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Suppression of prostaglandin E_2-induced MUC5AC overproduction by RGS4 in the airway

Kyoung Seob Song,[1] Yeon Ho Choi,[3,4] Jong-Mu Kim,[3,4] Hyunjae Lee,[3,4] Tae-Jin Lee,[5] and Joo-Heon Yoon[1,2,3,4]

[1]*The Airway Mucus Institute,* [2]*Department of Otorhinolaryngology,* [3]*Brain Korea 21 for Medical Sciences, and* [4]*Research Center for Human Natural Defense System, Yonsei University College of Medicine, Seoul; and* [5]*Department of Anatomy, College of Medicine, Yeungnam University, Taegu, Korea*

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Song KS, Choi YH, Kim JM, Lee H, Lee TJ, Yoon JH. Suppression of prostaglandin E_2-induced MUC5AC overproduction by RGS4 in the airway. *Am J Physiol Lung Cell Mol Physiol* 296: L684–L692, 2009. First published February 6, 2009; doi:10.1152/ajplung.90396.2008.—The mechanism by which E-prostanoid (EP) receptor is critically involved in PGE_2-induced mucin 5AC (*MUC5AC*) gene expression in the airway has been unclear. Furthermore, there have been little reports regarding the negative regulatory mechanism and/or proteins that affect PGE_2-induced MUC5AC overproduction. In the present study, we found that PGE_2 induced *MUC5AC* gene expression in a dose-dependent manner (EC_{50}: 73.31 ± 3.13 nM) and that the $EP_{2/4}$-specific agonist, misoprostol, increased *MUC5AC* mRNA level, whereas the $EP_{1/3}$-specific agonist, sulprostone, had no effect. Interestingly, the cAMP concentration (685.1 ± 14.9 pM) of the EC_{50} value of EP_4-mediated cAMP production was much higher than that of EP_2 (462.33 ± 23.79 pM), suggesting that EP_4 has higher sensitivity to PGE_2 compared with EP_2. Moreover, PGE_2-induced Muc5ac overproduction was much increased in *regulator of G protein signaling* (*Rgs*) *4* knockout (KO) mice compared with wild-type mice at both transcriptional and translational levels, and it was dramatically suppressed in *Rgs4* KO mice that had been infected with lentivirus expressing RGS4 (lenti::RGS4) compared with lentivirus expressing enhanced green fluorescent protein (lenti::eGFP). Finally, we demonstrate that PGE_2 can induce MUC5AC overproduction via the EP_4 receptor and that RGS4 may have suppressive effects in controlling MUC5AC overexpression in the airway. These findings may provide a molecular paradigm for the development of novel drugs for respiratory diseases.

inflammation; mucin 5AC; EP_4; regulator of G protein signaling-4

MUCINS are heavily glycosylated, high molecular weight glycoproteins produced by the epithelia of the respiratory, gastrointestinal, and reproductive tracts (25, 35). Twenty-one mucin genes have been identified; however, it has been unclear how mucins are regulated by various stimulants and are secreted in respiratory diseases (6). Of these, mucin 5AC (MUC5AC) is one of the major *mucin* genes in the human respiratory tract, and *MUC5AC* mRNA levels are known to be upregulated by proinflammatory cytokines, LPS, neutrophil elastase, cigarette smoking, or reactive oxygen species (15, 19, 31, 32, 36) via various signaling pathways. Nonetheless, there have been few reports on the negative regulatory mechanisms that affect stimulant-induced *MUC5AC* gene expression in the airway.

PGE_2, one of the major prostaglandins produced, is a versatile eicosanoid that regulates key responses in numerous physiological and pathological processes, including angiogenesis, tumorigenesis, immune modulation, and inflammatory airway diseases (7, 33). PGE_2 exerts its biological functions through E-prostanoid (EP) cell surface receptors. $EP_{1\text{-}4}$ receptors couple to $G\alpha_q$, $G\alpha_s$, $G\alpha_i$, and $G\alpha_s$, respectively, and can activate or inhibit several signaling proteins resulting in the increased formation of secondary messengers. Recently, Gray et al. (14) reported EP_2 and EP_4 may be the major receptors for IL-1β-induced *MUC5AC* gene expression by producing PGE_2 in the normal human tracheobronchial epithelial cells, and Kim et al. (20) suggested that EP_4 is essential for PGE_2-induced MUC5AC secretion in normal human nasal epithelial (NHNE) cells. However, the exact mechanism by which EP receptor is critically involved in PGE_2-induced *MUC5AC* gene expression has been unclear. Furthermore, we wanted to find a negative regulator of PGE_2-induced *MUC5AC* gene expression at the EP receptor level.

We thought a member of regulators of G protein signaling (RGS) proteins may play as a negative regulator of PGE_2-induced *MUC5AC* gene expression since RGS proteins can stimulate the G protein GTPase activity and thereby produce desensitization (deactivation). RGS proteins are defined by a shared 120-amino acid domain that binds directly to activated Gα subunits (3) and act as tightly regulated modulators and integrators of G protein signaling. A protein domain, known as the RGS box, harbors GTPase-accelerating proteins (GAP) activity and is the defining feature of RGS proteins. Outside the RGS box, the presence of other protein domains diversifies the range of protein interactions and regulatory activities of RGS proteins. Binding of RGS proteins to active Gα (GTP-bound) can interfere with effector-binding proteins, thereby blocking activation and downstream signaling (16). In addition, the expression of RGS2, -3, -5, and -16 subtypes, mainly members of R4 RGS subfamily, has been defected in the lung (22, 26). In the airway, which RGS protein has a critical role and how the interplay between EP receptor and RGS leads to the alteration of MUC5AC overproduction remain poorly understood.

Address for reprint requests and other correspondence: J.-H. Yoon, Dept. of Otorhinolaryngology, Yonsei Univ. College of Medicine, 134 Shinchon-Dong, Seodaemun-gu, Seoul 120-752, Korea (e-mail: jhyoon@yuhs.ac).

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In the present study, we examined the mechanism by which PGE_2 increases MUC5AC overproduction at the receptor level. We show that the EP_4 receptor plays as an important role in PGE_2-induced MUC5AC overproduction in the airway. In addition, RGS4 suppressed PGE_2-induced MUC5AC overproduction both in vitro and in vivo by binding to EP_4-coupled $G\alpha_s$ depending on GTPγS.

