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Upregulation of *MUC5AC* Gene Expression by IL-4 Through CREB in Human Airway Epithelial Cells

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ABSTRACT

Mucus hypersecretion is an important characteristic feature of the pathogenesis of allergy. Although interleukin (IL)-4 is known to be an inflammatory mediator in respiratory diseases, the mechanism by which IL-4 induces *MUC5AC* gene expression has not been fully explored. The aim of this study was to investigate the mechanism by which IL-4 induces *MUC5AC* gene expression in the airway. We examined the role of mitogen-activated protein kinase (MAPK) signaling on *MUC5AC* gene expression in airway epithelium. We showed that phosphorylation of ERK1/2 increased after treatment of cells with IL-4, whereas phosphorylation of p38 and JNK was not detected. In addition, pharmacologic and genetic inhibition of ERK1/2 abolished IL-4-induced *MUC5AC* gene expression. Moreover, we investigated the activation of p90 ribosomal S6 kinase 1 (RSK1) as a downstream signaling target of ERK1/2 in IL-4 signaling. The activation of RSK1 was prevented by pretreatment with PD98059 or plasmid expressing a MEK1 dominant-negative mutant. We also found that RSK1 mediated the IL-4-induced phosphorylation of cAMP response element-binding protein (CREB) and the transcription of *MUC5AC*. Furthermore, the cAMP-response element (CRE) in the *MUC5AC* promoter appears to be important for IL-4-induced *MUC5AC* gene expression in NCI-H292 cells. J. Cell. Biochem. 108: 974–981, 2009.

KEY WORDS: ALLERGEN; ASTHMA; IL-4; MUC5AC; CREB

Understanding the mechanisms that lead to increased mucus secretion in respiratory diseases is important for improving future therapies. Mucus hypersecretion in airway epithelium is a major characteristic of a number of respiratory diseases. MUC5AC and MUC5B are generally known to be major airway mucins [Hovenberg et al., 1996; Thornton et al., 1997; Wickstrom et al., 1998]. Biochemical studies on mucin genes are difficult because of their large molecular size and heavy glycosylation [Voynow et al., 1999]. MUC5AC is clearly expressed in nasal epithelial cells, nasal polyps, nasal turbinates, primary bronchial epithelium, and middle ear mucosa [Audie et al., 1993; Voynow and Rose, 1994; Aust et al., 1997; Voynow et al., 1998; Moon et al., 2000]. However, the exact mechanism of *MUC5AC* gene expression has yet to be determined.

Allergic pulmonary inflammation and airway hyperreactivity in asthma models is closely related in Th2 cytokines interleukin (IL)-4 and IL-13 via signaling dependent upon the IL-4 receptor-α chain [Karras et al., 2007]. IL-13 also has been implicated in multiple pathologies of allergy and asthma, including elevated serum IgE via regulation, increased airway eosinophilia, the development of lung remodeling, and promotion of the secretory phenotype of airway epithelium [Chatila, 2004]. These facts emphasize the possibility of developing therapeutic inhibitors that inhibit both IL-4 and IL-13 signaling to control respiratory diseases. MUC5AC expression is increased in mice overexpressing IL-4 compared to transgenic-negative control [Temann et al., 1997]. However, the mechanism of IL-4-mediated MUC5AC expression and the signal molecule(s) involved, especially in the downstream signaling of mitogen-activated protein kinase (MAPK), have not yet been elucidated.

cAMP response element-binding protein (CREB) is an effector for a variety of receptors, such as those for growth factors, hormones, retinoids, cytokines, and prostaglandins [Aggarwal et al., 2008]. It can be activated via multiple pathways by various upstream kinases,

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including protein kinase A (PKA) [Gonzalez et al., 1989; Colbran et al., 1992], PKC [Yamamoto et al., 1988], MAPK–activated protein-2 [Tan et al., 1996], and Akt [Du and Montminy, 1998]. In addition, ribosomal S6 kinase (p90RSK) and mitogen and stress-activated kinase (MSK) are important upstream activators of CREB [Song et al., 2003a,b]. To date, the transcriptional regulation of *MUC5AC* gene expression by inflammatory mediators is known to involve transcription factors such as CREB, SP1 [Van Seuningen et al., 2001], AP1, NF-κB [Chen et al., 2004], or GRE [Chen et al., 2006]. However, cAMP response element (CRE) *cis*-sequences in the promoter region of *MUC5AC* through which IL-4 may upregulate *MUC5AC* gene expression have not yet been identified.

Because increased *MUC5AC* gene expression during inflammation plays an important role in the pathogenesis of airway diseases such as asthma, we evaluated whether IL-4 up-regulates *MUC5AC* gene expression by activating specific signal transduction pathways. Here, we show that sequential Ras, ERK1/2, and RSK1 are essential for IL-4-induced *MUC5AC* gene expression in airway epithelial cells. Moreover, we show also that CRE in the *MUC5AC* promoter appears to be important for IL-4-induced *MUC5AC* gene expression.

MATERIALS AND METHODS

MATERIALS

PD98059, SB203580, and anti-α-tubulin antibody were purchased from Calbiochem (San Diego, CA). All phospho-specific antibodies were purchased from Cell Signaling (Beverly, MA). All primers and siRNAs were synthesized by Bioneer (Daejeon, Korea): CREB, UCAAGGAGGCCUUCCUACA (dTdT), and negative control CCUACGCCACCAAUUUCGU (dTdT).

CELL CULTURES

The human lung mucoepidermoid carcinoma cell line (NCI-H292) cells were purchased from the American Type Culture Collection (CRL-1848; Manassas, VA) and cultured in RPMI-1640 (Gibco BRL; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). For serum deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and recultured in RPMI-1640 with 0.2% FBS.

