MUC8 gene expression is overexpressed in nasal polypl epithelium and is also increased by treatment with inflammatory mediators in nasal epithelial cells. These data suggest that MUC8 may be one of important mucin genes expressed in human airway. However, the mechanisms of various inflammatory mediator-induced MUC8 gene expression in normal nasal epithelial cells remain unclear. We examined the mechanism by which prostaglandin E2 (PGE2), an arachidonic acid metabolite, increases MUC8 gene expression levels. Here, we show that ERK mitogen-activated protein kinase is essential for PGE2-induced MUC8 gene expression in normal human nasal epithelial cells and that p90 ribosomal S6 protein kinase 1 (RSK1) mediates the PGE2-induced phosphorylation of cAMP-response element binding protein. Our results also indicate that cAMP-response element at the −803 region of the MUC8 promoter is an important site of PGE2-induced MUC8 gene expression. In conclusion, this study gives insights into the molecular mechanism of PGE2-induced MUC8 gene expression in human airway epithelial cells.

Mechanical proteins produced by airway epithelial cells are essential components of airway mucus, which plays an important role in the protection of the airways from bacterial and viral attack. Mucins are highly glycosylated, high molecular weight glycoproteins and major components of the mucus produced by respiratory tract epithelia. In the airway, virtually all forms of airway inflammation are associated with the overproduction of mucus, which can lead to airway obstruction (1).

Twenty mucin genes have been identified; however, it is not known which mucins are secreted in the various airway diseases. Thus, in terms of treatment for mucin hypersecretion, it is crucial that we identify the genes responsible for high viscosity mucin in airway diseases and develop means of reducing mucin production. The mucins are usually subdivided into two groups based on domain, namely, the membrane-bound and secreted mucins. Specifically, MUC2 (2), MUC5AC (3), MUC5B (4), MUC6 (5), MUC7 (6), MUC9 (7), and MUC19 (8) are secreted mucins, and MUC1 (9), MUC3 (10), MUC4 (11), MUC11 (12), MUC12 (12), MUC13 (13), MUC17 (14), MUC18 (15), and MUC20 (16) are membrane-bound mucins. However, the other mucin genes including MUC8 (17) have not been fully characterized.

MUC8 may be an important airway mucin because its mRNA levels are up-regulated in chronic sinusitis with polyps (18) and by inflammatory mediators (19). In addition, because MUC8 protein is expressed in ciliated cells (20), it may be related to the differentiation or function of ciliated cells in airway epithelial cells. However, because only short partial sequences of the MUC8 gene have been reported, molecular studies have been limited. In a previous study, we completely sequenced MUC8 cDNA (GenBank™ accession number BK005559) and found that MUC8 protein contains secreted and membrane-bound mucin domains using the SMART program.

Prostaglandins are arachidonic acid metabolites with a wide range of biological actions. Moreover, it is known that cyclooxygenase converts arachidonic acid to prostaglandin H2, which is further metabolized to various prostaglandins and thromboxanes (22). These species are produced in a wide variety of tissues and function as lipid mediators. In particular, PGE2 mediates IL-1β-induced MUC5AC gene expression in human airway epithelium (23). Because mucin hypersecretion is a hallmark of airway inflammation, it is important that we determine how PGE2 regulates airway mucin gene expression.

In the present study, we examined the mechanism by which PGE2 increases MUC8 gene expression levels. We found that extracellular signal-regulated kinase mitogen-activated protein kinase (ERK MAPK) is essential for PGE2-induced MUC8 gene expression in normal human nasal epithelial (NHNE) cells. We also found that p90 ribosomal S6 protein kinase 1 (RSK1) mediates the PGE2-induced phosphorylation of cAMP
response element binding protein (CREB). In addition, transcriptional activities of cloned MUC8 promoter regions showed that CRE in MUC8 promoter is an important site of PGE2-induced MUC8 gene expression. Taken together, these studies provide insights into the mechanism of PGE2-induced MUC8 gene expression and extend our understanding of mucin gene overexpression during airway mucosal inflammation.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Epithelia were isolated by scrapings from the inferior turbinates of healthy adult volunteers. None of volunteers had any history of allergic symptoms, nasal polyps, or asthma. They did not have a history of smoking and did not take any medicines for the past 6 months. The volunteers' permission and the approval from the Institutional Review Board at Yonsei University College of Medicine were obtained for the use of the specimens. The epithelial cells from the turbinate tissues were treated with 1% Pronase (Type XIV protease, Sigma-Aldrich Chemical Co., St. Louis, MO) for 18 to 20 h at 4 °C. To remove fibroblasts, endothelial cells, and myoepithelial cells, isolated cells were placed in a plastic dish and cultured for 1 h at 37 °C. Isolated epithelial clusters were divided into single cells by incubating them with 0.25% trypsin/EDTA. Passage-2, NHNE cells were seeded in 0.5 ml of culture medium (24.5 mM, 0.45-μm pore size Transwell clear [Corning] culture inserts. Cells were cultured in a 1:1 mixture of bronchial epithelial cell growth medium (Clonetics):Dulbecco's modified Eagle's medium (Invitrogen) containing all supplements described previously (24). Cultures were grown submerged, and culture medium was changed on the first day and every other day thereafter. The human lung mucoperiosteal carcinoma cell line, NCI-H292, was purchased from the American Type Culture Collection (catalog no. CRL-1848, Manassas, VA) and cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) in the presence of penicillin-streptomycin at 37 °C in a 5% CO2 humidified chamber. We obtained A549 cell from ATCC (catalog no. CCL-185, Manassas, VA).