MATERIALS AND METHODS

Materials. All chemical compounds were purchased from Cayman Chemical (Ann Arbor, MI). $G\alpha_s$ and Gβ antibodies were purchased from Calbiochem (Merck; Darmstadt, Germany), RGS4 and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and MUC5AC antibody was purchased from Lab Vision (Fremont, CA). The cDNA expression constructs encoding EP_4, $G\alpha_s$, $G\alpha_s$ Q227L, RGS2, RGS3, RGS4, and RGS5 were purchased from the UMR cDNA Resource Center (Rolla, MO). All of small interfering RNAs (siRNAs) were synthesized from Bioneer (Daejeon, Korea): EP_4, 5′-UUCUUUCCAGGCUAUGUAU (dTdT)-3′; RGS4, 5′-GGAUCAGCUGUGAAGAGUA (dTdT)-3′; and negative control, 5′-CCUACGCCACCAAUUUCGU (dTdT)-3′.

Cell cultures. NHNE cells (2×10^5 cells/well) were seeded in 0.5 ml of culture medium on Transwell clear culture inserts (24.5 mm, 0.45-μm pore size; Costar, Cambridge, MA). Cells were cultured in a 1:1 mixture of BEGM and DMEM containing all the supplements previously described (39). Human tissue was maintained in accordance with the guidelines and approval of the institutional review board of Yonsei University, Seoul, Korea. Consent from both patients and the local ethics committee was obtained for use of the specimens. The human lung mucoepidermoid carcinoma cell line (NCI-H292) was purchased from the American Type Culture Collection (CRL-1848; Manassas, VA) and cultured in RPMI 1640 (Invitrogen; Carlsbad, CA) supplemented with 10% fetal bovine serum in the presence of penicillin-streptomycin at 37°C in a humidified chamber with 5% CO_2.

Cell transfection. Cells were plated in 6-well plates 1 day before transfection with plasmid DNA (1 μg/well) or siRNA (100 pmol/well) using FuGENE 6 (Roche; Indianapolis, IN) according to the manufacturer's instructions. Approximately 24 h after transfection, cells were maintained in 0.2% serum RPMI media for 16–18 h before treatment with PGE_2 and then harvested 24 h after treatment.

Measurement of cAMP level. Cells were plated in 6-well plates 1 day before transfection. Approximately 24 h after transfection, transfected cells were replated at 2,000/well in 96-well plates and maintained in 0.2% serum RPMI media for 16–18 h before treatment with PGE_2. cAMP production was measured according to the manufacturer's instructions (Promega; Madison, WI).

PCR. After reverse transcription, RT-PCR was performed to amplify DNA fragments. All of EP primers (18) were synthesized from Bioneer. PCR products were run for 35 cycles using the amplification conditions (18). Real-time PCR was performed using a Bio-Rad iCycler iQ Detection System (Bio-Rad, Hercules, CA) with iQ SYBR Green Supermix. The following primers were used: human MUC5AC, forward 5′-CAGCCACGTCCCCTTCAATA-3′ and reverse 5′-ACCGCATTTGGGCATCC-3′; β_2-microglobulin, forward 5′-CGCTCCGTGGCCTTAGC-3′ and reverse 5′-GAGTACGCTGGATAGCCTCCA-3′; mouse Muc5ac primers, forward 5′-CCATGCAGAGTCCTCAGAACAA-3′ and reverse 5′-TTACTGGAAAGGCCCAAGCA-3′; and mouse GAPDH primers, forward 5′-TGTGTCCGTCGTGGATCTGA-3′ and reverse 5′-CCTGCTTCACCACCTTCTTGAT-3′ (34). Parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The result was normalized against β_2-microglobulin as an internal control (mouse GAPDH for *Muc5ac* mRNA), and the relative quantity of *MUC5AC* mRNA was determined using a MUC5AC standard curve.

Western blot analysis, dot blot analysis, and immunoprecipitation. These methods were described previously (32). NCI-H292 cells were grown to confluence in 6-well plates. After treatment with PGE_2, the cells were lysed with 2× lysis buffer [250 mM Tris·HCl (pH 6.5), 2% SDS, 4% 2-mercaptoethanol, 0.02% bromphenol blue, 10% glycerol]. Equal amounts of whole cell lysates from protein samples were resolved by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA). For dot blot analysis, 125 μl of protein lysates were directly diluted with dot blot buffer (600 mM KSCN, 10 mM EDTA, pH 8.0) in 96-well plates. After serial dilution, lysates were applied by vacuum using a dot blot apparatus. The membrane were incubated with MUC5AC antibody as primary antibody and then incubated with anti-mouse horseradish peroxidase IgG. The signal was detected by enhanced chemiluminescence kit, and the relative fold increase was calculated by dividing stimulant-treated signal into control signal since there is no commercially available MUC5AC protein for standardization. The data were represented as means ± SD of triplicate cultures from the independent experiments. For immunoprecipitation, cells were transfected with either wild-type $G\alpha_s$ or dominant negative $G\alpha_s$ Q227L mutant and RGS4-expressing constructs using FuGENE 6. Following transfection, cells were washed with ice-cold PBS and harvested by scraping into lysis buffer [25 mM HEPES, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, protease inhibitor tablet (Complete Mini; Roche)], sonicated (4 times each for 5 s), and centrifuged at 12,000 *g* for 15 min. Supernatant lysates (230 μl) were precleared with GammaBind G Sepharose (GE Healthcare; Uppsala, Sweden) for 30 min at 4°C. Following centrifugation, anti-$G\alpha_s$ antisera were added to precleared lysates, incubated for 14 h at 4°C, and added GammaBind G Sepharose.

Mice, genotyping, and tracheotomy. Six- to eight-week-old C57BL/6 mice and RGS4 knockout (KO) mice (B6-129P2-*Rgs4*^{tm1Dgen}/J; The Jackson Laboratory; Bar Harbor, ME) were maintained in accordance with the guidelines and under approval of the Animal Care Committee of Yonsei University, Seoul, Korea. Mice were anesthetized with Zoletil (30 mg/kg). Genotyping of *Rgs4*$^{tm1Dgen/tm1Dgen}$ mice was performed by PCR on genomic tail DNA using an endogenous gene-specific primer (5′-GGACATGAAACATCGGCTGGGGTTC-3′), an endogenous and targeted gene-specific primer (5′-CCATCTTGACCCAAATCTGGCTCAG-3′), and targeted neo primer (5′-GGGCCAGCTCATTCCTCCCACTCAT-3′), which produced a 226-bp and a 484-bp band from the wild-type and the targeted allele, respectively. For intratracheal instillation, the trachea was surgically exposed after anesthetization by making an incision in the neck skin. A microsyringe carrying a 31-gauge needle filled with PGE_2 solution was used for injection into the exposed trachea. PGE_2 (50 μl; 0.5, 1.0, or 5.0 mg/kg) was injected into the lumen of each trachea. After injection, the skin was sutured. At 1 day after injection, the mouse was killed, and the trachea was removed (34).

