anti-human AP2α antibodies were purchased from Cell Signaling (Beverly, MA). RO-31-8220, PD98059, and anti-α-tubulin antibody were purchased from Calbiochem (San Diego, CA). SP600125 and SB239063 were purchased from Sigma (St. Louis, MO).

CELL CULTURE

After approval of the study protocol by the Institutional Review Board of the Yonsei University College of Medicine, human nasal specimens were obtained. Human nasal polyps were obtained from seven patients (5 men and 2 women, aged 27–60 years) with chronic sinusitis, and normal nasal mucosa specimens were obtained from seven healthy subjects (4 men and 3 women, aged 22–34 years). None of the patients had a history of asthma, aspirin sensitivity, or cystic fibrosis. Also, none had been on a regimen of intranasal medication or antibiotic treatment, and they had negative results on allergic skin-prick test. None of healthy subjects had any history of allergic symptoms, nasal polyps, or asthma. They did not have a history of smoking and did not take any medicine. We used the seven patient and healthy subject tissues for the immunohistochemistry. Because the number of epithelial cells from one patient is not enough to obtain PMA-treated or viral infected sample, we pooled samples from seven patients. Epithelia were isolated from scrapings of the inferior turbinate or nasal polyp. Epithelial cells were treated with 1% Pronase (Type XIV protease, Sigma–Aldrich Chemical Co., St. Louis, MO) for 18–20 h at 4°C. To remove fibroblasts, endothelial cells, and myoepithelial cells, isolated cells were placed in a plastic dish and cultured for one h at 37°C. Isolated epithelial clusters were divided into single cells by incubating them with 0.25% trypsin/EDTA. Passage-2, human nasal polyp epithelial cells were seeded in 0.5 ml of culture medium onto 24.5-mm, 0.45-μm pore size Transwell clear culture inserts. Cells were cultured in a 1:1 mixture of bronchial epithelial cell growth medium (Clonetics, CA) and Dulbecco's modified Eagle's medium (Invitrogen, San Diego, CA) containing all supplements. The cultures were grown submerged for the first 9 days, with the culture medium changed on day 1 and every other day thereafter. An air-liquid interface (ALI) was created on day 9 by removing the apical medium and feeding the cultures only from the basal compartment. The culture medium was changed daily after creating an ALI. Cells were treated 7 days after confluence for all experiments, indicating that primary cells were in a differentiated state at the time of treatment.

The human mucoepidermoid pulmonary carcinoma cell line NCI-H292 was purchased from the American Type Culture Collection (CRL-1848, Manassas, VA) and cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) in the

presence of penicillin–streptomycin (Invitrogen) at 37°C in a 5% CO_2 humidified chamber.

PMA TREATMENT

Cells were seeded into six-well plates, cultured for 24 h to confluency, and serum-starved in RPMI-1640 with 0.2% FBS for 24 h. Serum-starved medium was used throughout the experiments. Cells were treated with PMA (Sigma–Aldrich) as indicated in each experiment. For inhibition studies, cells were pretreated with media containing inhibitors for 30 min before replacing with control or PMA-containing medium. After 24 h, cells were collected to measure *MUC8* expression.

IMMUNOHISTOCHEMICAL STAINING FOR AP2α

The nasal polyp and normal nasal mucosa specimens were fixed with 4% paraformaldehyde for 24 h, cryoprotected with 12% and 18% sucrose, and stored in a deep freezer until use. The inserts were then divided into 10-μm sections, and the frozen sections were stained with anti-AP2α antibodies. Antibody staining was detected using peroxidase-conjugated anti-rabbit secondary antibody. Negative controls lacking a primary antibody contained an irrelevant antibody (purified rabbit IgG).

RNA ISOLATION AND REVERSE TRANSCRIPTASE PCR (RT–PCR)

Total RNA was extracted from cells using TRIzol® reagent (Invitrogen) following the manufacturer's instructions. Reverse transcription reactions were performed with 3 μg of total RNA, random hexamer primers (Applied Biosystems, Foster City, CA), AMV reverse transcriptase (Applied Biosystems), and RNase inhibitor (Applied Biosystems) in a final volume of 25 μl. The reverse transcription step ran for 30 min at 42°C and 5 min at 95°C. RT-PCR was performed with a MyCycler (Bio-Rad, CA) using the primers listed in Table I.

REAL-TIME QUANTITATIVE PCR

TaqMan real-time quantitative PCR analysis of *MUC8* gene expression was carried out with an Applied Biosystems 7300 Fast Real-Time PCR system and normalized with respect to β2-microglobulin RNA as an endogenous control. The standard curve used for quantification was generated with serial 10-fold dilutions of pGEMT-MUC8. The thermocycler parameters were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate. Relative *MUC8* mRNA expression was obtained using a comparative threshold method. Each reaction consisted of 12.5 μl of 2× TaqMan PCR Universal PCR master mix (Applied Biosystems), 1 μg of cDNA, 800 nM of primers, and 200 nM of TaqMan hybridization probe in 25 μl. The probe was labeled with carboxylfluorescein at the 5′-end and quencher carboxytetramethylrhodamine at the 3′- end. Quantitative AP2α DNA analysis was performed using SYBR Green PCR Core Reagents (Applied Biosystems). One microgram of cDNA was placed in a mixture containing 2.5 μl of 10× SYBR Green buffer, 3 μl of 25 mM $MgCl_2$, 2 μl of 5 mM dNTP, 0.1 μl of Ampli Taq Gold, 0.2 μl of Amp Erase UNG (Applied Biosystems), and 800 nM of primers. All primers and probes are listed in Table I.

WESTERN BLOT ANALYSES

Cells were seeded into six-well plates and cultured for 24 h before serum starvation. After starving in 0.2% medium for 24 h, cells were treated with 50 ng/ml PMA and harvested with 2× lysis buffer (250 nM Tris–Cl, pH 6.5, 2% SDS, 4% β-mercaptoethanol, 0.02% bromophenol blue, and 10% glycerol). Lysates were resuspended in SDS–PAGE sample buffer (50 mM Tris–HCl, pH 6.0, 10% glycerol, 2% SDS, 100 mM β-mercaptoethanol, and 0.1% bromophenol blue), boiled for 10 min, and analyzed on 12% SDS–PAGE gels. Resolved proteins were transferred to a polyvinylidene difluoride membrane (Millipore, MA). Blots were blocked with 5% skim milk in Tris-buffered saline (TBS, 50 mM Tris–Cl, pH 7.5, and 150 mM NaCl) for 1 h at room temperature and incubated overnight with primary antibodies (1:1,000) in 0.5% Tween 20 in TBS (TTBS). After thorough washing with TTBS, blots were further incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (1:2,000) (Cell Signaling) in TTBS and visualized by ECL (Amersham Biosciences).

TRANSIENT-TRANSFECTION ASSAY

For each transfection, NCI-H292 cells were seeded into six-well plates and cultured for 24 h before transfection. Cells were transfected with 1 μg of SPRSV-AP2α (AP2α overexpression), Δ165 (AP2α-dominant negative), or SPRSV-NN (negative control; empty vector) for 24 h using FuGENE6 Transfection Reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Cells were then treated with 50 ng/ml PMA for 24 h and assayed by RT-PCR, real-time PCR, or Western blot analysis.