**Inhibitors, Antibodies, and Dominant Negative Vector**—PD98059 and anti-α-tubulin antibody were purchased from Calbiochem, and anti-phospho-ERK1/2 MAP kinase (Thr-202/Tyr-204) antibody, anti-phospho-p38 MAP kinase (Thr-180/Tyr-182) antibody, and anti-phospho-SAPK/c-Jun NH2-terminal kinase MAP kinase antibody [Thr-183/Tyr-185] antibody, anti-phospho-RSK1 (Ser-380) antibody, and anti-phospho-CREB (Ser-133) antibody were purchased from Cell Signaling. Plasmid encoding kinase deficient MEK1 mutant (pcDNA5-MEK1DN) was kindly provided by Dr. Jian-Dong Li (House Ear Institute, Los Angeles, CA).

**Real-time Quantitative PCR**—Primers and probes were designed using PerkinElmer Life Sciences Primer Express® software purchased from PE Biosystems. Commercial reagents (TaqMan PCR Universal PCR master mix, PE Biosystems) and conditions were applied according to the manufacturer's protocol. 1 μg of cDNA (reverse transcription mixture), oligonucleotides at a final concentration of 500 nM of primers, and 200 nM TaqMan hybridization probe were used in a 25-μl volume. The probe of real-time PCR was labeled with carboxylfluorescein at the 5′ end. The following primers and probes were used: MUC8, forward (5′-TAACCCAATGGCACTTCTTC-3′) and reverse (5′-GGAGTTAGGGCTGACCACAGAA-carboxytetramethylrhodamine). For electromobility shift assay, oligonucleotides corresponding to the consensus CRE sequence (5′-AGAGATGGTCGAAGCTGAGG-3′) and the MUC8 CRE sequence (5′-AACAGATACACTACATGGCACTTGGCCCG-3′) were synthesized, annealed, and end-labeled with γ-[32P]ATP using T4-polymerase (Promega). Nuclear extract was incubated at room temperature for 30 min with the 32P-labeled CRE probes in binding buffer (Promega). Oligo-nuclear protein complexes were separated from the probes by electrophoresis through 5% native polyacrylamide gel in 0.5x Tris borate-EDTA (TBE) buffer. Supershift experiment was conducted using 2 μl of anti-phospho-CREB antibody (Cell Signaling). The gel was dried and autoradiographed on a PhosphorImager.

**Transient Transfection and Luciferase Analysis**—The constructs of deleted promoter and point-mutated CRE site of MUC8 gene were reported in our previous study.1 NCI-H292 cells were transiently transfected with pGL3-basic, pGL3-MUC8 (+1644/+ 87), pGL3-MUC8 (-1190/+ 87), pGL3-MUC8 (-973/+ 87), pGL3-MUC8 (-549/+ 87), pGL3-MUC8 CREM1, and pGL3-MUC8 CREM2 constructs using a FuGENE6 Transfection Reagent (Roche Applied Science) according to the manufacturer's instructions, incubated for 48 h, treated with 10 nM of PGE2 for 24 h, harvested, and assayed for luciferase activity using a luciferase assay system (Promega) according to the manufacturer's instructions. β-Galactosidase activity was also assayed to standardize sample transfection efficiencies. To confirm that the luciferase activity of each construct was from PGE2, we assayed the activity of each construct in the absence of PGE2.