Virus production and in vivo intranasal instillation for infection. Vector construction and virus production of *LentiM1.2::RGS4-eGFP*, *LentiM1.2::eGFP*, and *small hairpin (sh) Lenti3.4R::RGS4* (5′-CTGAGAACCTTAGCCATCA-3′) were performed by Macrogen (Seoul, Korea). Viruses [5×10^5 transduction units (TU)/50 μl] were administered drop by drop to the right nostril of anesthetized mice using sequencing tips (27). Three days after infection, tracheotomy was performed.

Statistical analysis. The data represent the means ± SD for at least three independent experiments. Where appropriate, the statistical differences were assessed by Wilcoxon Mann-Whitney test. A P value <0.05 was considered statistically significant.

RESULTS

PGE_2-induced MUC5AC gene expression was mediated via the EP_4 receptor in NHNE cells. To determine whether PGE_2 increases *MUC5AC* gene expression in NHNE cells, real-time

PCR analysis was performed. As seen in Fig. 1*A*, PGE_2 induced *MUC5AC* gene expression in a dose-dependent manner with an EC_{50} value of 73.31 ± 3.13 nM. Since Kunikata et al. (21) reported that endogenous PGE_2 played an important feedback function in allergic inflammation, and we thought that newly synthesized endogenous prostaglandins by exogenous PGE_2 might influence the results, we wanted to rule out this effect. Accordingly, indomethacin, a nonselective cyclooxygenase-1 (COX-1) and COX-2 inhibitor, was employed to investigate the function of exogenous PGE_2. Cells were treated for 24 h with PGE_2 alone or pretreated indomethacin (3 μM) for 1 h before PGE_2 treatment. Indomethacin alone had no effect, and pretreatment of indomethacin did not affect PGE_2-induced *MUC5AC* gene expression (Fig. 1*B*). This result suggests that *MUC5AC* gene expression have been affected by exogenous PGE_2 but not endogenous PGE_2 in the present study. We next examined whether mRNA level of EP receptors was affected by PGE_2. All of EP receptors were expressed, and treatment with PGE_2 did not affect *EP receptor* gene expression in NHNE cells (Fig. 1*C*). To identify which EP receptors are involved in PGE_2-mediated *MUC5AC* gene expression, cells were treated for 24 h with the $EP_{1/3}$-selective agonist, sulprostone, or the $EP_{2/4}$-selective agonist, misoprostol (Fig. 1*D*; Ref. 14). Misoprostol (10 μM) increased *MUC5AC* mRNA level, whereas 10 μM sulprostone had no effect. This result indicates that EP_2 and/or EP_4 were (was) involved in PGE_2-induced *MUC5AC* gene expression. Moreover, to identify which EP receptor has a substantial role in *MUC5AC* gene expression, a nonselective EP_2 antagonist, AH6809 (20, 30), and a selective EP_4 antagonist, GW627368X (37, 38), were used (Fig. 1*E*). The increase in *MUC5AC* gene expression by PGE_2 treatment (7.33-fold) is decreased by ~42.1% on addition of AH6809 (4.25-fold), whereas GW627368X inhibited ~73.6% (1.94-fold) of it. This result suggests that both EP_2 and EP_4 receptors are probably related to PGE_2-induced *MUC5AC* gene expression in NHNE cells. However, in our previous study, EP_4 receptor played as a major receptor for PGE_2-induced MUC5AC production at the transcriptional and translational levels (20). Accordingly, we selected the EP_4 receptor as a major receptor in the present study. To complement this pharmacological approach, we used the genetic strategy of siRNA in NCI-H292 cells. As shown in Fig. 1*F*, siRNA EP_2 and siRNA EP_4 suppressed intracellular EP_2 and EP_4 mRNA level in NCI-H292 cells, respectively. Consistent with the pharmacological study, the increase in *MUC5AC* gene expression by PGE_2 treatment (7.19-fold) was decreased by ~53.0% on addition of siRNA EP_2 (3.42-fold), whereas siRNA EP_4 inhibited ~71.3% (2.09-fold) of increased *MUC5AC* gene expression. These results suggest that both EP_2 and EP_4 receptors are probably related to PGE_2-induced *MUC5AC* gene expression. However, the EP4 receptor served as a major receptor in PGE_2-induced MUC5AC gene expression.

PGE_2-mediated $G\alpha_s$ activity was dependent on the EP_4 receptor. Next, to identify which EP receptor has a critical role in PGE_2 signaling, the ligand selectivity of PGE_2 was examined using cAMP production assay. NCI-H292 cells were transiently transfected with EP_2, EP_4, or mock construct. PGE_2-induced cAMP production was higher in cells transfected with EP_4 construct compared with cells transfected with EP_2 construct (Fig. 2*A*). The cAMP concentration (685.1 ± 14.9 pM) of the EC_{50} value of EP_4-mediated cAMP production was much higher than that of EP_2 (462.33 ± 23.79 pM) (Fig. 2*B*), suggesting that EP_4 has higher sensitivity to PGE_2 compared with EP_2. It also indicated that that PGE_2 increases $G\alpha_s$ activity in a dose-dependent manner and that the EP_4 receptor appears to have more functional activity than the EP_2 receptor, at least in part in NCI-H292 cells. To verify that the concentration of cAMP increased by PGE_2 was mediated by EP_4, siRNA EP_4 was employed. Whereas PGE_2-induced cAMP production was higher in cells transfected with wild-type EP_4 construct compared with cells treated with PGE_2 alone, PGE_2-induced cAMP production was significantly suppressed by siRNA EP_4 construct in a dose-dependent manner (Fig. 2*C*). These results indicate that PGE_2-mediated $G\alpha_s$ activity was dependent on the EP_4 receptor.