TABLE I. Primers and Probes Used for PCR

Gene name	Primer sequences	Product size (bp)
Mucin 8 (RT-PCR)	Forward: ACAGGGTTTCTCCTCATTG Reverse: CGTTTATTCCAGCACTGTTC	239
Mucin 8 (real-time PCR)	Forward: TAACCCAATGCCACTCCTTC Reverse: GGAGTGTAACCTGGCTGCTC Probe: GGTTAGGGCTGACCACAGAA	202
Activator protein-2α (RT and real-time PCR) [Anttila et al., 2000]	Forward: GCCCGTGTCCCTGTCCAA Reverse: TGAGGAGCGAGAGGCGACC	132
β2-Microglobulin (RT-PCR)	Forward: TCGCGCTACTCTCTCTTTCTGG Reverse: GCTTACATGTCTCGATCCCACTTAA	366
β2-Microglobulin (real-time PCR)	Forward: CGCTCCGTGGCCTTAGC Reverse: GAGTACGCTGGATAGCCTCCA Probe: TGCTCGCGCTACTCTCTCTTTCTGGC	67

Dr. Trevor Williams, Yale University (New Haven, CT, USA) provided SPRSV-AP2α, Δ165, and SPRSV-NN plasmids [Williams and Tjian, 1991].

siRNA PREPARATION AND TRANSFECTION

AP2α siRNA (siAP2α) oligonucleotides (Stealth™ siRNA) were synthesized by Invitrogen. We screened AP2α mRNA (GenBank NM_001032280 and NM_003220) and selected three potential siRNA sequences with high knock-down probability values. The siRNA sequences in NM_001032280 started at bases 965, 1238, and 1594 (965: 5′-TCGCAAGATCCTTACTCCCACGTCA-3′ 1238: 5′-CCGCATGTAGA AGACCCGGG TATTA-3′ 1594: 5′-AGGGAGACG-TAAAGC TGC CAACGTT-3′). Stealth RNAi Negative Control Duplex (Medium GC, Invitrogen) was used as a siRNA negative control (siCon). The siPKC (catalog no.sc-29449), siERK1 (catalog no. sc-29307), and siERK2 (catalog no. sc-35335), and siRNA negative control (scrambled sequence, catalog no. sc-37007) were purchased from Santa Cruz Biotechnology. The sip38 (catalog no.6564) and siSAPK/JNK (catalog no. 6232) were purchased from Cell Signaling. siRNA transfection into NCI-H292 cells was carried out with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. NCI-H292 cells were seeded into six-well plates 1 day before transfection and co-transfected with 15 nM of each siRNA when the cells reached 30–50% confluence.

LENTIVIRUS SHRNA PREPARATION

The complete details of the pLL3.7 lentivirus vector construction are described online (http://web.mit.edu/jacks-lab/index.html). AP2α and mutant AP2α (negative control) shRNA target sequences were 5′-GGGTATTAACATCCCAGATCA-3′ and 5′-GG*A*TA*G*TA*C*CA*G*CC-*T*AGCTCC-3′ (italics represent mutations), respectively, which have no significant similarity to any other AP2 family mRNAs. 293T cells were seeded and cultured for 24 h until 80–90% confluent and co-transfected with pLL3.7 and packaging vectors. The resulting supernatant was collected after 48 h. The virus was recovered by ultracentrifugation for 5 h at 25,000 rpm in a Beckman SW28 rotor and resuspended in PBS (200 μl). Titers were determined by infecting 293T cells with serial dilutions of concentrated lentivirus. GFP expression of the infected cells was determined by flow cytometry 48 h after infection; the titer was approximately 5×10^5 viral particles/μl.

STATISTICAL ANALYSIS

Data are presented as means ± SD of at least three independent experiments. Where appropriate, statistical differences were assessed by Wilcoxon Mann–Whitney tests. A P value less than 0.05 was considered statistically significant.

RESULTS

AP2α EXPRESSION IN NORMAL NASAL MUCOSA AND NASAL POLYPS

Several lines of evidence revealed that AP2α plays a critical role in epithelial cell specific transcriptional regulation. A number of epithelial cells express AP2α protein, which is involved in epithelial gene expression [Hennig et al., 1996; Batsche et al., 1998; Anttila et al., 2000]. There have been no reports about the role of AP2α in nasal epithelial cells. To determine whether AP2α is a critical factor in nasal epithelial cells, we first examined AP2α expression in normal nasal mucosa and nasal polyp tissue by Western blot analysis. AP2α was overexpressed in all nasal polyps and weakly expressed in the normal nasal mucosa (Fig. 1A). The mean AP2α expression level was 21-fold higher in nasal polyp tissue than in normal nasal mucosa (Fig. 1B). We also investigated AP2α expression in normal nasal mucosa and nasal polyps by immunohistochemical analysis (Fig. 1C). AP2α was highly expressed throughout the epithelium of nasal polyp tissue and minimally expressed in normal nasal mucosa. These data show that AP2α may be one transcription factor activated in chronic sinusitis with nasal polyps.

PMA UPREGULATES *MUC8* GENE EXPRESSION IN NCI-H292 CELLS

PMA has been suggested as an inducer of *MUC2*, *MUC5AC*, and *MUC5B* gene expression. In this study, we investigated the effect of PMA on *MUC8* gene expression because PMA activates AP2α. NCI-H292 cells were treated with 5 to 50 ng/ml PMA for 24 h. PMA induced *MUC8* gene expression in a dose-dependent manner (Fig. 2A). Based on this observation, 50 ng/ml of PMA was used for all subsequent experiments. To determine whether PMA induced *MUC8* gene expression in a time-dependent manner, we examined *MUC8* gene expression at different time points (Fig. 2B). Stimulation with 50 ng/ml of PMA led to a significant increase in *MUC8* gene expression, with a peak 6–24 h after PMA stimulation.

AP2α IS INVOLVED IN PMA-INDUCED *MUC8* GENE EXPRESSION

We next evaluated AP2α mRNA expression in NCI-H292 cells stimulated with PMA for 10 min to 24 h. AP2α mRNA expression levels peaked at 1 h and decreased after 12 h (Fig. 3A). Western blot analysis showed a marked increase in AP2α protein levels after 1 h of treatment. Figure 3B shows a time-dependent AP2α increase, which returned to baseline level at 24 h.

To determine whether AP2α plays a role in *MUC8* gene expression, siRNA knocked down AP2α in cells. To do so cells were transiently transfected with AP2α siRNA or control siRNA. The effects of the siRNA were analyzed by Western blot analysis and RT-PCR. AP2α siRNA suppressed PMA-induced AP2α (Fig. 3C). Quantitative real-time PCR showed that AP2α siRNA also suppressed *MUC8* gene expression (Fig. 3D). This finding indicates that AP2α is required for PMA-induced *MUC8* gene expression.