RT-PCR

Total RNA was isolated using TRIzol (Gibco BRL) from cells treated with IL-4 (50 ng/ml). cDNA was synthesized with random hexamers (Roche, Branchburg, NJ) using MMLV reverse transcriptase (Roche). The oligonucleotide primers for PCR were designed based on the Genbank sequence of MUC5AC as previously described [Song et al., 2003b].

WESTERN BLOT ANALYSIS

For Western blot analysis, NCI-H292 cells were grown to confluence in six-well plates. After 15, 30, or 45 min treatment with IL-4, the cells were lysed with 2× lysis buffer [250 mM Tris–Cl (pH 6.5), 2% SDS, 4% β-mercaptoethanol, 0.02% BPB, 10% glycerol]. Equal amounts of whole cell lysates were resolved by 10–15% SDS–PAGE and transferred to a polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA).

IN VITRO p38 KINASE ASSAY

p38 kinase activity was measured using a p38 MAPK assay kit (Cell Signaling) according to the manufacturer's recommendations. Briefly, confluent cells were rendered quiescent for 24 h and then incubated with or without 20 μM SB203580 for 2 h prior to being stimulated with IL-4 for 15 min. Cells lysates were scraped off the dish with 500 μl lysis buffer and 1 mM PMSF, sonicated four times for 5 s each on ice and centrifuged for 10 min at 4°C; the supernatants were then transferred to a new tube. Cell lysates (400 μg) and 20 μl of immobilized phospho-p38 MAPK monoclonal antibody were incubated with gentle rocking overnight at 4°C. After being washed twice with lysis and kinase buffer, resuspended pellet with kinase buffer containing 200 μM ATP and 2 μg of activating transcription factor 2 (ATF2) fusion protein, incubated 30 min at 30°C, and then immunoblotted with phospho-ATF2 antibody.

PLASMIDS, TRANSIENT TRANSFECTION, AND LUCIFERASE ASSAY

Cells were transiently transfected with plasmids using the FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions. Deletion mutants covering the promoter regions of *MUC5AC* were generated by PCR [Song et al., 2003b]. Cells were incubated for 48 h, harvested, and assayed for luciferase activity using a luciferase assay system (Promega; Madison, WI) according to the manufacturer's instructions. Luciferase values were normalized to β-galactosidase. Transfection experiments were performed in duplicate and repeated at least three times.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

ChIP assays were performed with the EZ ChIP Assay kit and protocol (Millipore, Billerica, MA). A total of 4.5×10^7 cells were fixed in 1% formaldehyde at room temperature for 20 min. Isolated nuclei were lysed followed by chromatin shearing with the Enzymatic Shearing kit (Active Motif, Carlsbad, CA). A rabbit IgG antibody (Sigma, St. Louis, MO) was used as the control. After reverse cross-linking and DNA purification, DNA from input (1:20 diluted) or immunoprecipitated samples were assayed by PCR, and the products were separated by agarose gel electrophoresis. The CRE (−912 to −792) primers used for ChIP analysis PCR reaction are as follows: forward (5′-CATTTCTGGATCTTGGTGGC-3′) and reverse (5′-ACCATGCAGC-CCCCTCCCAG-3′), with the PCR product being 120 bp. We also designed the primers for the negative controls (−3,382 to −3,222) to amplify the DNA fragment upstream of the CRE element: forward (5′-GCCCCACTGACATAACCACCTGGC-3′) and reverse (5′-GGTCT-GACTCGACCAGGTGTAGCC-3′), yielding a PCR product of 160 bp.

ELECTROPHORETIC MOBILITY SHIFT ANALYSIS (EMSA)

Cells were washed with ice-cold PBS and pelleted. Pellets were then resuspended in nuclear extraction buffer I [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5% Nonidet P-40, 1 mM PMSF, 2 μg/ml leupeptin, and 2 μg/ml aprotinin], incubated for 15 min on ice, and vortexed vigorously. Nuclei were pelleted, resuspended in nuclear extraction buffer II [20 mM HEPES (pH 7.9), 20% glycerol, 420 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 0.1 mM

PMSF, 2 μg/ml leupeptin, and 2 μg/ml aprotinin], and vigorously vortexed. The nuclear extracts were then centrifuged for 15 min at 4°C, and the supernatants were stored at −70°C. For EMSA, oligonucleotides corresponding to CRE-specific sequences in the *MUC5AC* promoter region −878 to −871 (5′-AGAGATTGCCT-GACTTGAAGAGCTAG-3′) were synthesized, annealed, and end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclear extract was incubated at room temperature for 30 min with the ^{32}P-labeled CRE probe in binding buffer [20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris–Cl (pH 7.5), and 0.25 mg/ml poly(d*I* − d*C*)]. DNA–nuclear protein complexes were separated from the DNA probe by electrophoresis through 5% non-denaturing polyacrylamide gels in 0.5× Tris borate EDTA (TBE) buffer. Supershift experiments were conducted using 4 μl of anti-CREB antibody. The gel was dried and autoradiographed using an intensifying screen at −70°C.

STATISTICAL ANALYSIS

The data are presented as the mean ± 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

IL-4 CAN INDUCE GENE EXPRESSION OF MUC5AC IN NCI-H292 CELLS

To determine if IL-4 can induce *MUC5AC* gene expression within NCI-H292 cells, we carried out RT-PCR after treatment with IL-4 for 24 h (Fig. 1). *MUC5AC* mRNA expression was increased after treatment with 50 ng/ml of IL-4. There was no corresponding change in the expression of the internal control, β_2-microglobulin. These data show that IL-4 could specifically induce the gene expression of *MUC5AC* in NCI-H292 cells.