RGS4 could regulate PGE_2-induced MUC5AC gene expression by interaction with $G\alpha_s$ in a GTPγS-dependent manner. The main aim of this study is to identify specific molecule(s) that suppresses PGE_2-induced MUC5AC overproduction during airway mucosal inflammation. We therefore screened inhibitory G protein molecules that attenuate G protein signaling. We checked the inhibitory effects of RGS proteins containing GTPase activity on *MUC5AC* gene expression. It has been reported that mainly R4 RGS family proteins (RGS2, -3, -5, and -16), containing only RGS domain, are expressed in the lung (22, 26). Of these RGS proteins, because RGS16 has been upregulated in germinal center and activated T cells, suggesting that it has a role in adaptive immunity (8), we have excluded RGS16 from this study, and selected RGS2, -3, -4, and -5 proteins. Since these RGS subtype constructs have been tagged with hemagglutinin (HA), Western blot analysis was performed with anti-HA antibody. No change was detected in the expression of HA in cells transfected with each RGS construct (Fig. 3*A*, *top*). Of the RGS proteins tested, only ectopic RGS4 expression inhibited PGE_2-induced *MUC5AC* gene expression (Fig. 3*B*, *bottom*). To determine a potential role for RGS4 in PGE_2-mediated *MUC5AC* gene expression, siRNA RGS4 was employed. Whereas wild-type RGS4 dramatically suppressed PGE_2-induced *MUC5AC* gene expression, siRNA RGS4 much increased PGE_2-induced *MUC5AC* gene expression compared with PGE_2 alone (Fig. 3*B*). This phenomenon is due to the inhibition of endogenous RGS4 expression. In addition, to investigate a potential role for the RGS4 in $G\alpha_s$-mediated signal processing, we asked whether RGS4 bound to $G\alpha_s$ as a component of the G protein complex. If so, we thought that RGS4 could negatively regulate *MUC5AC* gene expression by binding to the $G\alpha_s$-mediated signaling complex. To verify this possibility, NHNE cells were infected with lentiviruses (multiplicity of infection 30) expressing wild-type RGS4 and shRGS4 (Fig. 3*C*). On *day 3* after infection, RGS4 and $G\alpha_s$ were coimmunoprecipitated by the addition of GTPγS, and this reaction was disrupted by the addition of GDP. Gβ was dissociated from the $G\alpha_s$-RGS4 complex by addition of GTPγS in cell lysates and was used as a positive control for GTP (Fig. 3*C*). These results suggest that RGS4 has an inhibitory effect on PGE_2-induced *MUC5AC* gene expression by binding to $G\alpha_s$ in vitro.

RGS4 plays as a negative regulator molecule for PGE_2-dependent Muc5ac gene expression in vivo. To determine the in vivo relevance of in vitro results, approximately 6- to 8-wk-old male C57BL/6 mice were tested. The trachea of mice