INVOLVEMENT OF ERK1/2 MAPK IN PMA-INDUCED *MUC8* AND AP2α EXPRESSION

We determined which MAPK subfamily members are actively involved in PMA-induced *MUC8* gene expression. As shown in Figure 4A, ERK1/2 MAPK was maximally activated at 10 min and decreased after 20 min. PMA weakly activated both c-Jun N-terminal kinase (JNK) and p38 at 10 min. To examine the involvement of ERK1/2, JNK, and p38 in PMA-induced *MUC8* gene expression, cells were exposed to PD98059 (a MEK1 inhibitor), SP600125 (a JNK inhibitor), or SB-239063 (a p38 inhibitor) prior to PMA treatment. Pretreatment with PD98059 for 1 h clearly inhibited PMA-induced ERK1/2 MAPK and significantly suppressed PMA-induced *MUC8* gene expression in a dose-dependent manner

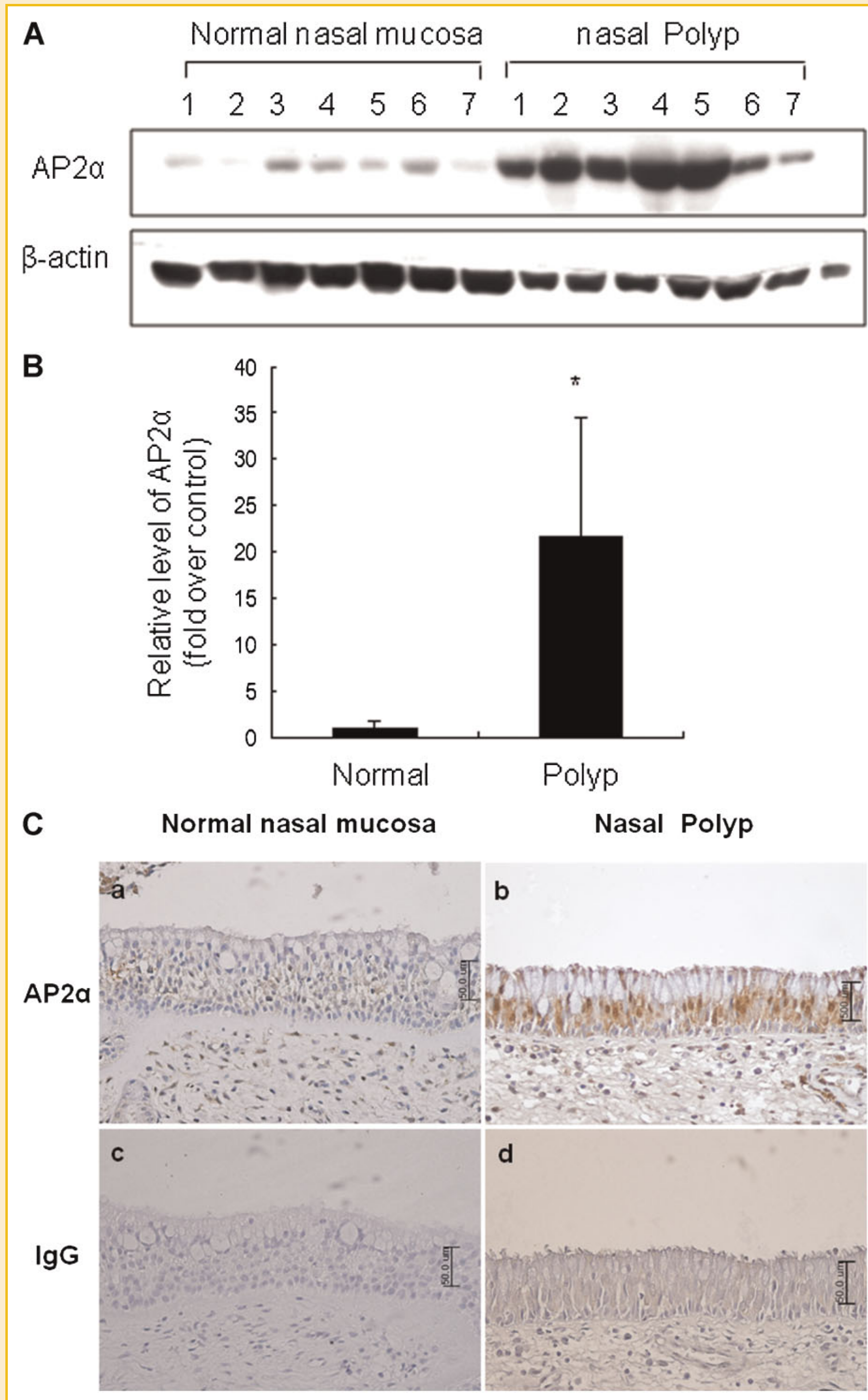

Fig. 1. AP2α expression in normal nasal mucosa and nasal polyps Normal nasal mucosa and nasal polyps were examined for AP2α expression. A Western blot of AP2α protein in normal nasal mucosa and nasal polyp tissue from seven patients is shown. Each lane contained β-actin as a loading control to normalize the AP2α protein levels (A). Graph of densitometric analysis. AP2α band intensities were normalized to β-actin. Mean ± SD AP2α expressions of all nasal polyp samples were normalized to β-actin and expressed as fold intensity over normal nasal mucosa (B). $^{*}P < 0.05$ compared to normal nasal mucosa. Immunohistochemical staining of AP2α expression in normal nasal mucosa and nasal polyps (C). Immunostaining of AP2α in normal nasal mucosa (a) and nasal polyp tissue (b). Immunostaining of IgG (negative control) in normal nasal mucosa (c) and nasal polyp tissue (d). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

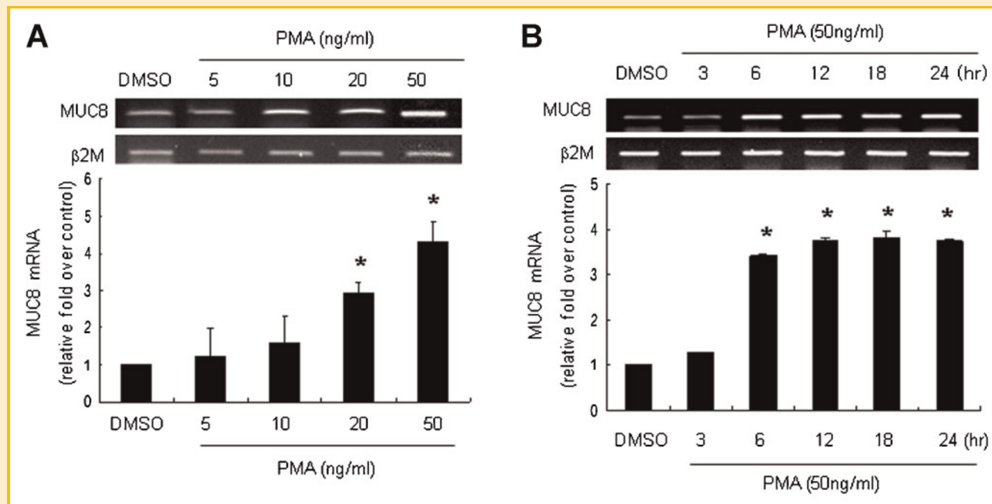

Fig. 2. Effects of PMA on *MUC8* gene expression NCI-H292 cells. Confluent cells were treated with increasing doses of PMA (5, 10, 20, and 50 ng/ml) for 24 h (A). Confluent cells were treated with PMA (50 ng/ml) for 3, 6, 12, 18, or 24 h (B). Total RNA was prepared and subjected to RT-PCR (upper panel) and quantitative real-time PCR (lower panel) with β2-M (beta2-microglobulin) as an internal control. $^{*}P < 0.05$ compared to control (DMSO). Data are presented as mean ± SD values of three independent experiments. Figures are representative of three independent experiments.