ERK1/2 ACTIVATED BY THE RAS-MEK1 PATHWAY IS ESSENTIAL FOR IL-4-INDUCED MUC5AC GENE EXPRESSION

To determine which MAPK signal pathway is involved in cells stimulated by IL-4, we performed Western blotting. ERK1/2 showed maximum activation at 15 min, and this effect decreased at 45 min (Fig. 2A). No change was detected in the phosphorylation of p38 and JNK. To further investigate the role of ERK1/2 in IL-4-induced *MUC5AC* gene expression, we pretreated cells with 20 μM PD98059, MEK1 inhibitor, for 2 h before treatment for 15 min with IL-4, and then analyzed protein expression (Fig. 2B). IL-1β has been used as a positive control for SB203580 [Song et al., 2003b]. IL-4 treatment clearly increased ERK1/2 activity, but pretreatment with PD98059 abrogated the effect of IL-4 completely. Moreover, to test for cross-talk between ERK1/2 and p38 for IL-4 signaling, we also used SB203580, p38 inhibitor, before performing Western blot analysis with anti-phospho ERK1/2 antibody. SB203580 did not affect the phosphorylation of ERK1/2, and PD98059 did not affect the phosphorylation of ATF2, which acts as a substrate of p38 for the in vitro p38 kinase assay (Fig. 2C). IL-1β has been used as a positive control for SB203580. We have previously suggested that inhibition of either ERK1/2 or p38 abolishes IL-1β- and TNFα-induced *MUC5AC* gene expression [Song et al., 2003b], suggesting that IL-4 signaling may be closely related only to ERK1/2, and not p38. Accordingly, we investigated the effect of pretreating cells with PD98059 or SB203580 before IL-4 exposure on *MUC5AC* mRNA expression. Pretreatment with PD98059 completely inhibited *MUC5AC* gene expression (Fig. 2D), whereas pretreatment with SB203580 did not. IL-1β has been used as a positive control for SB203580. Thus, the activation of ERK1/2 appears to be closely related to the signaling pathways activated by IL-4. To examine whether the sequential Ras/MEK1/ERK1/2 pathway plays a role in IL-4-induced *MUC5AC* gene expression, we performed transient transfection with plasmid expressing dominant-negative mutant Ras (Ras N17) or mutant MEK1. The overexpression of either Ras N17 or MEK1 DN significantly decreased IL-4-induced *MUC5AC* gene expression (Fig. 2E). This result indicates that activation of ERK1/2 via MEK1 by IL-4 might be a Ras-dependent pathway that induces *MUC5AC* gene expression.

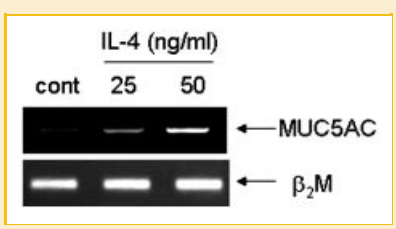

Fig. 1. Effect of IL-4 on *MUC5AC* gene expression. Confluent cells were rendered quiescent in RPMI-1640 with 0.2% FBS for 24 h prior to treatment with IL-4 for 24 h, and cell lysates were harvested for RT-PCR. β-2-Microglobulin (β_2M) was employed as an internal control. The figures shown are representative of three independent experiments.

IL-4-INDUCED ACTIVATION OF RSKI MEDIATED BY ERK1/2

To determine which molecules are involved during the downstream signaling of ERK1/2 within the signal pathway induced by IL-4, we investigated p90 ribosomal S6 kinase 1 (RSK1). RSK1 can be activated by ERK1/2 [Song et al., 2003a]. The phosphorylation of RSK1 reached a maximum after 30 min and decreased after 60 min of IL-4 stimulation (Fig. 3A). Moreover, pretreatment with ERK1/2 inhibitor, PD98059, inhibited the IL-4-induced phosphorylation of RSK1 (Fig. 3B), suggesting that ERK1/2 is able to regulate the phosphorylation of RSK1. In addition, we examined whether the activation of ERK1/2-RSK1 pathway may be essential for IL-4-activated CREB led to induce *MUC5AC* gene expression in NCI-H292 cells, because RSK1 is an upstream protein of CREB that plays as an important transcription factor, inducing various cellular phenomena [Song et al., 2003a]. PD98059 was treated for 2 h in prior to the treatment of IL-4. Phosphorylation of CREB was dramatically diminished by ERK1/2 inhibitor and dominant-negative mutant RSK1 (D205N), whereas overexpression of wild-type RSK1 increased IL-4-induced CREB phosphorylation, suggesting that ERK1/2-RSK1 pathway might be essential for IL-4-induced CREB activation. Consistently, IL-4-induced *MUC5AC* gene expression was increased by wild-type RSK1 and significantly inhibited by RSK1 D205N