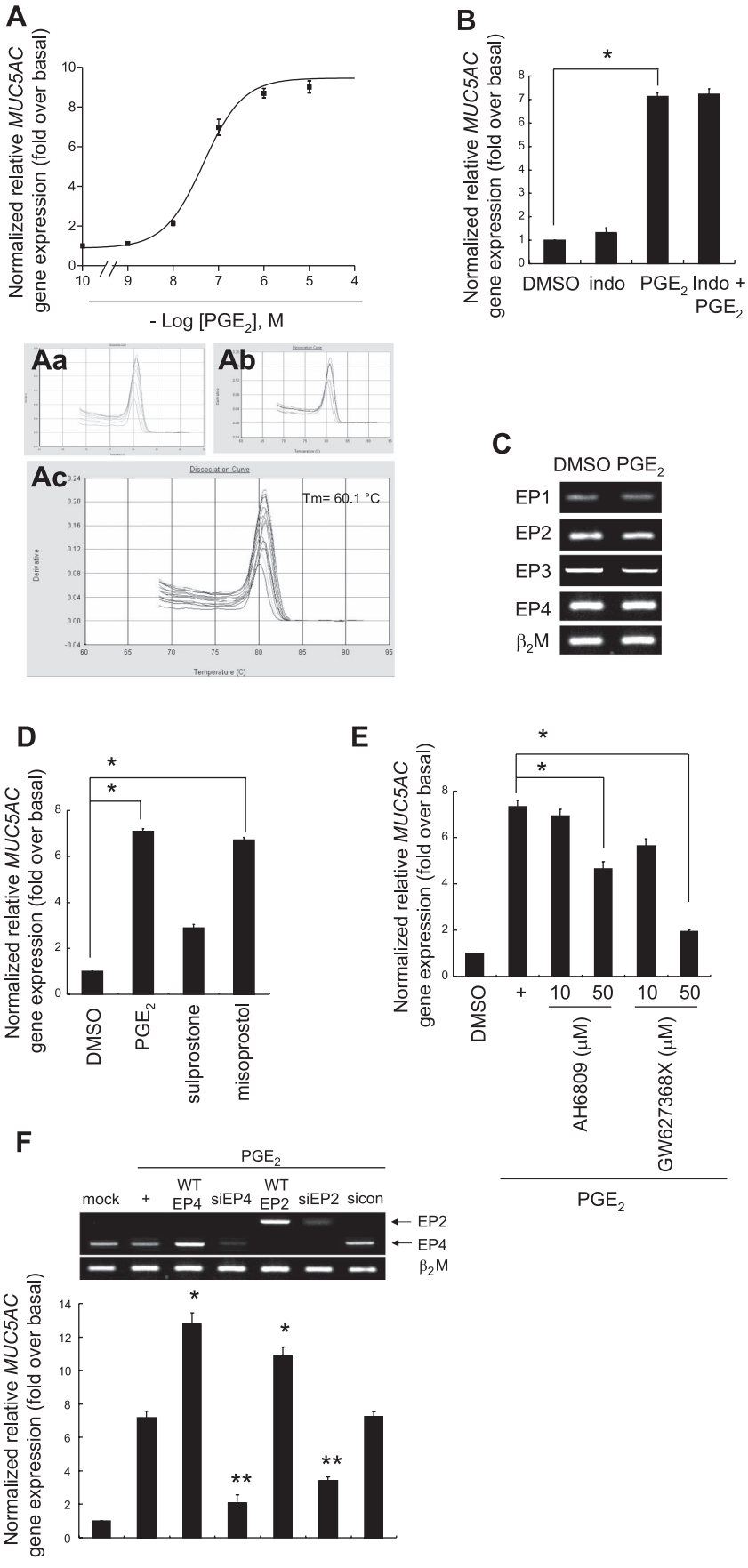

Fig. 1. PGE_2 induces *MUC5AC* gene expression via the E-prostanoid (EP) receptor 4 (EP_4) in normal human nasal epithelial (NHNE) and human lung mucoepidermoid carcinoma (NCI-H292) cells. *A*: NHNE cells were treated for 24 h with several concentrations of PGE_2. Cell lysates were harvested for real-time quantitative PCR. Corresponding dissociation curves for standard (0.01 fg to 1 ng; *Aa*) and samples (*Ab*) were amplified using specific primers with SYBR Green dye. These curves were merged on the same panel (*Ac*). *B*: NHNE cells were treated for 1 h with 3 μM indomethacin (indo), a cyclooxygenase-1 (COX-1) and COX-2 inhibitor, before treatment for 24 h with 100 nM PGE_2. Cell lysates were harvested for real-time quantitative PCR. $*P < 0.05$ compared with control. *C*: NHNE cells were treated with 100 nM PGE_2 for 24 h. The presence of EP receptors mRNA was detected by RT-PCR with specific EP receptors primers, respectively. *D*: NHNE cells were treated for 24 h with 100 nM PGE_2, 10 μM $EP_{1/3}$ agonist, sulprostone, or 10 μM $EP_{2/4}$ agonist, misoprostol. Cell lysates were performed real-time quantitative PCR. $*P < 0.05$ compared with control. *E*: NHNE cells were treated for 1 h with either 10 or 50 μM EP_2 antagonist, AH6809, or 10 or 50 μM EP_4 antagonist, GW627368X, before treatment for 24 h with 100 nM PGE_2. Cell lysates were harvested for real-time quantitative PCR. $*P < 0.05$ compared with PGE_2 alone. *F*: NCI-H292 cells transfected with construct encoding wild-type EP_2 or EP_4, small interfering RNA (siRNA) EP_2 or EP_4, or siRNA control were treated for 24 h with 100 nM PGE_2. Cell lysates were harvested for PCR. $*P < 0.05$ compared with PGE_2 alone and $**P < 0.05$ compared with overexpressed EP_2 or EP_4. All data shown are representative of 3 independent experiments. Tm, melting temperature; β_2M, β_2-microglobulin; WT, wild-type; siEP2, siRNA EP_2; sicon, siRNA control.

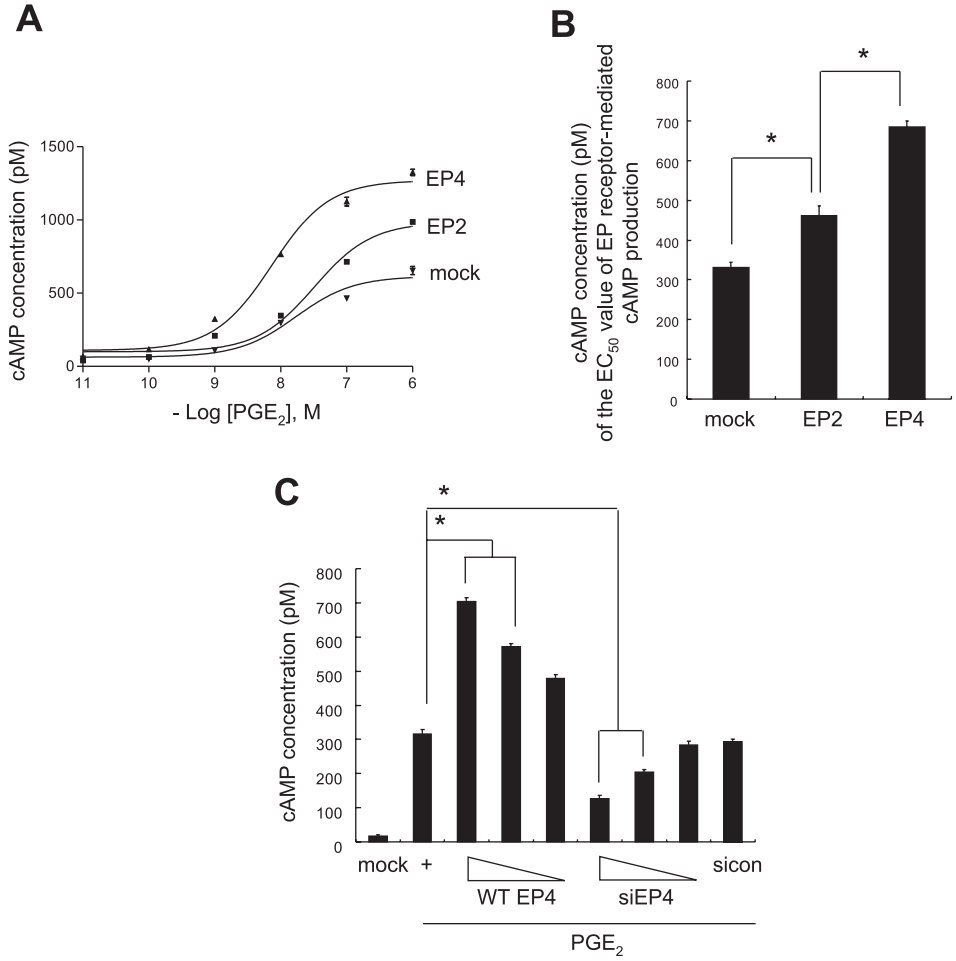

Fig. 2. EP_4 is essential for PGE_2-induced *MUC5AC* gene expression through $G\alpha_s$ activation. *A*: cells were transiently transfected with construct expressing wild-type EP_4, wild-type EP_3, or pcDNA3.1 as the transfection control. Cells were serum-starved overnight and then treated with the indicated concentrations of PGE_2 for 6 h, after which the cAMP production was measured. The values shown are means ± SD of experiments performed in triplicate. *B*: we compared the cAMP concentrations of the EC_{50} values of EP_2- and EP_4-mediated cAMP production. $^*P < 0.05$ compared with PGE_2 concentration of EC_{50} values of EP_2. *C*: cells were transiently transfected with constructs expressing either 0.01, 0.1, or 1.0 μg/well of wild-type EP_4 or 1, 10, 100 pmol/well of siRNA EP_4. Six hours after PGE_2 treatment (10 nM), the luciferase activity was measured and normalized to the X-gal activity. $^*P < 0.05$ compared with PGE_2 alone. All data shown are representative of 3 independent experiments.