(Fig. 4B). In this study, pretreatment with 10 μM SP 600125 and 10 μM SB-239063 failed to inhibit PMA-induced *MUC8* gene expression (Fig. 4C). To further confirm the inhibitors results, cells were transiently transfected with ERK1, ERK2, p38, JNK or control siRNA. Western blot analysis showed that ERK1 and ERK2 siRNA constructs clearly inhibited ERK1 or ERK2 MAPK (Fig. 4D, upper panel) and p38 and JNK siRNA constructs also clearly inhibited p38 and JNK MAPK (Fig. 4E, upper panel). In addition, ERK1 or ERK2 siRNA significantly suppressed PMA-induced *MUC8* gene expression (Fig. 4D, lower panel). On the other side, p38 and JNK siRNA constructs not affected *MUC8* gene expression (Fig. 4E, lower panel). These results demonstrate that ERK1 or ERK2 MAPK is essential for PMA-induced *MUC8* gene expression in NCI-H292 cells.

To identify the molecules involved in the upstream signaling of ERK1/2 MAPK in PMA-induced *MUC8* gene expression, we investigated the role of protein kinase C (PKC) in initiating PMA-induced

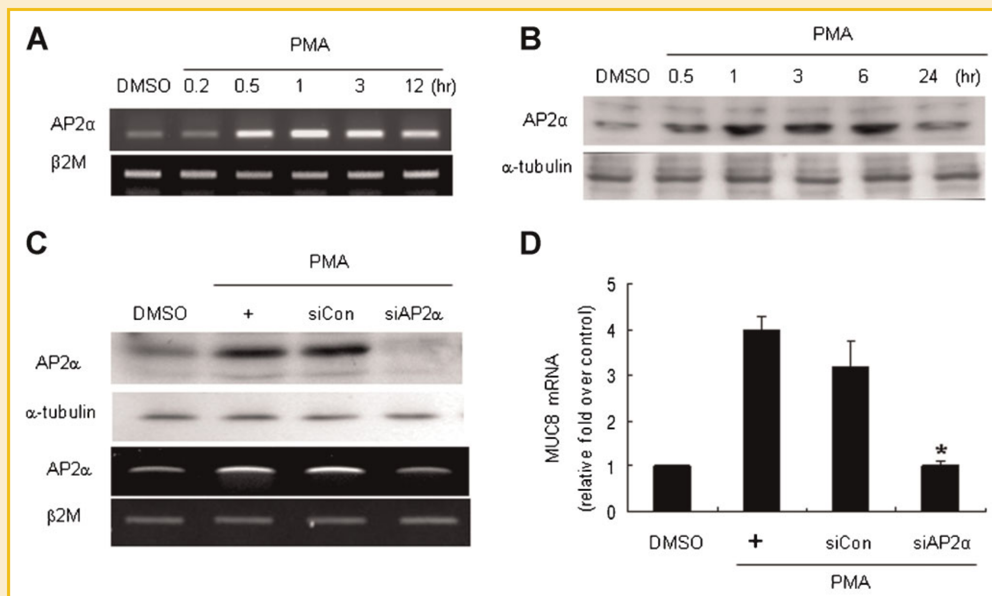

Fig. 3. AP2α involvement in PMA-mediated *MUC8* gene expression in NCI-H292 cells. Confluent cells were stimulated with PMA for the indicated times, and total cell lysates were collected for AP2α RT-PCR (A) and Western blot analysis (B). Cells were transiently transfected with either siAP2α or control siRNA. Twenty-four hours after transfection, cells were stimulated with PMA for 3 h (for Western blot analysis), 1 h (for RT-PCR), or 24 h (for quantitative real-time PCR) (C and D). Figures shown are representative of three independent experiments. $^{*}P < 0.05$ compared with PMA treatment only. Data are presented as mean ± SD values of three independent experiments.