**RESULTS**

PGE2 Can Induce MUC8 Gene Expression—To determine whether PGE2 can induce MUC8 gene expression within NHNE cells, we carried out reverse transcription-PCR after treating cells with various concentrations of PGE2. As the dose of PGE2 was increased from 1 to 1000 nM, MUC8 gene expression was observed to gradually increase from 10 nM. As shown in Fig. 1A, 10 nM PGE2 significantly induced MUC8 gene expression. However, no corresponding change was found in
PGE2 Induces MUC8 Gene Expression via ERK MAP Kinase Signaling—To investigate which MAP kinase signal pathway is activated in NHNE cells, we performed Western blot analysis using phosphospecific antibodies. ERK MAP kinase was maximally activated at 5 min, and this effect decreased after 20 min (Fig. 2A). However, no change was detected in the activations of phospho-p38 and c-Jun NH2-terminal kinase. IL-1β-treated NCI-H292 cells (25) and A549 cells (26) were used as positive controls for p38 and c-Jun NH2-terminal kinase, and the maximum effect is at 5 min (A). Confluent cells were pretreated for 1 h with 20 μM PD98059 and then stimulated for 24 h with PGE2 prior to collection of total RNA for real-time quantitative PCR. Pretreated cells were stimulated for 5 min with PGE2 prior to collection of cell lysates for Western blot analysis (B). The cells were transiently transfected with MEK1-dominant negative (MEK1-DN) construct and stimulated with PGE2 for 24 h prior to real-time quantitative PCR. The figures shown are representative of three independent experiments. Transfected cells were stimulated with PGE2 for 5 min prior to Western blot analysis (C).

Effect of RSK1 on PGE2-induced MUC8 Gene Expression—To identify the molecules involved in the downstream signaling of ERK MAP kinase in PGE2-induced MUC8 gene expression, we investigated RSK1 and MSK1. RSK1 has been reported to be activated by ERK MAP kinase in IL-1β-induced MUC8 gene expression (27). In this study, the overexpression of MEK1 DN was found to suppress PGE2-induced RSK1 activity (Fig. 2C). This result shows that PGE2-induced RSK1 activation is via MEK1/ERK MAP kinase. And in NHNE cells, MSK1 was found to be activated by ERK MAP kinase in IL-1β-induced MUC8 gene expression (28). However, MSK1 did not affect PGE2-induced MUC8 gene expression (data not shown). The phosphorylation of RSK1 by PGE2 peaked at 5 min and then decreased at 20 min (Fig. 3A). To determine whether RSK1 plays an important role in PGE2-induced MUC8 gene expression, we performed an RSK1 mutant study; RSK1 is a candidate mediator of cytokine-induced CREB phosphorylation at Ser-133. Overexpression of RSK1 DN (D205N, a dominant negative construct encoding RSK1 protein with an Asp-205 phosphorylation site mutated to Asn-205) suppressed PGE2-induced CREB and RSK1 phosphorylation (Fig. 3B), and PGE2-induced MUC8 gene expression was significantly suppressed by RSK1 DN. These results show that RSK1 is required for PGE2-induced MUC8 gene expression.

Effect of CREB on PGE2-induced MUC8 Gene Expression—To determine whether CREB plays a role in MUC8 gene expression, we performed Western blot analysis using anti phospho-CREB antibody. The phosphorylation of CREB by PGE2 peaked at 10 min and then decreased at 60 min (Fig. 4A). Furthermore, PGE2-induced MUC8 gene expression was significantly suppressed in cells transfected with plasmid encoding the expression of the internal control, β 2-microglobulin. To determine whether PGE2 induced MUC8 gene expression in a time-dependent manner, we examined MUC8 expression after various exposures to 10 nM PGE2 (Fig. 1B). MUC8 gene expression was found to be significantly increased after 24 h of exposure to PGE2. These results show that MUC8 gene expression was significantly elevated by PGE2 treatment in NHNE cells. Ten nM PGE2 was used in subsequent experiments.
ing CREB DN (S133A, a dominant negative construct encoding CREB protein with its Ser-133 phosphorylation site mutated to Ala-133 (Fig. 4B)). These findings suggest that the activation of CREB is essential for PGE2-induced MUC8 gene expression via ERK MAP kinase and RSK1.

Identification of the CREB-CRE binding complex formed in response to PGE2—In a previous study, we cloned MUC8 promoter (encompassing -1644 to +87) to pGL3-basic, a luciferase reporter vector. The CRE site is located at -803 of the MUC8 promoter. To analyze the DNA binding activity of PGE2-activated CREB, we performed an electromobility shift assay using the nuclear extracts of PGE2 treated NCI-H292 cells. As shown in Fig. 5, the activities of consensus CRE (CREc) and MUC8 specific CRE (CREs) oligonucleotides remarkably increased in response to PGE2. To identify specific CRE binding complexes, competition and supershift analysis were performed using 50-, and 100-fold excesses of non-radio-labeled (cold) CREc oligonucleotide and anti-phospho-CREB antibody, respectively. The specific band was found to be selectively inhibited by a consensus CRE competitor and to be supershifted by anti-phospho-CREB antibody (Fig. 5). These results indicate that activated CREB binds to a cis-acting element, CRE, in the MUC8 promoter.