was surgically exposed under general anesthesia and aseptic conditions, and 50 μl of 0.1, 1.0, and 5.0 mg/kg PGE_2 was intratracheally instilled with a 31-gauge needle. After 24 h, PCR and dot blotting for Muc5ac expression were performed using the trachea from the killed mice (Fig. 4, *A* and *B*). PGE_2 increased MUC5AC expression in vivo at both the transcriptional and translational levels. These results were consistent with the in vitro results. In addition, to investigate whether RGS4 has an effect on PGE_2-induced Muc5ac expression in vivo, *Rgs4* KO mice were employed. After *Rgs4*$^{tm1Dgen/tm1Dgen}$ mice were genotyped by PCR with genomic tail DNA using specific primers, which produced a 226-bp and a 484-bp band from the wild-type and the targeted allele, respectively (data not shown), tracheotomy was performed for PGE_2 instillation. Twenty-four hours later, total RNA and tissue lysates were purified from the trachea of *Rgs4* KO mice. PGE_2-induced Muc5ac expression was much increased in *Rgs4* KO mice compared with wild-type mice at both the transcriptional and translational levels (Fig. 4, *C* and *D*), indicating that RGS4 probably has a suppression effect on PGE_2-induced Muc5ac overproduction in the airway. We thought that when lenti::RGS4 virus administrate into *Rgs4* KO mice, it may restore lost RGS function in PGE_2-induced Muc5ac overproduction in the airway. Lentivirus expression system was used. Viruses (5×10^5 TU/50 μl) were administrated in the right nostril of anesthetized mice drop by drop using sequencing tips (27). Three days after infection, the trachea was surgically exposed to instill PGE_2. RGS4 was well-expressed in *Rgs4* KO mice that had been infected with lenti::RGS4-eGFP (Fig. 4*E*, *top*). PGE_2 instillation to the trachea of *Rgs4* KO mice that had been infected with lenti::RGS4-eGFP resulted in a fourfold decrease of Muc5ac expression compared with lenti::Mock-eGFP (Fig. 4*E*, *bottom*), indicating that administration of RGS4 to *Rgs4* KO mice airway restores its suppressive function in *Rgs4* KO mice. These results were consistent with in vitro results (Fig. 3*B*), and an inhibitory effect of RGS4 on Muc5ac overproduction was revealed by knocking out RGS4. Next, to investigate whether EP_4 is responsible for PGE_2-induced *Muc5ac* gene expression in vivo, wild-type mice were injected with the EP_4-specific agonist, PGE_1-OH, and expression level of *Muc5ac* mRNA was determined. After the mice were preinjected with 1.0, 2.5, and 5.0 mg/kg GW627368X (ip) for 1 h, PGE_1-OH or PGE_2 was instilled intratracheally. The EP_4 agonist increased *Muc5ac* mRNA level, whereas the mRNA level strongly inhibited GW627368X in a dose-dependent manner, and PGE_2-induced mRNA level was also inhibited by GW627368X (Fig. 4*F*), indicating that EP_4 receptor is essential for PGE_2-induced Muc5ac overproduction. These results suggest that PGE_2 binds to the EP_4 receptor thereby inducing MUC5AC overproduction, and RGS4 may be a key negative regulator during airway mucosal inflammation.

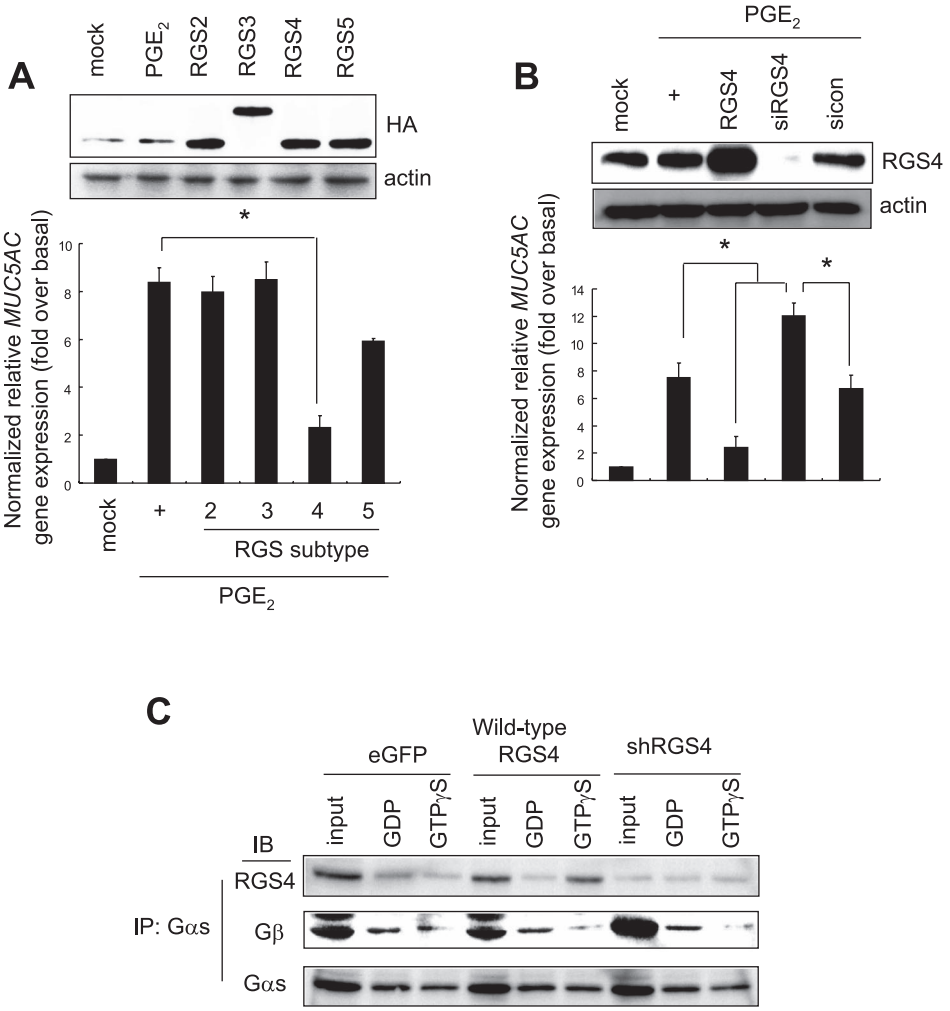

Fig. 3. Regulator of G protein signaling-4 (RGS4) plays as a suppressor of PGE_2-induced *MUC5AC* gene expression by interacting with $G\alpha_s$ depending on GTPγS in vitro. *A*: NCI-H292 cells were transiently transfected with construct expressing wild-type RGS2, -3, -4, or -5, respectively. Confluent cells were serum-starved overnight and then treated with 100 nM PGE_2 for 24 h followed by Western blotting with hemagglutinin (HA) antibody (*top*) and PCR (*bottom*). $*P <$ 0.05 compared with PGE_2 alone. *B*: cells were transiently transfected with construct expressing wild-type RGS4, siRNA RGS4 (siRGS4), or siRNA control. Cells were serum-starved and treated with 100 nM PGE_2 for 24 h, after which cell lysates were harvested for Western blot analysis with anti-RGS4 IgG (*top*) and real-time PCR (*bottom*). $*P < 0.05$ compared with PGE_2 alone. *C*: 3 days after NHNE cells were infected with lentivirus expressing enhanced green fluorescent protein (eGFP), wild-type RGS4, or small hairpin (sh) RGS4, cell lysates were preincubated with 100 μM GDP or 100 μM GTPγS/25 mM $MgCl_2$ at 24°C for 30 min before immunoprecipitation (IP) against $G\alpha_s$. The input lane contains <⟩ volume used for IP. IB, immunoblotting. Gβ was used as positive control for GTPγS. Data shown are representative of 3 independent experiments.