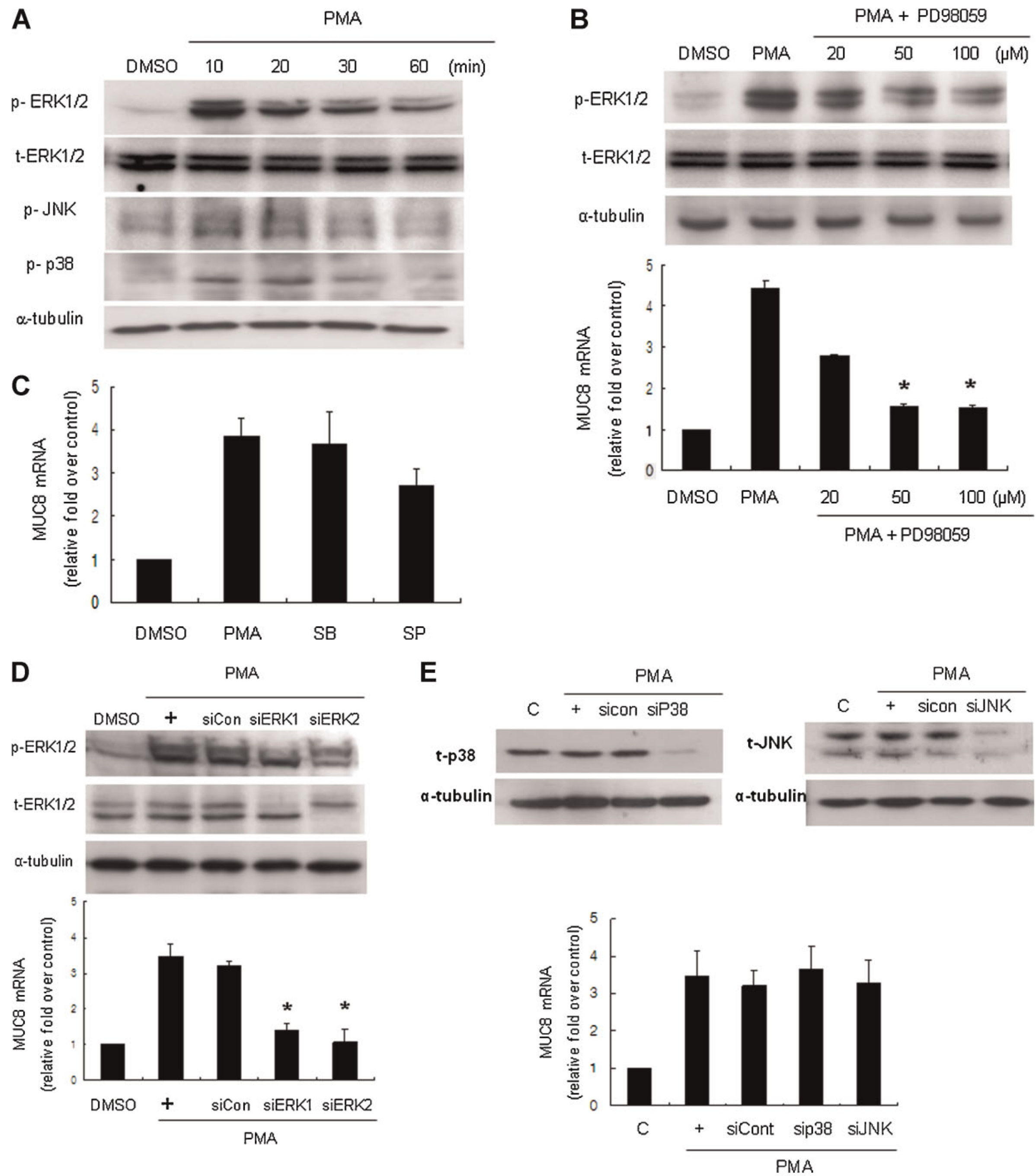

Fig. 4. ERK1/2 MAPK involvement in *MUC8* and AP2α induction by PMA. Confluent cells were treated with PMA for the indicated periods of time (10, 20, 30, or 60 min), and 30 μg/lane of total cell lysate protein was subjected to Western blot analysis. Blotted proteins were probed with antibodies against phosphorylated or non-phosphorylated MAP kinases (ERK1/2, JNK, and p38) and α-tubulin (control) as indicated (A). Confluent cells were pretreated for 1 h with PD98059. Pretreated cells were stimulated with PMA for 10 min to detect ERK1/2 phosphorylation (B, upper panel) or 24 h for *MUC8* quantitative real-time PCR (B, lower panel). After pretreatment with SB239063 and SP600125 for one hr, cells were stimulated with PMA for 24 h and *MUC8* gene expression was measured using quantitative real-time PCR (C). Cells were transiently transfected with siERK1, siERK2, or control siRNA (siCon) and stimulated with PMA for 10 min to detect ERK1/2 phosphorylation (D, upper panel) or 24 h for *MUC8* quantitative real-time PCR (D, lower panel). Cells were transiently transfected with siCon, sip38, and siJNK and stimulated with PMA for 10 min to detect total p38 and JNK (E, upper panel) or 24 h for *MUC8* quantitative real-time PCR (E, lower panel). Confluent cells were pretreated for 1 h with RO-31-8220 (10 μM) and then stimulated with PMA for 10 min to detect ERK1/2 phosphorylation (F, upper panel) or 24 h for *MUC8* quantitative real-time PCR (F, lower panel). Cells were transiently transfected with siCon or siPKC and stimulated with PMA for 10 min for Western blot analysis (G, upper panel) or 24 h for *MUC8* quantitative real-time PCR (G, lower panel). After pretreatment with PD98059 for 1 h, cells were stimulated with PMA for 3 h to detect AP2 α activation (H). Cells were transiently transfected with siERK1, siERK2, or siCon and stimulated with PMA for 3 h to detect AP2α activation (I). (Figures shown are representative of three independent experiments. $^{*}P < 0.05$ compared to PMA treatment only. Data are presented as mean ± SD values of three independent experiments.

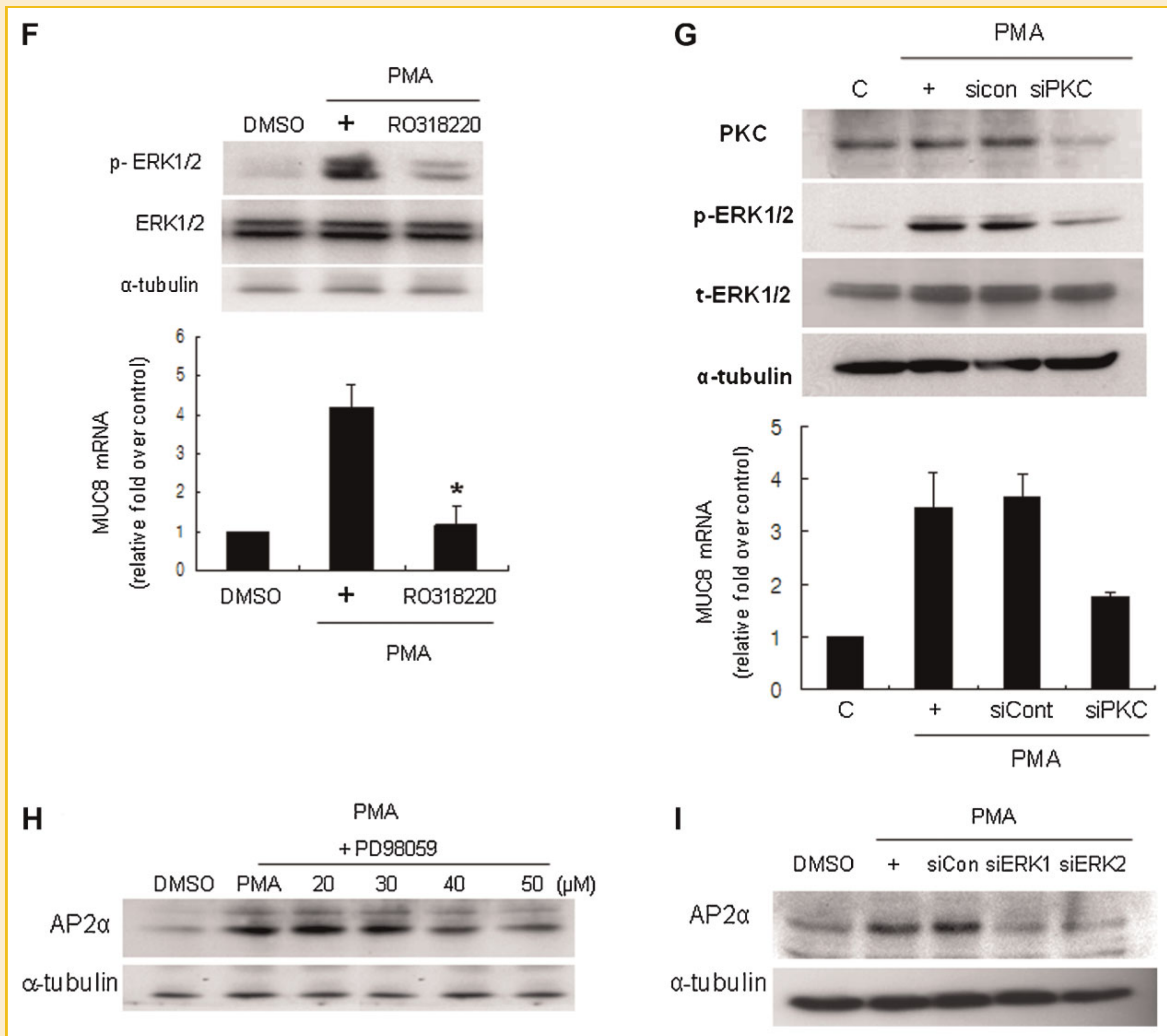

Fig. 4. (*Continued*)

MUC8 gene expression. PMA has been reported to induce PKC activation in several cell types and is known to regulate ERK1/2 activity [Nagle and Blumberg, 1980; Lee et al., 2002; Hewson et al., 2004]. To determine the role of PKC, NCI-H292 cells were pretreated with 10 μM RO-31-8220 (a PKC inhibitor) for 1 h before PMA stimulation, followed by Western blot analysis with anti-phospho-ERK1/2 antibody (Fig. 4F, upper panel) or quantitative real-time PCR for *MUC8* gene expression (Fig. 4F, lower panel). In this study, RO-31-8220 significantly inhibited PMA-induced ERK1/2 phosphorylation and *MUC8* gene expression. To further confirm the RO-31-8220 results, cells were transiently transfected with PKC or control siRNA. Western blot analysis showed that ERK1/2 phosphorylation was clearly inhibited by PKC siRNA (Fig. 4G, upper panel). In addition, PKC siRNA significantly suppressed PMA-induced *MUC8* gene expression (Fig. 4G, lower panel). These results show that PMA-induced ERK1/2 phosphorylation and *MUC8* gene expression occur via PKC activation in NCI-H292 cells.