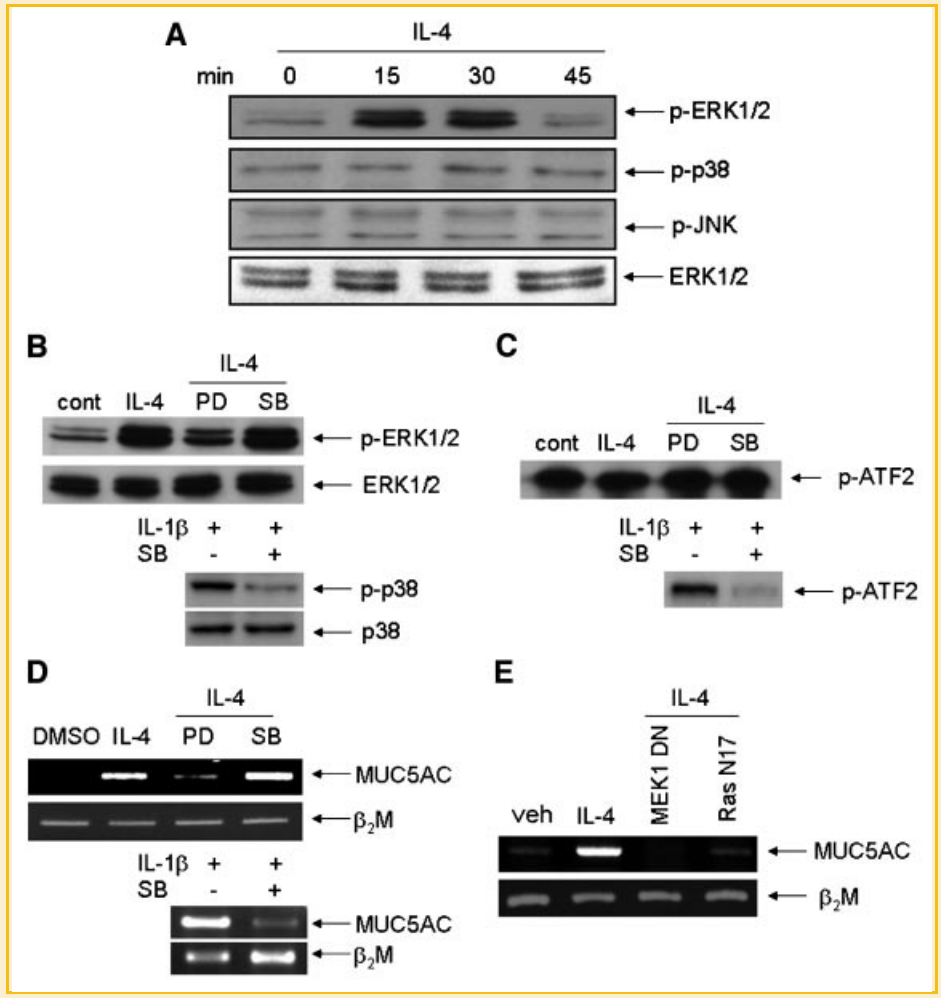

Fig. 2. Effect of ERK1/2 on *MUC5AC* gene expression. A: Confluent cells were treated with IL-4 (50 ng/ml) for 15, 30, or 45 min and cell lysates were harvested for Western blot analysis using phospho-specific antibodies. Total ERK1/2 was used as loading control. Cells were pretreated for 15 min with IL-4 prior to collection of total proteins for kinase assays. Cont: control. Representative kinase assays show the phosphorylation of ERK1/2 by PD98059 (B) and ATF2 as an exogenous substrate with p38 immunoprecipitated from IL-4-treated cells (C). IL-1β (10 ng/ml) has been used as a positive control for SB203580. D: Cells were treated with PD98059 or SB253580 for 2 h, and then stimulated for 24 h with IL-4 prior to collection of total RNA for RT-PCR analysis of *MUC5AC* mRNA expression. IL-1β (10 ng/ml) has been used as a positive control for SB203580. E: Cells were transiently transfected with dominant negative mutant construct and stimulated with IL-4 for 24 h prior to RT-PCR. The figures shown are representative of three independent experiments. Veh: vehicle transfection.

(Fig. 3C). These results provide evidence that RSK1 is required for IL-4-induced both CREB activation and *MUC5AC* gene expression.

CREB IS ESSENTIAL FOR IL-4-INDUCED MUC5AC GENE EXPRESSION

To understand the signaling proteins downstream of RSK1 for IL-4 signaling, we investigated CREB. Transient phosphorylation of CREB reached a maximum peak around 10–30 min after IL-4 treatment (Fig. 4A). In addition, both IL-4-induced CREB phosphorylation and *MUC5AC* gene expression were significantly decreased in cells transfected with a siRNA-CREB construct (Fig. 4B). Our results implicated CREB in IL-4-induced *MUC5AC* gene expression. To identify the IL-4-responsive region in the *MUC5AC* promoter, cells were transiently transfected with deletion mutants and treated with IL-4 for 24 h. As shown in Figure 4C, IL-4-induced luciferase activity of the −929/−1 region of the *MUC5AC* promoter was more increased compared to that of −776/−1 and −486/−1 regions, indicating that the −929/−776 region of the *MUC5AC* promoter may be essential for the response to IL-4. We further investigated whether ERK1/2, RSK1, and CREB pathway may affect directly IL-4-induced the transcriptional activation of *MUC5AC* gene in *MUC5AC* promoter and whether CRE site (TGACTTGA; −878/−871) within the −929/−776 region of the *MUC5AC* promoter [Song et al., 2003b] acts critically as a *cis*-element. The cells were pretreated with PD98059 or cotransfection was performed with dominant negative RSK1 construct, wild-type CREB, siRNA-CREB, or siRNA-control construct and a −929/−1 construct to examine its effect on *MUC5AC* transcription activity. PD98059, dominant negative RSK1 mutant, or siRNA-CREB decreased dramatically the luciferase activity, whereas wild-type CREB increased the luciferase activity of the −929/−1 region of the *MUC5AC* promoter (Fig. 4D). This result indicated that activation of *MUC5AC* transcription by IL-4 was