DISCUSSION

PGE_2 is frequently observed at high concentrations at the sites of inflammation and plays physiological roles in the regulation of mucosal immunity (9). Secreted PGE_2 stimulates epithelial cells in an autocrine and paracrine fashion. Epithelial cells thereby increase mucin secretion and *mucin* gene expression, including MUC5AC and MUC8 (20). Interestingly, even though the importance of PGE_2 in human airway epithelium is well-established (29), the molecular mechanism by which PGE_2-induced MUC5AC overproduction is regulated is still unclear. The focus of the present study was to identify molecular mechanism(s) and regulatory molecule(s) for PGE_2-induced MUC5AC overproduction.

In the present study, we showed that PGE_2 induced *MUC5AC* mRNA expression in NHNE cells, whereas endogenously produced or endocrinal PGE_2 did not affect *MUC5AC* gene expression during exogenous PGE_2 treatment (Fig. 1*B*). Interestingly, even if the half-life of PGE_2 is very short (10), PGE_2 had an effect in the early stage during the 24-h treatment period. The fact that *MUC5AC* gene expression increases at 12 h after the beginning of PGE_2 exposure can be attributed to PGE_2 binding to its receptors in the early stage. It seems that the kinetics of PGE_2 in the early stage is sufficient to induce *MUC5AC* gene expression because activated receptor propagates specific signaling to amplify physiological PGE_2 signaling. The biological actions of PGE_2 are mediated by seventh transmembrane EP receptors (23). Using RT-PCR, we found that all EP receptor mRNAs were expressed and PGE_2 treatment did not affect *EP receptor* gene expression in NHNE cells (Fig. 1*C*). In the airway, EP_2 and EP_4 receptors have been known as major receptors for *MUC5AC* gene expression and secretion (14, 20). Consistent with this result, the $EP_{2/4}$-selective agonist (misoprostol) had a significant effect on *MUC5AC* gene expression (Fig. 1*D*), whereas the $EP_{1/3}$-selective agonist (sulprostone) had no effect. These results suggest that either the simultaneous induction of the two different $G\alpha_s$-coupled PGE_2 receptors plays a pivotal role for PGE_2 during airway mucosal inflammation or both EP_2 and EP_4 may cooperatively work as a compensatory system for *MUC5AC* gene expression.

Among these receptors, however, the EP_4-specific antagonist has much more inhibited PGE_2-induced *MUC5AC* gene expression than the EP_2 antagonist (Fig. 1*E*). Even if the pharmacokinetics of the two different drugs has been considered deeply, this suggests that EP_4 may be more closely related to PGE_2 signaling at least in part in NHNE cells. Consistent with the pharmacological data, knockdown of EP_4 suppressed PGE_2-induced *MUC5AC* gene expression (Fig. 1*F*). We wondered what is different between EP_2 and EP_4 despite them both