Furthermore, cells treated with either PD98059 or ERK 1- or ERK2-specific siRNA showed significantly suppressed PMA-induced AP2α expression. As shown in Figure 4H, PD98059 reduced PMA-induced AP2α protein in a dose-dependent manner. Twenty-four hours after siRNA transfection, cells were stimulated with PMA for 3 h and harvested for RNA. Transfection with ERK1 or ERK2 siRNA, but not control siRNA, suppressed AP2α mRNA expression (Fig. 4I). These results indicate that ERK1 or ERK2 is responsible for AP2α activation and *MUC8* gene expression in response to PMA.

AP2α OVEREXPRESSION UPREGULATES *MUC8* GENE EXPRESSION IN NCI-H292 CELLS

To examine the function of AP2α in *MUC8* gene expression, we used wild-type AP2α or mutant AP2α (dominant negative, N-terminal deletion) expression vectors in NCI-H292 cells. Efficient transfection

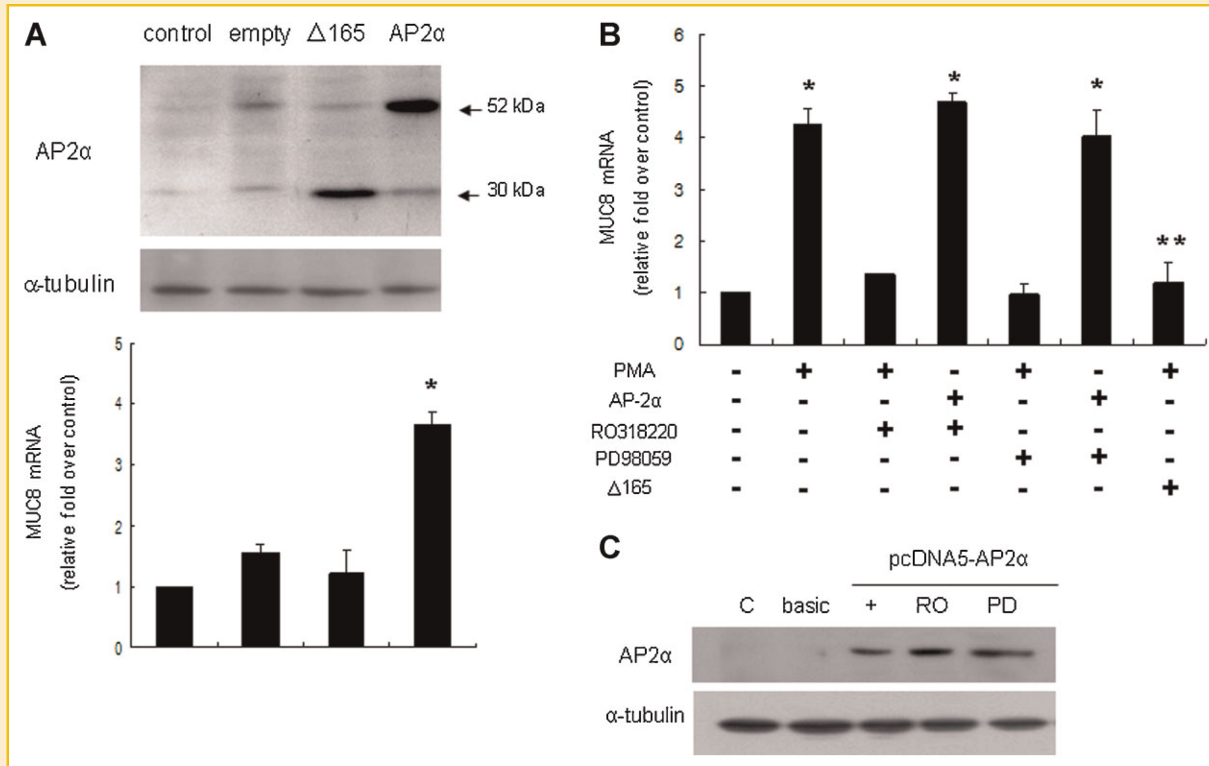

Fig. 5. AP2α overexpression upregulates *MUC8* gene expression in NCI-H292 cells. Confluent cells were transiently transfected with SPRSV-NN (empty), wild-type, or mutant (Δ165, N terminal 165 aa deletion) AP2α expression vectors. Twenty-four hours after transfection, total cell lysates were collected, and Western blot analysis of AP2α was performed (A, upper panel), or total RNA was prepared, and *MUC8* gene expression was measured using quantitative real-time PCR (A, lower panel). Twenty-four hours after transfection, cells were pretreated with RO318200 and PD98059 for 1 h and stimulated with PMA for quantitative real-time PCR (B) or without PMA for Western blot analysis (C). Figures shown are representative of three independent experiments. $^{*}P < 0.05$ compared to control. $^{**}P < 0.05$ compared to PMA treatment only. Data are presented as mean ± SD values of three independent experiments.

of constitutively active AP2α (efficient AP2α expression) was confirmed by detecting wild-type (52 kDa) and mutant (30 kDa) AP2α proteins (Fig. 5A, upper panel). Overexpressing the wild-type AP2α gene affected *MUC8* mRNA expression as measured by quantitative real-time PCR, suggesting that AP2α activity is critical for *MUC8* gene expression (Fig. 5A, lower panel). Next, we evaluated *MUC8* gene expression induced by wild type AP2α under the influence of the inhibitors RO318200 and PD98059. Twenty-four hours after transfection with wild-type AP2α, NCI-H292 cells were pre-treated with inhibitor for 1 h and stimulated with PMA. RO318200 and PD98059 inhibited PMA-induced *MUC8* gene expression, but not in wild-type AP2α transfected cells (Fig. 5B). We also determined the effect of the AP2α dominant negative mutant on *MUC8* gene expression in PMA-treated NCI-H292 cells. The mutant-type AP2α vector has a DNA binding/dimerization domain but no transcriptional activation domain [Williams and Tjian, 1991]. Overexpressing mutant AP2α decreased *MUC8* gene expression (Fig. 5B). Pretreatment of RO318200 and PD98059 did not affect transgenes expression from AP2α vector (Fig. 5C). These findings indicate that transfected AP2α remains active downstream of the PKC/ERK1/2 pathway and influences *MUC8* gene expression. Taken together, these results demonstrate the key roles of AP2α in *MUC8* transcription.