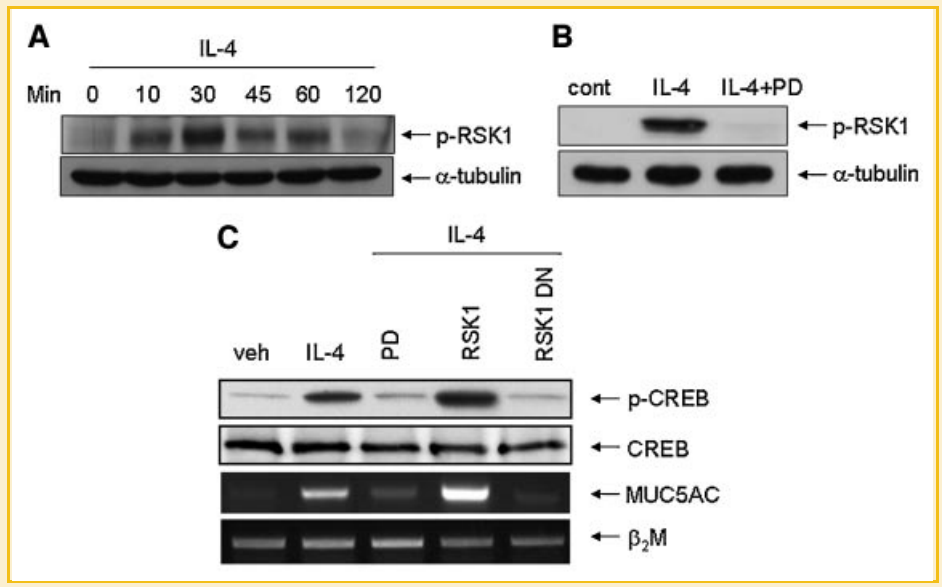

Fig. 3. Effect of RSK1 on IL-4-induced *MUC5AC* gene expression. A: Cells were stimulated for the indicated times with IL-4, and then total proteins were collected for Western blot analysis using phospho-RSK1 antibody. B: In other experiments, the cells were pretreated for 2 h with 20 μM PD98059 and the cells were then stimulated for 30 min prior to Western blot analysis with IL-4. C: The cells were pretreated for 2 h with 20 μM PD98059 or were transiently transfected with either a wild-type or mutant RSK1 D205N construct. The cells were stimulated with IL-4 for 30 min prior to Western blot analysis (upper panel) and for 24 h prior to RT-PCR (lower panel). The figures shown are representative of three independent experiments.

mediated by a sequential ERK1/2-RSK1-CREB pathway and that CREB might be led to the stimulation of CRE-mediated *MUC5AC* transcription. Moreover, we investigated whether CRE activation is required for IL-4-induced *MUC5AC* transcription by performing site-directed mutagenesis of the CREB-binding site. CRE site mutant constructs Mut1 and Mut2 decreased *MUC5AC* transcriptional activity compared to wild-type (Fig. 4E). These results suggest that CRE in the regulatory region of the *MUC5AC* promoter is implicated in the up-regulation of *MUC5AC* transcriptional activity stimulated by IL-4. To whether CREB binds to the CRE site in the *MUC5AC* promoter [Song et al., 2003b], we performed chromatin immunoprecipitation experiments. Chromatin was prepared from IL-4-treated cells, then immunoprecipitated with an anti-CREB or PLC antibody as a non-relevant antibody. PCR was performed on immunoprecipitated DNA after reversal of cross-linking using primers specific for the CRE recognition site. The control primers were for a site ~3,382 bp upstream of CRE [Song et al., 2003b; Song et al., 2008]. As shown in Figure 4F, the CRE site was specifically immunoprecipitated with the anti-CREB antibody, indicating that CREB activated by IL-4 led to an increase in the binding of CRE to the *MUC5AC* promoter. To further confirm whether CREB binds to the CRE site in the *MUC5AC* promoter [Song et al., 2003b], we performed electrophoretic mobility shift assay (EMSA) experiments using nuclear extracts from NCI-H292 cells after treatment of IL-4. For EMSA, oligonucleotides corresponding to CRE-specific sequences in the *MUC5AC* promoter region −878 to −871 (5′-AGAGATTGCCTGACTTGAAGAGCTAG-3′) were synthesized. As shown in Figure 4G, the activity of *MUC5AC* specific CRE remarkably increased in response to IL-4. To distinguish any specific CRE-binding complexes, competition and supershift analysis were performed using either 25- or 50-fold excesses of non-radiolabeled (cold) CRE oligonucleotide and anti-CREB antibody, respectively. The specific band was selectively inhibited by the cold-CRE oligonucleotide and was supershifted by anti-CREB antibody. These results indicated that activated CREB binds to a *cis*-acting element, CRE, in the *MUC5AC* promoter.

DISCUSSION

The mechanism of the regulation of MUC5AC secretion by external stimuli or cytokines in the airway is very important, and the exact understanding of the mechanism may provide new therapeutic strategies for the inhibition of the mucus hypersecretion in the airway. A few goblet cells were present in the airway of healthy subject, whereas they were increased dramatically in the airway of respiratory patients [Dabbagh et al., 1999]. In respiratory diseases, such as COPD, cystic fibrosis, and asthma, mucus hypersecretion has been frequently observed [Nadel, 2001]. Of 21 human *mucin* genes which identified until now, MUC5AC has been known as a major airway mucin because it highly expressed in the goblet cells [Song et al., 2003b] and it is regulated by proinflammatory cytokines, H_2O_2, LPS, IL-9, retinoic acid [Takeyama et al., 2000; Reader et al., 2003; Kim et al., 2007; Yan et al., 2008], or transforming growth factor-α [Jonckheere et al., 2004] in human airway epithelium. Although efforts have been made to identify the intracellular signaling pathways triggered by these cytokines, the signal transduction mechanisms, from the cytoplasm to the nucleus, by which these cytokines induce *MUC5AC* gene expression are still unclear.