CRE Is Required for PGE2-induced MUC8 Transcription—Various deletion clones such as -1190 to +87, -973 to +87, and -549 to +87 were constructed based on the above -1644 to +87 clone. NCI-H292 cells were then transiently transfected with the various deletion mutants and treated with PGE2 (10 nM) for 24 h, respectively. As shown in Fig. 6A, PGE2 selectively increased the luciferase activity of the -973 to -549 region of the MUC8 promoter. However, its effect was reduced on fragments covering the -549 to +87 region, indicating that
MAP kinase activation is required for PGE2-induced MUC8 gene expression (Fig. 2A), although several studies (29–31) have suggested that more than one MAP kinase is necessary for the signal transduction of various inflammatory mediators. Moreover, the activation of ERK MAP kinase by various stimulants mainly occurs through MEK1. Thus, in this study we investigated whether ERK MAP kinase activation by PGE2 is MEK1-dependent or -independent. The overexpression of MEK1 mutant significantly reduced PGE2-induced ERK MAP kinase phosphorylation and MUC8 gene expression (Fig. 2B), showing that the ERK MAP kinase activation required to induce MUC8 gene expression in human airway epithelial cells occurs via an MEK1-dependent pathway. However, the dominant negative effect of MEK1 did not completely block the MUC8 gene expression as compared with control. This result can be explained by two possibilities. One is the low transfection rate and the other is that PGE2 stimulation may affect MUC8 gene expression not only through the MAP kinase pathway but also through other pathways, such as G-protein-activated pathway (32) and phospholipase C pathway (33).

To date, the signal molecules involved in the downstream signaling of ERK MAP kinase for PGE2-induced MUC8 gene expression have not been identified. However, recently it was reported that IL-1β-induced MUC8 gene expression is mediated by sequential ERK MAPK/RSK1/CREB activation in airway epithelial cells (27). In the present study, we found that RSK1 and CREB are also important downstream molecules of ERK MAP kinase activation in PGE2-induced MUC8 gene expression (Figs. 3 and 4). MSK1 and RSK1 are known substrates of CREB in NHNE cells (27, 28). However, MSK1 did not affect PGE2-induced MUC8 gene expression (data not shown). On the other hand, RSK1 phosphorylates several transcription factors, e.g., CREB (34), c-Fos (35), CCAAT/enhancer binding protein (36), nuclear factor-κB (37), and the estrogen receptor (38), and interacts with transcriptional coactivator CREB-binding protein (also known as p300) (39). Moreover, PGE2 receptors, such as EP1–4, are coupled to cAMP upregulation (40), which implies CREB activation. Also, CREB is a known potent regulator of the expression of mucin genes (MUC2, MUC5AC, MUC5B, and MUC6) in the p15 arm of chromosome 11 (11p15) (41). In a previous study, we reported that CREB was involved in IL-1β-induced MUC8 gene expression (27), but at that time because the MUC8 promoter sequence was not known we could not determine whether CREB binds to a MUC8 specific promoter. However, we already cloned the MUC8 promoter region.1 In the present study, our results show that the −973 region of the MUC8 promoter is involved in response to PGE2 and that CRE in the −803 region of the MUC8 promoter is important for MUC8 gene up-regulation by PGE2. Gerritsen et al. (42) reported that p800 and CREB-binding protein act as co-activators of p65 transactivation and may play an important role in the cytokine-induced expression of various immune and inflammation genes. These findings suggest that CREB may interact directly or indirectly with other transcription factor(s) and that non-DNA binding transcriptional co-activators, such as p300 and CREB-binding protein, which were believed to function as bridging proteins between DNA-binding transcription factors and basal transcription factors, play a role as integrators of diverse signaling pathways leading to MUC8 gene expression.

Thus, to induce MUC8 gene expression, IL-1β transduces through the IL-1β receptor/Ras/Raf/ERK/RSK1/CREB cascade pathway (27), and PGE2 transduces through the EP1–4/MEK1/ERK/RSK1/CREB cascade pathway. Although these two substances stimulate their own membrane receptors, they seem to
MUC5AC gene by IL-1 regulated exocytosis. Gray et al. (23) reported that the induction of MUC5AC gene by IL-1β involves COX2-generated PGE2. From these reports, we cannot exclude the possibility that IL-1β stimulates PGE2 secretion and that secreted PGE2 induces MUC8 gene expression in an autocrine manner.

In summary, our results demonstrate that ERK MAP kinase is essential for PGE2-induced MUC8 gene expression and that the activations of RSK1 and CREB are required for the intracellular mechanisms that mediate MUC8 gene expression. This study also demonstrated that CRE in the MUC8 promoter may play a role in these processes by binding CREB. Further analysis of the signal pathways activated by various stimulators may yield deeper insights into the signaling mechanism of MUC8 gene expression.

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