EFFECTS OF PMA ON *MUC8* GENE EXPRESSION IN HUMAN NASAL POLYP EPITHELIAL CELLS

We examined *MUC8* induction by PMA in human nasal polyp epithelial cells. Cells were treated with 2–50 ng/ml PMA for 12 h. *MUC8* gene expression gradually increased and plateaued at 10 ng/ml, giving a 4.3-fold increase (Fig. 6A). Based on this observation, 10 ng/ml of PMA was used for all subsequent experiments in cells. In addition, to determine whether PMA induced *MUC8* gene expression in a time-dependent manner, we examined *MUC8* gene expression at different time points (Fig. 6B). Stimulation with 10 ng/ml of PMA induced *MUC8* in a time-dependent manner. Taken together, these results suggest that human nasal polyp epithelial cells are more sensitive to PMA induction of *MUC8* gene expression than are NCI-H292 cells. Pretreatment with RO-31-8220 or PD98059 inhibited ERK1/2 phosphorylation and PMA-induced *MUC8* gene expression (Fig. 6C, upper and lower panels), implicating PKC and ERK1/2 as important signaling molecules in PMA-induced *MUC8* gene expression, even in human polyp airway epithelial cells.

EFFICIENTLY SILENCING ENDOGENOUS AP2α BLOCKS *MUC8* GENE EXPRESSION IN HUMAN NASAL POLYP EPITHELIAL CELLS

To assess the role of AP2α in *MUC8* gene expression in human nasal polyp epithelial cells, we used lentivirus shRNA to delete AP2α.

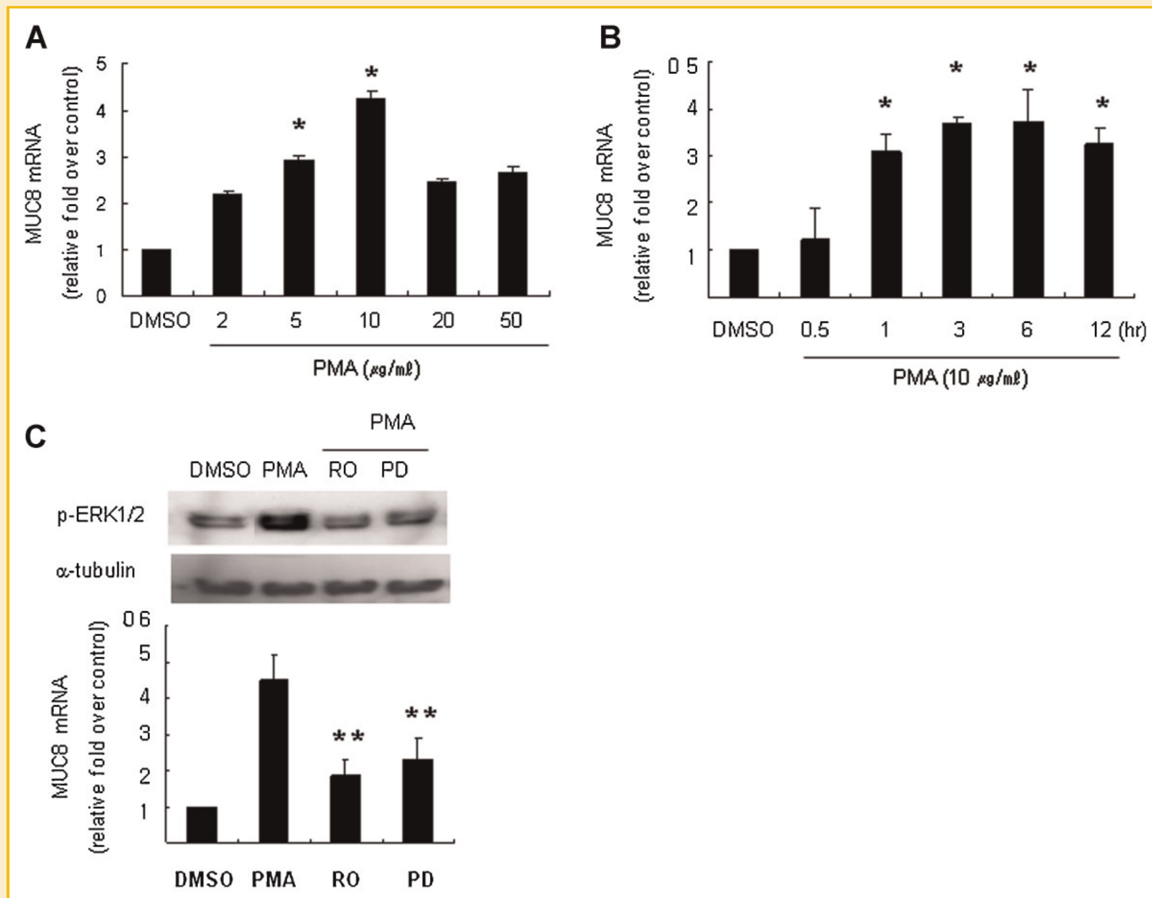

Fig. 6. Effects of PMA on *MUC8* gene expression in Human nasal polyp epithelial cells. Cells were treated with increasing doses of PMA (2, 5, 10, 20, and 50 ng/ml) for 12 h (A). Cells were treated with PMA (10 ng/ml) for 0.5, 1, 3, 6, or 12 h (B). Total RNA was prepared and subjected to quantitative real-time PCR. Cells were pretreated with RO-31-8220 (10 μM) or PD98059 for 1 h and then stimulated for 10 min with PMA prior to collecting total cell lysates for Western blot analysis using anti-phospho-ERK1/2 antibody (C, upper panel). Inhibitor-pretreated cells were stimulated for 12 h with PMA prior to preparing total RNA and measuring *MUC8* gene expression using quantitative real-time PCR (C, lower panel). Figures shown are representative of three independent experiments. $^{*}P < 0.05$ compared to control (DMSO). $^{**}P < 0.05$ compared to PMA treatment only. Data are presented as mean ± SD values of three independent experiments.

Because the AP2α siRNA sequences were effective in NCI-H292 cells, we used the partially overlapping siRNA sequences to make the lentivirus shRNA oligonucleotide. Cells were infected with shAP2α lentivirus or control shRNA (mutant shAP2α) lentivirus 1 day before confluence. We determined that transgene expression could be maintained throughout cell differentiation. The vector co-expresses green fluorescent protein (GFP) as a reporter gene. As shown in Figure 7A, 7 days after reaching confluence, GFP expression was readily detected in both wild-type and mutant shAP2α-infected cells. We confirmed that shAP2α significantly suppressed endogenous AP2α expression by Western blot analysis, whereas control shRNA did not affect endogenous AP2α expression (Fig. 7B, upper panel). We next examined whether AP2α knock-down affects *MUC8* gene expression in cells. Seven days after reaching confluence, shRNA-infected cells were prepared for quantitative real-time PCR. shAP2α markedly reduced endogenous *MUC8* gene expression (Fig. 7B, lower panel). These results suggest that endogenous AP2α is essential to *MUC8* expression.