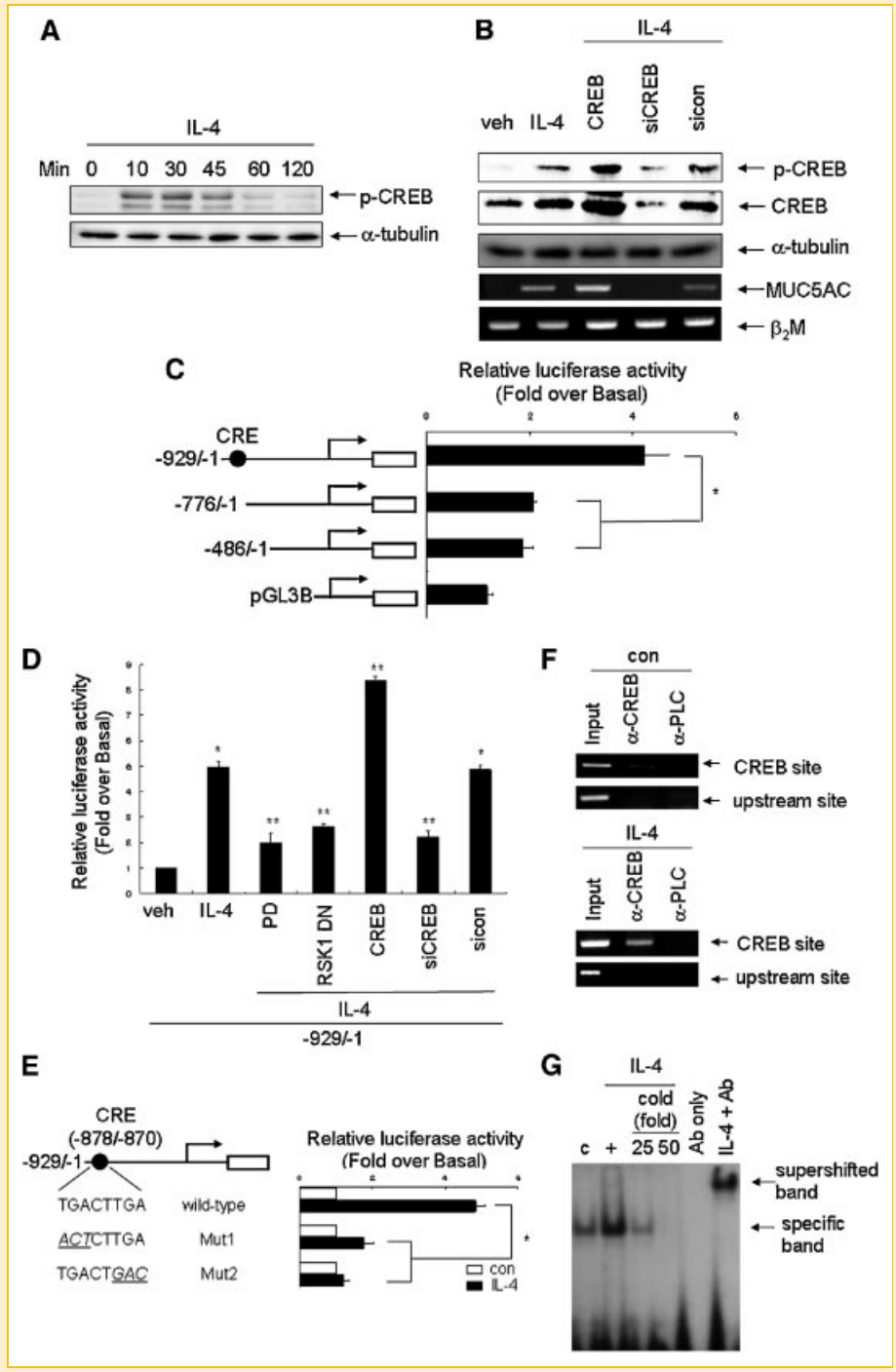

Fig. 4. IL-4-induced activation of CRE-mediated *MUC5AC* transcription via a *cis*-acting regulatory CRE motif. A: Cells were treated for the indicated times with IL-4, and then total proteins were collected for Western blot analysis using phospho-CREB antibody. B: Cells were transiently transfected with wild-type CREB, siRNA-CREB, or siRNA-control construct and stimulated with IL-4 for 30 min prior to Western blot analysis (upper panel) and for 24 h prior to RT-PCR (lower panel). C: Cells were pretreated with PD98059 or transiently transfected with several *MUC5AC* promoter luciferase reporter constructs and stimulated with IL-4 for 24 h. Luciferase activity was then assessed in IL-4-treated cells. $^{*}P < 0.05$ compared to $-929/-1$ reporter construct. Cells were cotransfected with wild-type CREB, siRNA-CREB, or siRNA-control construct and a reporter construct of the $-929/-1$ region of the *MUC5AC* promoter (D) and wild-type CREB, CRE site mutant 1 construct (Mut1), or CRE site mutant 2 construct (Mut2) (E). The displayed luciferase activities were corrected for transfection efficiency using the β-galactosidase activity of the cell lysates to standardize the values. The values shown are mean ± SD of experiments performed in triplicate. Figures shown are representative of three independent experiments. $^{*}P < 0.05$ compared to control and $^{**}P < 0.05$ compared to IL-4 only treatment. F: Chromatin prepared from cells with/without IL-4 treatment was immunoprecipitated using two different antisera and PCR was performed on DNA purified from input chromatin (Input) or immunoprecipitated chromatin, using primer pairs surrounding the CRE site or an upstream site of CRE site in the *MUC5AC* promoter. PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining. Anti-PLC antibody was used as a negative control. These figures are representative of three independent experiments. G: Cells were stimulated for 1 h with IL-4. Nuclear protein extracts from IL-4-treated NCI-H292 cells were subjected to EMSA. Nuclear proteins were incubated with [γ-^{32}P] labeled CRE oligonucleotides, either 25- or 50-fold excess of cold CRE probe or anti-CREB antibody before EMSA. The lane "antibody only" was indicated that CREB antibody was not incubated with nuclear extracts and that was utilized as a negative control. The labeled nuclear proteins were separated by electrophoresis on 5% polyacrylamide gels, and the gels were dried and exposed to autoradiography at -70°C overnight.