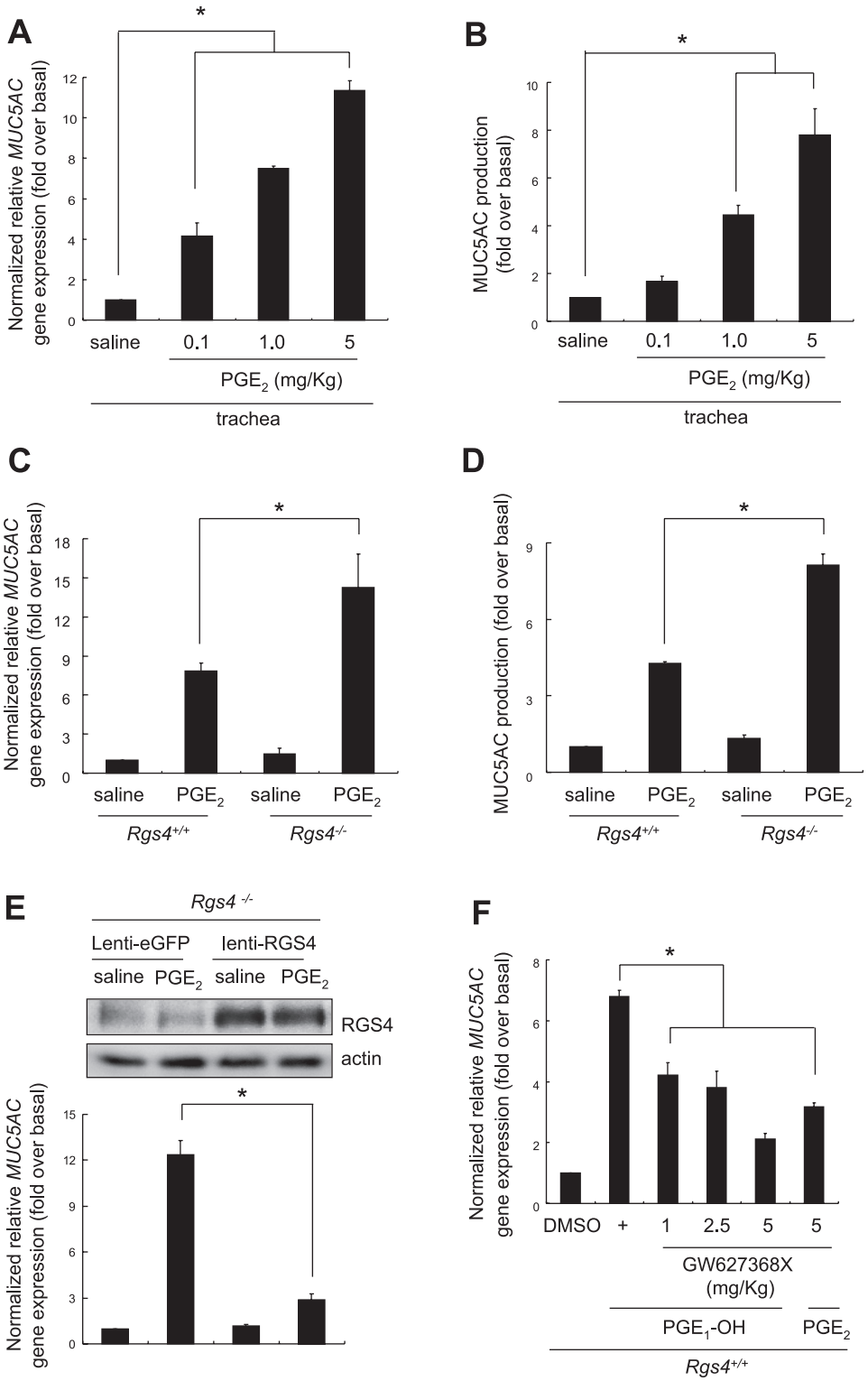

Fig. 4. RGS4-dependent attenuation of MUC5AC overproduction in the airway. *A*: 50 μl of 0.1, 1.0, or 5 mg/kg PGE_2 or saline was instilled in the trachea of C57BL/6 mice. Twenty-four hours later, mouse tracheal tissue was processed for real-time PCR and dot blot analysis with anti-MUC5AC IgG (*B*). Saline was used as a vehicle. $*P < 0.05$ compared with saline-treated mice. *C*: 50 μl of 1.0 mg/kg PGE_2 or saline was instilled inside the trachea of the genotyped mice. Twenty-four hours later, trachea was processed for real-time PCR and dot blot analysis with anti-MUC5AC IgG (*D*). $*P < 0.05$ compared with PGE_2-treated wild-type mice. *E*: either lenti::eGFP or lenti::RGS4-eGFP [5×10^5 transduction units (TU)/50 μl] was administered drop by drop to the right nostril. Three days after infection, 50 μl of 1.0 mg/kg PGE_2 or saline was instilled inside the trachea. Twenty-four hours later, tracheas were processed for PCR. $*P < 0.05$ compared with PGE_2-treated *Rgs4* knockout mice infected by lenti::eGFP. *F*: genotyped mice were injected with DMSO as a vehicle for different dosages of the EP_4 antagonist, GW627368X (1–5 mg/kg ip), for 1 h before instillation of the EP_4-specific agonist, PGE_1-OH (50 μl of 5 mg/kg it), and PGE_2 (50 μl of 1.0 mg/kg). Tracheas from killed mice were harvested for real-time PCR. $*P < 0.05$ compared with PGE_1-OH-treated wild-type mice. All data shown are representative of 3 independent experiments.

being the same $G\alpha_s$-coupled receptors. One reason may be due to a difference in their ligand selectivity of PGE_2. Despite almost the same mRNA expression levels of the receptors, cAMP production of the EC_{50} value of EP_4-mediated cAMP production was about 685.1 ± 14.9 pM (Fig. 2*A*), whereas that of EP_2-mediated cAMP production was 462.33 ± 23.79 pM following PGE_2 treatment (Fig. 2*B*). This indicates that the higher cAMP concentration of the EC_{50} value of EP_4-mediated cAMP production is because of high selectivity of PGE_2 to convey signaling efficiently to coupled signaling pathways. Consistent with our results, Fujino et al. (11, 12) reported that EP_4 receptor strengthens an association of the PGE_2 to induce T cell factor and early growth response factor-1 in inflammation. Another possible reason that there is a difference in

biological actions of EP_2 and EP_4 may be due to posttranslational modifications, such as glycosylation, hydroxylation, methylation, nucleotidylation, and phosphorylation. Our thinking was supported by several studies that a charged residue(s) in the seventh transmembrane domain and posttranslational modification sites were involved in receptor activation and desensitization (1, 2, 13, 24).

The main finding of this study is that RGS4 suppressed PGE_2-induced $G\alpha_s$-mediated signaling in vitro and in vivo by enhancing GTPase acceleration. Until now, there has been no report of a relationship between PGE_2 receptors and RGS in mucin-related literature. Despite that the characterized main function of RGS proteins is GTPase-activating proteins for $G\alpha_{i/o}$, $G\alpha_q$, or $G\alpha_{12/13}$ classes of G proteins, no interaction between RGS and $G\alpha_s$ has been found (40). Recently, Castellone et al. (4) reported that the $G\alpha_s$-GTP bound axin protein contained an RGS domain, and thus PGE_2 was able to promote colon cancer cell growth. In addition, Zheng et al. (40) suggested that the RGS domain of RGS-PX1 specifically interacted with $G\alpha_s$, thereby accelerating its GTP hydrolysis, which leads to an attenuation of $G\alpha_s$-mediated signaling in bovine brain lysates. In the present study, RGS4 bound $G\alpha_s$-GTP, thereby attenuating PGE_2-induced MUC5AC overproduction. In addition, the dynamics of heterotrimeric G protein signaling complex are regulated by RGS4 protein in GTP-dependent manner (Fig. 3*C*). To address the role of RGS4 protein in the pathogenesis of anti-inflammation in the airway, a gain/loss-of-function study in an animal model must be considered. PGE_2 instillation into *Rgs4* KO mice much increased Muc5ac overproduction compared with that in wild-type mice (Fig. 4, *C* and *D*), and Rgs4 function has been restored and *Muc5ac* gene expression was suppressed after administration of RGS4-expressing lentivirus into *Rgs4* KO mice (Fig. 4*E*). These results demonstrate that RGS4, a classical R4 RGS superfamily lacking any additional well-defined domains (3), negatively regulates the $G\alpha_s$-mediated signaling that induced Muc5ac production during airway mucosal inflammation. These results, however, raise an interesting question. Why does only RGS4 function in the airway epithelium? We just thought that specificity may be mediated in part through selectivity of RGS proteins for particular G protein substrates (17) or that posttranslational modification of NH_2-terminal domain except palmitoylation may be required for the plasma membrane association of RGS4. In fact, although RGS2 does not have an inhibitory function (Fig. 3*A*), it is similar in its NH_2-terminal structure to RGS4, including an amphipathic α-helical membrane targeting domain (5, 17). In addition, although it has been known that RGS4 and RGS2 are the most likely to be involved in regulation of signaling by receptors coupled to $G\alpha_q$ pathways (28), RGS4 inhibited $G\alpha_s$ pathway in the present study. These findings support our hypothesis that either the difference of the physiological functions of RGS2 and RGS4 may due to posttranslational modifications on NH_2- or COOH-terminal domain, excluding RGS box, or that some mediating (bridging) protein(s) may regulate the ability of RGS4 protein like a small switching molecule when it (they) senses the multiplicity of different signaling pathways during airway inflammation. In the present study, we used mice trachea to detect differences in Muc5ac overproduction in $Rgs4^{+/+}$ and $Rgs4^{-/-}$ mice but not lung and selected 24 h as Muc5ac overproduction peaked at 24 h. Although 24 h may be sufficient to express Muc5ac in goblet cells before inducing inflammatory cell infiltration or mucous metaplasia, it may be not enough to induce inflammatory cell infiltration or mucous metaplasia in the airway.

Taken together, these findings suggest that PGE_2 may interact directly with EP_4 receptor, resulting in increased MUC5AC overproduction. The ability of RGS4 proteins to interact with $G\alpha_s$ is important to negatively regulate PGE_2-induced MUC5AC overproduction in the airway. As more mechanistic insights are obtained, identification of an RGS accessory molecule that regulates RGS function may be considered.

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