DISCUSSION

Previous studies have suggested that *MUC8* is an important mucin gene because inflammatory mediators upregulate *MUC8* gene expression in vitro, and human nasal polyp epithelia upregulate *MUC8* gene expression. Extensive work has shown that *MUC8* may play an important role in mucus hypersecretion pathogenesis in polyp epithelium. Lopez-Ferrer et al. report that human bronchial epithelium overexpress *MUC8* protein [Lopez-Ferrer et al., 2001]. Moreover, Martinez-Anton et al. [2006] indicate that both nasal polyp epithelia and submucosal glands highly express *MUC8* proteins. At the same time, the molecular mechanism of *MUC8* gene overexpression in nasal polyp epithelium remains poorly understood. In this study, we observed AP2α expression in nasal polyp tissue and in normal nasal mucosa. Since AP2α levels were much greater in nasal polyp epithelium, we sought to ascertain whether increased AP2α might lead to *MUC8* gene expression. We found that PMA increased *MUC8* expression in NCI-H292 cells by activating

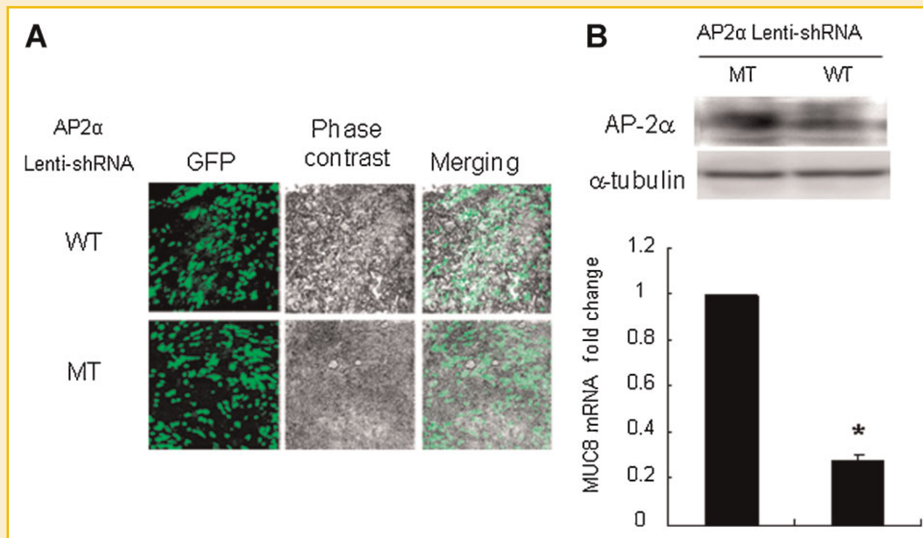

Fig. 7. Lentivirus-mediated shAP2α efficiently blocks *MUC8* gene expression in Human nasal polyp epithelial cells. Lentivirus provides efficient delivery of exogenous genes into Cells. Cells were infected with wild-type AP2α lentivirus or mutant shRNA lentivirus. Seven days after confluence, GFP expression and phase contrast images of the same area were taken. Cells were lysed for AP2α Western blot analysis (A), or total RNA was prepared for real-time PCR (B). $^{*}P < 0.05$ compared to mutant shRNA lentivirus. Data are presented as mean ± SD of three independent experiments. The figures are representative of three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

AP2α. In addition, we showed that PMA increased AP2α mRNA and protein in a dose- and time-dependent manner, although several studies have suggested that phorbol esters and cyclic AMP induce AP2α activity independent of protein synthesis [Imagawa et al., 1987]. In contrast, retinoic acid induces AP2α activity by increasing AP2α mRNA levels in human teratocarcinoma cells [Luscher et al., 1989; Buettner et al., 1993]. These different patterns of AP2α activity may depend on cell type- and tissue-specific PMA regulation. Further work is necessary to explore these regulatory mechanisms. Specific AP2α knock-down with siRNA significantly reduced PMA-induced *MUC8* gene expression (Fig. 3C), showing that AP2α activation is required to induce *MUC8* gene expression in human airway epithelial cells.

Next, we tried to identify the molecules involving in *MUC8* and AP2α expression after PMA treatment. PKC-mediated activation of the MEK/ERK MAPK signaling pathway and its role in regulating mucin gene expression have been studied [Nagle and Blumberg, 1980; Lee et al., 2002; Chang et al., 2005; Woo et al., 2005], but the exact order of events leading to AP2α activation has not been fully elucidated. To identify which MAP kinase pathways were involved in *MUC8* and AP2α expression, various kinase inhibitors were tested for their ability to reduce *MUC8* and AP2α transcription. Only ERK1/2 MAPK activation was required for PMA-induced *MUC8* and AP2α gene expression, although p-38 and JNK MAPK were weakly phosphorylated by PMA (Fig. 4A). Specific ERK1 and ERK2 knock-downs with siRNA completely blocked PMA-induced *MUC8* and AP2α gene expression, compared to the control, showing that ERK1/2 MAPK activation is required to induce AP2α activation and *MUC8* gene expression in NCI-H292 cells.

To confirm AP2α involvement in mediating the effects of PMA, we transfected cells with a dominant-negative AP2α, which significantly inhibited PMA-induced *MUC8* gene expression. In addition, overexpressing wild-type AP2α significantly increased *MUC8* gene expression (Fig. 5). These findings indicate that AP2α regulates *MUC8* gene expression.

We also tested the effect of PMA on *MUC8* gene expression in human nasal polyp epithelial cells (Fig. 6). This study showed that both human nasal polyp epithelial cells and NCI-H292 cells employ the same mechanisms for PMA-induced *MUC8* gene expression.

To date, a positive influence of AP2α has been reported in mammary epithelial cells. A number of studies have reported that AP2α has a tumor suppressor activity, and its activity is of prognostic relevance in invasive breast cancer. A loss of AP2α expression may be associated with malignant transformation and tumor progression in malignant melanoma [Jean et al., 1998; Gee et al., 1999; Bar-Eli, 2001; Tellez et al., 2003]. In addition, re-expressing AP2α in breast cancer and metastatic melanoma cells results in enhanced chemosensitivity, decreased tumorigenicity, and inhibited metastatic potential [Kim et al., 2000; Wajapeyee et al., 2005]. Although much is known about the positive regulation of AP2α genes, this study demonstrated that AP2α transcription activity was required for *MUC8* gene expression, suggesting that AP2α may induce inflammation in airway epithelium.

Furthermore, to examine whether AP2α mediates the transcription of endogenous *MUC8* in nasal polyp epithelial cells, we used lentivirus shRNA to delete AP2α from nasal polyp epithelial cells. This method is highly efficient because the lentivirus integrates into the genome [Ershler et al., 2003; Rubinson et al., 2003], allowing permanent AP2α shRNA expression, leading to long-term AP2α protein knock-down. After shRNA-mediated AP2α knockdown, *MUC8* gene expression was significantly lowered in nasal polyp epithelial cells.

Taken together, our results demonstrated that PMA induces PKC activation and ERK1/2 MAPK phosphorylation which are essential

for PMA-induced *MUC8* gene expression. The activation of AP2α is required to mediate *MUC8* gene expression. Especially, human nasal polyp epithelial cells over-express AP2α, playing an important role in *MUC8* gene expression. From these results, we conclude that AP2α plays a crucial role in *MUC8* gene expression in human airway epithelial cells. These results suggest that AP2α may be one of the major molecule activated in polyp epithelium and that they may play an important role in the pathogenesis of *MUC8* hypersecretion in chronic sinusitis with polyps. Further detailed analysis of the mechanisms of AP2α regulation in *MUC8* expression may yield deeper insights into nasal polyposis and mucus hypersecretion in chronic sisusitis and lead to therapeutic opportunities for treating chronic sinusitis.

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