A better understanding of the biochemical characteristics of IL-4 will provide additional insights into the molecular signaling mechanism that leads to induce asthma. However, the level of MUC5AC expression was significantly increased by IL-4 administration in vivo, but it was not altered by IL-4 in NCI-H292 cells [Dabbagh et al., 1999]. The main reason why IL-4 could not induce MUC5AC expression in NCI-H292 cells might be 10 ng/ml of IL-4 was less sufficient for inducing MUC5AC expression in the cells. According to our result (Fig. 1), 25 ng/ml of IL-4 could not induce *MUC5AC* gene expression, indicating that even though NCI-H292 cells expressed IL-4R [Dabbagh et al., 1999], activating IL-4R was needed to more concentrated IL-4 than 25 ng/ml of IL-4. These cells were therefore responded to more concentrated IL-4 through its receptor and were mediated physiological phenomena in NCI-H292 cells. Our results show that the biological activity of MEK1/2, and consequently the function of ERK1/2, is determined by Ras small G-protein in airway epithelial cells (Fig. 2E). Furthermore, we further examined how IL-4 induces *MUC5AC* gene expression in the nucleus. We previously suggested mitogen-and stress-activated protein (MSK) 1 and RSK1 as intranuclear molecules involved in cytokine-induced *mucin* gene expression [Song et al., 2003a,b]. Since these proteins are tightly regulated by MAPK in cells, effects of their roles on IL-4-mediated proinflammatory signaling also need to be elucidated. As seen in Figure 3, ERK1/2-RSK1 pathway was involved in the IL-4-mediated signaling pathway, but MSK1 was not (data not shown), suggesting that the signaling pathways leading to *MUC5AC* gene expression are distinct, depending on the type of stimulant and cells used.

To date, signaling mediators involved in the downstream signaling of ERK1/2 for IL-4-induced *MUC5AC* gene expression have not been fully understood. Previously, we suggested that CREB may play an important role in cytokine-induced *mucin* gene expression [Song et al., 2003a,b]. We also found that CREB is responsible for IL-4-induced *MUC5AC* gene expression through the ERK1/2 pathway via Ras-dependent signaling. This finding is consistent with our previous studies in which we have shown involvement of the ERK1/2 pathway in the transcriptional activation of *MUC5AC* gene expression by several cytokines [Song et al., 2003a,b], which in turn, CREB may be a key regulator of inflammation in airway, much like NF-κB. However, the CREB-binding partner for the increased transcriptional activity by IL-4 has been yet unclear. Several papers have proposed that interactions between CREB, CREB-binding protein (CBP), and NF-κB regulated each functions for altering physiological phenomena [Parry and Mackman, 1997; Shenkar et al., 2001; Yalcin et al., 2003; Xu et al., 2007]. These findings are consistent with our speculation that CREB may interact directly or indirectly with other transcription factor(s) that function as bridging mediators between DNA-binding transcription factors and either enhancer, coactivator, or suppressor factor, which form a transcriptional complex for diverse signaling pathways in the regulation of *MUC5AC* gene expression [Song et al., 2003a].

In summary, we found that ERK1/2, but not p38 and JNK signaling, is essential for IL-4-induced *MUC5AC* gene expression in the Ras-dependent manner. We also suggest that CRE in the *MUC5AC* promoter might play a critical role in these processes by binding CREB. On the basis of our findings, we speculate that activation of the ERK1/2, RSK1, and CREB signaling cascades are crucial aspects of the intracellular signaling mechanisms that mediate *MUC5AC* gene expression induced by IL-4. We show that the mucus overproduction seen in asthma may be a result of IL-4-mediated increased *MUC5AC* gene expression within the inflamed lung. Further analysis of the signaling pathways activated by various cytokines may provide deeper insights into the pathogenesis of allergic airway diseases.

REFERENCES

Aggarwal S, Kim SW, Ryu SH, Chung WC, Koo JS. 2008. Growth suppression of lung cancer cells by targeting cyclic AMP response element-binding protein. Cancer Res 68:981–988.

Audie JP, Janin A, Porchet N, Copin MC, Gosselin B, Aubert JP. 1993. Expression of human mucin genes in respiratory, digestive, and reproductive tracts ascertained by in situ hybridization. J Histochem Cytochem 41:1479–1485.

Aust MR, Madsen CS, Jennings A, Kasperbauer JL, Gendler SJ. 1997. Mucin mRNA expression in normal and vasomotor inferior turbinates. Am J Rhinol 11:293–302.

Chatila TA. 2004. Interleukin-4 receptor signaling pathways in asthma pathogenesis. Trends Mol Med 10:493–499.

Chen R, Lim JH, Jono H, Gu XX, Kim YS, Basbaum CB, Murphy TF, Li JD. 2004. Nontypeable Haemophilus influenzae lipoprotein P6 induces MUC5AC mucin transcription via TLR2-TAK1-dependent p38 MAPK-AP1 and IKK-beta-IkappaBalpha-NF-kappaB signaling pathways. Biochem Biophys Res Commun 324:1087–1094.

Chen Y, Nickola TJ, DiFronzo NL, Colberg-Poley AM, Rose MC. 2006. Dexamethasone-mediated repression of MUC5AC gene expression in human lung epithelial cells. Am J Respir Cell Mol Biol 34:338–347.

Colbran JL, Roach PJ, Fiol CJ, Dixon JE, Andrisani OM, Corbin JD. 1992. cAMP-dependent protein kinase, but not the cGMP-dependent enzyme, rapidly phosphorylates delta-CREB, and a synthetic delta-CREB peptide. Biochem Cell Biol 70:1277–1282.

Dabbagh K, Takeyama K, Lee HM, Ueki IF, Lausier JA, Nadel JA. 1999. IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo. J Immunol 162:6233–6237.

Du K, Montminy M. 1998. CREB is a regulatory target for the protein kinase Akt/PKB. J Biol Chem 273:32377–32379.

Gonzalez GA, Yamamoto KK, Fischer WH, Karr D, Menzel P, Biggs W, Vale WW, Montminy MR. 1989. A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature 337:749–752.

Hovenberg HW, Davies JR, Herrmann A, Linden CJ, Carlstedt I. 1996. MUC5AC, but not MUC2, is a prominent mucin in respiratory secretions. Glycoconj J 13:839–847.

Jonckheere N, Van Der Sluis M, Velghe A, Buisine MP, Sutmuller M, Ducourouble MP, Pigny P, Büller HA, Aubert JP, Einerhand AW, Van Seuningen I. 2004. Transcriptional activation of the murine Muc5ac mucin gene in epithelial cancer cells by TGF-beta/Smad4 signalling pathway is potentiated by Sp1. Biochem J 377:797–808.

Karras JG, Crosby JR, Guha M, Tung D, Miller DA, Gaarde WA, Geary RS, Monia BP, Gregory SA. 2007. Anti-inflammatory activity of inhaled IL-4 receptor-alpha antisense oligonucleotide in mice. Am J Respir Cell Mol Biol 36:276–285.

Kim SW, Hong JS, Ryu SH, Chung WC, Yoon JH, Koo JS. 2007. Regulation of mucin gene expression by CREB via a nonclassical retinoic acid signaling pathway. Mol Cell Biol 27:6933–6947.

Moon SK, Yoo JH, Kim HN, Lim DJ, Chung MH. 2000. Effects of retinoic acid, triiodothyronine and hydrocortisone on mucin and lysozyme expression in cultured human middle ear epithelial cells. Acta Otolaryngol 120:944–949.

Nadel JA. 2001. Role of epidermal growth factor receptor activation in regulating mucin synthesis. Respir Res 2:85–89.

Parry GC, Mackman N. 1997. Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-kappaB-mediated transcription. J Immunol 159:5450–5456.

Reader JR, Hyde DM, Schelegle ES, Aldrich MC, Stoddard AM, McLane MP, Levitt RC, Tepper JS. 2003. Interleukin-9 induces mucous cell metaplasia independent of inflammation. Am J Respir Cell Mol Biol 28:664–672.

Shenkar R, Yum HK, Arcaroli J, Kupfner J, Abraham E. 2001. Interactions between CBP, NF-kappaB, and CREB in the lungs after hemorrhage and endotoxemia. Am J Physiol Lung Cell Mol Physiol 281:L418–L426.

Song KS, Seong JK, Chung KC, Lee WJ, Kim CH, Cho KN, Kang CD, Koo JS, Yoon JH. 2003a. Induction of MUC8 gene expression by interleukin-1 beta is mediated by a sequential ERK MAPK/RSK1/CREB cascade pathway in human airway epithelial cells. J Biol Chem 278:34890–34896.

Song KS, Lee WJ, Chung KC, Koo JS, Yang EJ, Choi JY, Yoon JH. 2003b. Interleukin-1 beta and tumor necrosis factor-alpha induce MUC5AC overexpression through a mechanism involving ERK/p38 mitogen-activated protein kinases-MSK1-CREB activation in human airway epithelial cells. J Biol Chem 278:23243–23250.

Song KS, Lee TJ, Kim K, Chung KC, Yoon JH. 2008. cAMP-responding element-binding protein and c-Ets1 interact in the regulation of ATP-dependent MUC5AC gene expression. J Biol Chem 283:26869–26878.

Takeyama K, Dabbagh K, Shim JJ, Dao-Pick T, Ueki IF, Nadel JA. 2000. Oxidative stress causes mucin synthesis via transactivation of epidermal growth factor receptor: Role of neutrophils. J Immunol 164:1546–1552.

Tan Y, Rouse J, Zhang A, Cariati S, Cohen P, Comb MJ. 1996. FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. EMBO J 15:4629–4642.

Temann UA, Prasad B, Gallup MW, Basbaum C, Ho SB, Flavell RA, Rankin JA. 1997. A novel role for murine IL-4 in vivo: Induction of MUC5AC gene expression and mucin hypersecretion. Am J Respir Cell Mol Biol 16:471–478.

Thornton DJ, Howard M, Khan N, Sheehan JK. 1997. Identification of two glycoforms of the MUC5B mucin in human respiratory mucus. Evidence for a cysteine-rich sequence repeated within the molecule. J Biol Chem 272:9561–9566.

Van Seuningen I, Pigny P, Perrais M, Porchet N, Aubert JP. 2001. Transcriptional regulation of the 11p15 mucin genes. Towards new biological tools in human therapy, in inflammatory diseases and cancer? Front Biosci 6:D1216–D1234.

Voynow JA, Rose MC. 1994. Quantitation of mucin mRNA in respiratory and intestinal epithelial cells. Am J Respir Cell Mol Biol 11:742–750.

Voynow JA, Selby DM, Rose MC. 1998. Mucin gene expression (MUC1, MUC2, and MUC5/5AC) in nasal epithelial cells of cystic fibrosis, allergic rhinitis, and normal individuals. Lung 176:345–354.

Voynow JA, Young LR, Wang Y, Horger T, Rose MC, Fischer BM. 1999. Neutrophil elastase increases MUC5AC mRNA and protein expression in respiratory epithelial cells. Am J Physiol 276:L835–L843.

Wickstrom C, Davies JR, Eriksen GV, Veerman EC, Carstedt I. 1998. MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: Identification of glycoforms and C-terminal cleavage. Biochem J 334:685–693.

Xu W, Kasper LH, Lerach S, Jeevan T, Brindle PK. 2007. Individual CREB-target genes dictate usage of distinct cAMP-responsive coactivation mechanisms. EMBO J 26:2890–2903.

Yalcin A, Koulich E, Mohamed S, Liu L, D'Mello SR. 2003. Apoptosis in cerebellar granule neurons is associated with reduced interaction between CREB-binding protein and NF-kappaB. J Neurochem 84:397–408.

Yamamoto KK, Gonzalez GA, Biggs W, Montminy MR. 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature 334:494–498.

Yan F, Li W, Jono H, Li Q, Zhang S, Li JD, Shen H. 2008. Reactive oxygen species regulate *Pseudomonas aeruginosa* lipopolysaccharide-induced MUC5AC mucin expression via PKC-NADPH oxidase-ROS-TGF-alpha signaling pathways in human airway epithelial cells. Biochem Biophys Res Commun 366:513–519.